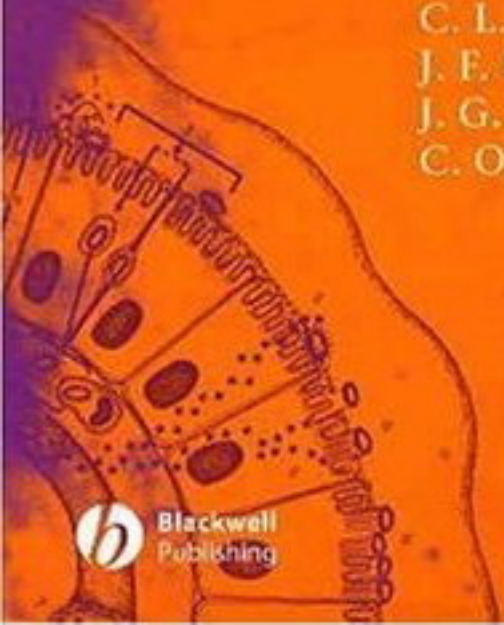
Pathogenesis of Bacterial Infections in Animals

Third Edition

C. L. Gyles
J. F. Prescott
J. G. Songer
C. O. Thoen



Blackwell Publishing

Pathogenesis of Bacterial Infections in Animals

Third Edition

Pathogenesis of Bacterial Infections in Animals

Third Edition

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

Carlton L. Gyles, DVM, MSc, PhD, is professor of veterinary microbiology at Ontario Veterinary College, University of Guelph, Ontario, Canada.

John F. Prescott, MA, Vet MB, PhD, is chair of pathobiology and professor of veterinary microbiology at Ontario Veterinary College, University of Guelph, Ontario, Canada.

J. Glenn Songer, PhD, is professor of veterinary science and microbiology, University of Arizona, Tucson, Arizona.

Charles O. Thoen, DVM, PhD, is former chair and professor of microbiology, immunology, and preventive medicine, Iowa State University, Ames, Iowa.

Blackwell Publishing Professional
2121 State Avenue, Ames, Iowa 50014, USA

Orders: 1-800-862-6657
Office: 1-515-292-0140
Fax: 1-515-292-3348
Web site: www.blackwellprofessional.com

Blackwell Publishing Ltd
9600 Garsington Road, Oxford OX4 2DQ, UK
Tel.: +44 (0)1865 776868

Blackwell Publishing Asia
550 Swanston Street, Carlton, Victoria 3053, Australia
Tel.: +61 (0)3 8359 1011

Printed on acid-free paper in the United States of America

Library of Congress Cataloging-in-Publication Data

Pathogenesis of bacterial infections in animals / edited by Carlton L. Gyles … [et al.].—3rd ed.
p. ; cm.
Includes bibliographical references and index.
ISBN-13: 978-0-8138-2939-5
ISBN-10: 0-8138-2939-9 (alk. paper)
1. Bacterial diseases in animals.
[DNLM: 1. Bacterial Infections—veterinary.
2. Bacteria—pathogenicity. SF 780.3 P297 2004]
I. Gyles, C. L. (Carlton L.), 1940–

SF780.3.P37 2004
636.089'692—dc22
2004002510

The last digit is the print number: 9 8 7 6 5 4 3 2

Contents

Contributors

Thomas Adam Institut fur Mikrobiologie und Hygiene, Medizinische Fakultat der Humboldt-Universitat, Charite, Dorotheenstr., Berlin, Germany

Ben Adler Bacterial Pathogenesis Research Group, Department of Microbiology, School of Biomedical Sciences, Monash University, VIC 3800, Australia

Craig Altier Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh NC 27606, USA

Arthur A. Andersen National Animal Disease Center, Agricultural Research Service, Ames, IA 50010, USA

Hylton Bark School of Veterinary Medicine, The Hebrew University of Jerusalem, P. O. Box 12 Rehovot, 76100, Israel

Raul G. Barletta Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, NE, USA

David A. Bemis Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA

Stephen J. Billington Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, AZ 85721, USA

Patrick Boerlin Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

Janine T. Bossé Molecular Infectious Disease Group, Department of Academic Paediatrics, Imperial College London, St. Mary's Campus, London W2 1PG, United Kingdom

John D. Boyce Bacterial Pathogenesis Research Group, Department of Microbiology, School of Biomedical Sciences, Monash University, VIC 3800, Australia

Glenn F. Browning School of Veterinary Science, The University of Melbourne, Parkville, Victoria 3010, Australia

N. B. Buller Western Australian Department of Agriculture, Baron-Hay Court, South Perth, Western Australia 6151, Australia

Rebecca Chafel Department of Microbiology and Molecular Biology, Tufts University, Boston, Massachusetts, USA

Norman F. Cheville National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA 50010, USA

Lynette Corbeil Department of Pathology, University of California at San Diego Medical Center, 2000 West Arbor Drive, San Diego, CA 92103, USA

Charles Czuprynski Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706-1102, USA

Luc A. Devriese Faculty of Veterinary Medicine, Ghent University, Salisburylann 133, B 9820 Merelbeke, Belgium

Terry C. Dixon Department of Pediatrics, Duke University Medical Center, Durham, NC, USA

Chris Dupont, Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

John M. Fairbrother Faculté de médecine vétérinaire, Université de Montréal, 3200 Sicotte CP5000, St. Hyacinthe, Quebec, J2S 7C6 Canada

Jacinta L. Farn University of Melbourne, Department of Microbiology and Immunology, Parkville, Victoria 3052, Australia

Brad Fenwick Department of Diagnostic Medicine/ Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA

Connie J. Gebhart Department of Veterinary PathoBiology, College of Veterinary Medicine, University of Minnesota, St. Paul, MN, USA

Roberto M. C. Guedes Department of Veterinary PathoBiology, College of Veterinary Medicine, University of Minnesota, St. Paul, MN, USA

Carlton L. Gyles Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Freddy Haesebrouck Faculty of Veterinary Medicine, Ghent University, Salisburylann 133, B 9820 Merelbeke, Belgium

Tom A. Halsey Department of Microbiology, College of Veterinary Medicine, North Carolina State University, Raleigh NC 27606, USA

David J. Hampson School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia

Shimon Harrus School of Veterinary Medicine, The Hebrew University of Jerusalem, P.O. Box 12 Rehovot, 76100, Israel

Katleen Hermans Faculty of Veterinary Medicine, Ghent University, Salisburylann 133, B 9820 Merelbeke, Belgium

Thomas J. Inzana, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Lynn A. Joens Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, AZ 85721, USA

B. Helen Jost Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, AZ 85721, USA

Joseph S. Lam Canadian Bacterial Diseases Network, Department of Microbiology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

Steve J. Libby Department of Microbiology, College of Veterinary Medicine, North Carolina State University, Raleigh NC 27606, USA

Reggie Y. C. Lo Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Jan I. MacInnes Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Suman Mahan University of Florida/USAID/ SADC Heartwater Research Project, Department of Pathobiology, College of Veterinary Medicine, Gainesville, FL 32610-0880, USA

Mauricia Matewish Canadian Bacterial Diseases Network, Department of Microbiology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

Joan Mecsas Department of Microbiology and Molecular Biology, Tufts University, Boston, MA, USA

Wojtek P. Michalski CSIRO Livestock Industries, Australian Animal Health Laboratory, Protein Biochemistry and Proteomics Group, Geelong, Victoria 3220, Australia

Alejandro de la Peña-Moctezuma Departamento de Microbiología e Inmunología, Facultad de Medi-

cina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Cd. Universitaria, Coyoacán, 04510, México, D.F. México

T. G. Nagaraja Department of Diagnostic Medicine/ Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66502-5606, USA

Steven C. Olsen National Animal Disease Center Agricultural Research Service US Department of Agriculture, Ames, IA 50010, USA

Jennifer Potter Department of Microbiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, USA

John F. Prescott Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Jun Ren Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Yoshihiro Shimoji National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

J. Glenn Songer Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, AZ 85721, USA

Charles O. Thoen Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University Ames, IA 50011, USA

John F. Timoney Department of Veterinary Medicine, 108 Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099, USA

Trevor Waner Israel Institute for Biological Research, PO Box 19, Ness Ziona, 74100, Israel

Matthew A. Weiner Department of Pediatrics, Duke University Medical Center, Durham, NC, USA

Kevin L. Whithear School of Veterinary Science, The University of Melbourne, Parkville, Victoria 3010, Australia

Robert H. Whitlock Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, 426 Dean Drive Cedar Croft, Kennett Square, PA 19348, USA

Ian Wilkie Veterinary Pathology and Anatomy, School of Veterinary Science and Animal Production, The University of Queensland, QLD, 4072, Australia

Preface

The third edition of *Pathogenesis of Bacterial Infections in Animals* is much more than an updated version of the second edition. Two new editors and 47 new authors bring new perspectives and considerable expertise to the book. For most chapters, 2 to 5 authors have combined their efforts to ensure that the book provides a balanced and authoritative account of pathogenesis. For the other chapters, we were fortunate to find individuals whose breadth and depth of knowledge on a group of pathogens allowed them to carry out the task single-handedly.

We have added two introductory chapters to provide an overview of mechanisms in pathogenesis and a review of evolutionary changes that impact on pathogenesis. Readers will be able to further explore the themes and the evolution of virulence as they read individual chapters. The chapter on *Nocardia*, *Actinomyces*, and *Dermatophilus* was omitted from this edition as there have not been great strides in knowledge of pathogenesis of these bacteria in recent years.

This book is possible because of the dedication of a vast number of people. Most important are the researchers whose curiosity, commitment to discovery, and hard work have produced a vast body of knowledge of the interactions of pathogenic bacteria and their hosts. The writers of the chapters in this book are part of this group and have used their own work and insights as well as the work of colleagues to produce interesting accounts of pathogenesis. Authors were allowed considerable freedom to tell the pathogenesis story in a way that they felt was most effective for the particular organism or group of organisms. The editors reviewed the chapters and have ensured that there is consistency of approach, despite the variation in styles of writers and in state of knowledge of pathogenesis of various bacteria. Several publishers and colleagues kindly gave permission for the use of illustrations in the book and we are grateful to them.

This is the age of things molecular. Therefore, it is not surprising that molecular pathogenesis is a major aspect in almost every chapter. Bacterial and host molecules that participate in the disease process are being identified at a rapid pace, and our understanding of their precise roles in pathogenesis is improving daily. This is a satisfying experience as more and more pieces of the puzzle are put together, and these advances are leading to new ways of preventing and controlling disease. Care has been taken to ensure that our fascination with molecular events has not led to the neglect of other aspects of pathogenesis that are critical for appreciation of the big picture. This has been possible because of the selection of authors who are active in cutting-edge research and have expertise in the whole range of interactions between host, parasite, and environment that constitute pathogenesis.

This book is unique. It brings together expertise from both medical and animal health research and describes events at the molecular, cellular, organ, and whole-animal levels. This is a truly international production with contributors from Australia, Belgium, Canada, Germany, Israel, Japan, Mexico, the United Kingdom, the United States, and Zimbabwe.

Finally, we wish to thank our publishers who have been extremely helpful in transforming the writings of 57 individuals into a beautiful book. Special thanks go to Tad Ringo, the project manager; Justin Eccles, designer; Carol Kromminga, copy editor; Cheryl Garton, publishing assistant; and Gretchen Van Houten, commissioning editor.

Pathogenesis of Bacterial Infections in Animals

Third Edition

1
Themes in Bacterial Pathogenic Mechanisms

C. L. Gyles and J. F. Prescott

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 should be of fundamental value in designing better and unprecedented ways to counter infectious diseases. This is happening already. 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).

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).

BASIC STEPS IN PATHOGENESIS 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; and (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. Binding may result 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. As many receptors are developmentally regulated, age specificity may also be determined by the receptor to which a pathogen binds.

Bacterial pathogens, including those associated with wound infections, may bind to extracellular matrix molecules such as fibronectin, collagen, laminin, or other proteins possessing RGD sequences for binding of eukaryotic cell-membrane integrins. Bacteria may use "invasins" to invade 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 cytoskeleton rearrangement. 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 zipperlike 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 entering through complement or other lectin-binding receptors and thus avoiding the oxidative burst that might otherwise kill them. Remarkably for these organisms, which subsequently interfere with normal macrophage phagosome maturation, the safest place in the body 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 with 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. The lack of available iron within the substance of the body that restricts the growth of many bacteria is often overcome by the iron-acquisition systems of pathogens. Many organisms, particularly those causing septicemia and pneumonia, have prominent, usually carbohydrate, capsules that help the organism resist phagocytosis in the absence of antibody. 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. Other 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 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, such as the IgA proteases of *Haemophilus somnus*, 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 not only result in a "cytokine storm" that confuses the immune system but also result 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 longer-term acquisition of the nutrients the bacterium needs to thrive and to continue its pathogenic lifestyle. Infection does not always lead to disease, which is 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, though that based on activity is 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 activation of host-cell pathways, leading to aberrant cell metabolism. Examples in *E. coli* include the heat-stable enterotoxin STa, which binds to the receptor for guanylyl cyclase, resulting in hypersecretion due to excessive levels of cGMP, and the CNF toxins, which activate Rho GTPases, resulting in cytoskeletal rearrangements. Other examples include the *Bacillus anthracis* EF toxin, the *Pasteurella multocida* PMT toxin, and the 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 AB (active-binding) two-component toxin molecules. Examples include the ADP-ribosyl transferases (e.g., the *E. coli* LT toxin), 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 probably represents an extreme case in which hyperresponsiveness to 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 homolog 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 assure 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 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 EHEC O157:H7, the bacteria are normal flora in the intestine of animals in which they do not cause disease, but they 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.

REFINING CONCEPTS OF VIRULENCE

Bacteria cause disease by a variety of virulence mechanisms in a highly complex process that usually involves penetrating host 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.

The impact of infection on the evolution of animal hosts can generally only be guessed at, but may have been profound. For example, the target of the *Vibrio cholerae* toxin (CT) and *Escherichia 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 current high frequency of the delta F508 mutation (1 in 25), homozygotes for which develop cystic fibrosis. It is interesting that 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 molecular Koch's postulates") (Falkow 1988) but were still largely pathogen centered and focused on a narrow range of virulence determinants such as

exotoxins of *Corynebacterium diphtheriae* and *Clostridium tetani*.

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-associated genes" that regulate essential virulence genes or are otherwise required for their expression, secretion, or processing, as well as "virulence lifestyle genes," which 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 from 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 bacterial survival and successful further spread 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.

CRITICAL HOST-BACTERIA COMMUNICATION

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 not surprising, therefore, 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, that investigates bacterial signal transduction as a tool to characterize host signaling pathways.

Bacteria have an astounding ability to sense their environment and to rapidly 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).

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 up-regulated while others are down-regulated 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 target for novel synthetic or natural inhibitory molecules.

A recent study by Hentzer and colleagues (2003) showed in 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 demon-

strated 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 >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 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 1/1,000th 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.

New regulatory signals that are critical for virulence expression are being identified. For example, the enterohemorrhagic *E. coli* 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. It is interesting that 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. 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 proinflammatory 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 shown to induce apoptosis include outer membrane proteins, LPS, lipomannans, lipoarabinomannans, 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 *Salmonella*, *Shigella*, *Escherichia*, *Yersinia, Campylobacter, Pseudomonas, Chlamydia, Mycobacterium*, *Staphylococcus, Streptococcus,* and *Listeria*. Apoptosis may provide benefits to the host by way of 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 pro-apoptotic molecules or inhibiting anti-apoptotic molecules (Grassme et al. 2001). Direct activation of pro-apoptotic signals is seen in infection by *Shigella flexneri*, *Salmonella*, and *S. aureus*. *S. 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 results in widening of 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 MAP kinase and MAP kinase kinases as well as 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 anti-apoptotic proteins. *P. aeruginosa* depends on its TTSS to up-regulate 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 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 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 release of IL-8 from intestinal epithelial cells. It is interesting that 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 up-regulation of costimulatory 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 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 activation of B cell proliferation, release of chemokines, and adjuvant effects of the PAMPs.

PROMISE OF GENOMICS

"The enormous influx of information from genome sequencing projects 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," say Whittam and Bumbaugh (2002). Examining differences in specific genes between a pathogen and a closely related nonpathogen, or between parent and its offspring with a specific null mutation, has been a valuable approach for identification of virulence genes. The rate of recognition of potential virulence genes is increasing dramatically as genome sequences are now available for over two hundred bacteria, and genomic data and microarray analysis are now frequently combined to rapidly identify hundreds of potential virulence factors simultaneously. However, these potential virulence

factors will need to be tested individually to assess their roles in virulence.

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 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 to use *in vitro* conditions to simulate the *in vivo* setting. One of the challenges in these studies is to simulate accurately the host microenvironment. Currently, it is common for only one or two aspects of that environment to be examined in simulations (e.g., temperature, iron concentration, pH). It is likely that much more complex simulations will be attempted in the future.

Comparative genomics involving comparison of ORFs of genomes has been a valuable starting point in identification of virulence genes. For example, a comparison of the genomes of pathogenic *Listeria monocytogenes* and the closely related nonpathogenic *L. innocua* identified 270 genes that were specific to *L. monocytogenes* and 149 that were specific to *L. innocua* (Buchrieser et al. 2003). These genes were present in coding regions that were scattered over the genomes but typically different in 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 STEC (M. A. Karmali, personal communication).

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 has 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 is essential for infection of the host and for disease.

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-ribosyltransferase activity were identified by this method in bacteria as diverse as *Streptococcus pyogenes*, *Mycobacterium avium*, *Salmonella* 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 function. Besides, presence of gene sequences does not necessarily mean that functional proteins are produced.

EVOLUTION OF PATHOGENS—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 is found on a variety of mobile genetic elements (bacteriophages, 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 (point mutations, rearrangements) of existing genes to improve survival in different niches (though this is also important). Clonal analysis of bacterial populations has been used to characterize the different times and populations in which these dramatic changes occurred.

For example, *Salmonella* evolved as a pathogen over the last one hundred 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 1 (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 non-host-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 marked even further 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 DT104 appear to have a broad host range. However, in contrast, in Rock doves, *S.* Typhimurium var. Copenhagen is considered to be a specifically adapted subtype, with DT2 and DT99 being isolated almost exclusively from this species in Europe and North America (Rabsch et al. 2002). Certain strains of *S.* Typhimurium, particularly DT40 and DT160, 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 through homologous recombination to 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 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.

Bacteriophages 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*. Phages 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, raising the suggestion that 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 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 *Salmonella 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*.

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). The *cadA* encodes lysine decarboxylase whose activity results in 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 to be 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.

REFERENCES

Ahmad-Nejad, P., H. Hacker, M. Rutz, S. Bauer, R. M. Vabulas, and H. Wagner. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur. J. Immunol. 32:1958–1968.

Bäumler, A. J., R. M. Tsolis, and F. Heffron. 2000. Virulence mechanisms of *Salmonella* and their genetic basis. In *Salmonella* in domestic animals. C. Wray and A. Wray (eds.). CABI Publishing, Wallingford, Oxon, pp. 57–72.

Bäumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams. 1998. Evolution of host adaptation in *Salmonella enterica*. Infection and Immunity 66:4579–4587.

Buchrieser, C., C. Rusniok, F. Kunst, P. Cossart, P. Glaser, and Listeria Consortium. 2003. Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria innocua*: clues for evolution and pathogenicity. FEMS Immunol. Med. Microbiol. 35:207–213.

Cannon, C. L., M. P. Kowalski, K. S. Stopak, and G. B. Pier. 2003. *Pseudomonas aeruginosa*-induced apoptosis is defective in respiratory epithelial cells expressing mutant cystic fibrosis transmembrane conductance regulator. Am. J. Respir. Cell Mol. Biol. 29:188–197.

Day, W. A., Jr., R. E. Fernandez, and A. T. Maurelli. 2001. Pathoadaptive mutations that enhance virulence: genetic organization of the *cadA* regions of *Shigella* spp. Infect. Immun. 69:7471–7480.

D'Hauteville, H., S. Khan, D. J. Maskell, A. Kussak, A. Weintraub, J. Mathison, R. J. Ulevitch, N. Wuscher, C. Parsot, and P. J. Sansonetti. 2002. Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. J. Immunol. 168:5240–5251.

Erwert, R. D., K. T. Eiting, J. C. Tupper, R. K. Winn, J. M. Harlan, and D. D. Bannerman. 2003. Shiga toxin induces decreased expression of the anti-apoptotic protein Mcl-1 concomitant with the onset of endothelial apoptosis. Microb. Pathog. 35:87–93.

Falkow, S. 1988. Molecular Koch's postulates applied to microbial pathogenicity. Rev. Infect. Dis. 10:S274–S276.

Figueroa-Bossi, N., S. Uzzau, D. Maloriol, and L. Bossi. 2001. Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in *Salmonella*. Mol. Microbiol. 39:260–271.

Finlay, B. B., and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61:136–169.

Grassme, H., V. Jendrossek, and E. Gulbins. 2001. Molecular mechanisms of bacteria induced apoptosis. Apoptosis 6:441–445.

Gruenheid, S., and B. B. Finlay. 2003. Microbial pathogenesis and cytoskeletal function. Nature 422:775–781.

Hauck, C. R. 2002. Cell adhesion receptors—signaling capacity and exploitation by bacterial pathogens. Med. Microbiol. Immunol. (Berl). 191:55–62.

Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby, and M. Givskov. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J.22:3803–3815.

Malish, H. R., N. L. Freeman, D. V. Zurawski, P. Chowrashi, J. C. Ayoob, J. W. Sanger, and J. M. Sanger. 2003. Potential role of the EPEC translocated intimin receptor (Tir) in host apoptotic events. Apoptosis 8:179–190.

Pallen, M. J., A. C. Lam, N. J. Loman, and A. McBride. 2001. An abundance of bacterial ADP-ribosyltransferases—implications for the origin of exotoxins and their human homologues. Trends Microbiol. 9:302–307.

Pier, G. B., M. Grout, T. Zaidi, G. Meluleni, S. S. Mueschenborn, G. Banting, R. Ratcliff, M. J. Evans, and W. H. Colledge. 1998. *Salmonella typhi* uses CFTR to enter intestinal epithelial cells. Nature 393:79–82.

Rabsch, W., H. L. Andrews, R. A. Kingsley, R. Prager, H. Tschäpe, L. G. Adams, and A. J. Bäumler. 2002. *Salmonella enterica* serotype Typhimurium and its host-adapted variants. Infect. Immun. 70:2249–2255.

Reid, S. D, C. J. Herbelin, A. C. Bumbaugh, R. K. Selander, and T. S. Whittam. 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature 406:64–67.

Sieling, P. A., and R. L. Modlin. 2002. Toll-like receptors: mammalian "taste receptors" for a smorgasbord of microbial invaders. Curr. Opin. Microbiol. 5:70–75.

Smith, R. S., and B. H. Iglewski. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Curr. Opin. Microbiol. 6:56–60.

Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper. 2003. Bacteria-host communication: the language of hormones. Proc. Natl. Acad. Sci. U S A 100:8951–8956.

Sperandio, V., A. G. Torres, J. A. Giron, and J. B. Kaper. 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. J. Bacteriol. 183:5187–5197.

Wagner, H. 2002. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Curr. Opin. Microbiol. 5:62–69.

Wassenaar, T. M., and W. Gaastra. 2001. Bacterial virulence: can we draw the line. FEMS Microbiol Lett. 201:1–7.

Whittam, T. S., and A. C. Bumbaugh. 2002. Inferences from whole-genome sequences of bacterial pathogens. Curr. Opin. Genet. Dev. 12:719–725.

Wilson, M., R. McNab, and B. Henderson. 2002. Bacterial disease mechanisms. Cambridge: Cambridge University Press.

2
Evolution of Bacterial Virulence

P. Boerlin

Animals and humans constitute a suitable environment for many microbial pathogens, but the deleterious effects of colonization and exploitation of the host may ultimately also affect the microorganism itself. Thus, pathogens have evolved 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 suggests that the general evolutionary trend of pathogenic microorganisms would be toward commensalism. High virulence levels would be a sign of recent colonization of a new host by a microbial species. The longer a microorganism persisted and associated with a host species, the less virulent it would become until it reached the climax of commensalisms. 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 consequently 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). However, the reality of infectious disease is still more complex, and this theory is not able to explain many cases of virulence and transmission patterns observed in the field (Levin 1996; Ebert and Bull 2003).

The factors that influence the evolution of virulence are manifold and many remain to be studied. For instance, the exact role of ecology in the encounters and interactions between hosts and pathogens has to be clarified. This is particularly true for opportunistic pathogens, in which virulence may be fortuitous. The influence of host variability 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, 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 are subject to evolutionary constraints (Pfennig 2001). Competition between microorganisms for a host or an ecological niche within this host is another important factor driving the evolution of virulence of some pathogens. Finally, the influence of coevolution of

host populations together with pathogen populations remains an as yet barely studied topic.

In light of all this, 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, including the development of strategies to manipulate the evolution of virulence (Ewald et al. 1998; Ebert and Bull 2003).

POPULATION STRUCTURES AND GENOME PLASTICITY: SEX IN BACTERIA

Three major modes of evolution are available to bacteria in order to maintain variability and adaptability to new environments (Moschhäuser et al. 2000): local sequence change through mutations, DNA rearrangements, and DNA acquisition (horizontal gene transfer, or HGT). These mechanisms seem to be facilitated by the presence of so-called mutator strains defective in their control of DNA replication fidelity in many natural bacterial populations (Rainey 1999; Giraud et al. 2001). Bacterial species vary greatly in their ability to accept foreign DNA and integrate it into their own genome. A broad diversity of genetic behaviors can therefore be observed in natural populations of bacterial pathogens, ranging from completely clonal to panmictic populations (i.e., populations with random exchange and rearrangement of genetic determinants), reminiscent of population structures found in eukaryotes with a sexual reproduction cycle (Spratt and Maiden 1999). Despite this apparent range of evolutionary behaviors, the advances brought in population genetics by the newest molecular biology tools show that bacteria rely mainly on HGT for the major steps in their evolution (Lawrence and Ochman 1998; Fitzgerald and Musser 2001), in particular with regard to the evolution of virulence (Ziehbuhr et al. 1999; Ochman et al. 2000). Mutations and internal rearrangements seem to be rather slow determinants of evolution in bacteria, mostly involved in microevolution and in fine-tuning or in the emergence through pathoadaptative mutations of clones with particular characteristics or distributions rather than in major macroevolutionary steps.

HGT is mediated by three major transfer mechanisms (for a general source of references on this topic, see Syvanen and Kado 2002): transformation (direct uptake of naked DNA by competent bacteria), transduction (infection through a bacteriophage), and conjugation (direct transfer of plasmid or chromosomal DNA between a donor and a recipient bacterium). Examples of all three mechanisms are known or suspected in relation to the transfer of virulence determinants. After transfer, the foreign DNA has first to be stabilized and replicated efficiently, and second to be expressed adequately. Finally, the acquired characteristics have to be fixed in the new bacterial host population. In the case of transformation, the first step is supposed to happen 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, and conjugative transposons 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 to be fixed in a new host than single genes (Lawrence 1997). This leads to the formation of so-called genomic islands (Hacker and Carniel 2001), a phenomenon frequently observed for virulence genes, which are regionally clustered within pathogenicity islands, which are discussed below. Mutations, rearrangements, and recombinations within the genome of the new host (for example, deletion of deleterious genes) are subsequently responsible for fine-tuning the expression of the newly acquired virulence characteristics (Ziehbuhr et al. 1999).

PATHOGENICITY ISLANDS: NOT A PLACE FOR FUN

Pathogenicity islands (PAIs) represent a particular case of genomic islands found in pathogenic bacteria. They were first discovered in uropathogenic and enteropathogenic *Escherichia coli* but have now been described in many other gram-negative and gram–positive pathogens from humans, animals, and plants. These include animal pathogenic species such as diverse categories of pathogenic *E. coli*, *Salmonella enterica*, several *Yersinia* species, *Dichelobacter nodosus*, *Bacteroides fragilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Listeria ivanovii*, and *Clostridium difficile* (Hacker and Kaper 2000). Due to the major role of HGT in the evolution and emergence of virulence, this list is expected to expand rapidly in the future. Diverse

virulence factors are encoded by PAIs, which include 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 (Hacker and Kaper 2000).

Beside specific virulence genes, PAIs usually share characteristics that are also common to other kinds of genomic islands (Hacker and Kaper 2000; table 2.1). Past horizontal transfer is generally evident in the G + C content and codon usage in PAIs, which differ from those of the core genomic DNA of their bacterial host. These differences tend to decrease over time and to totally disappear in very old PAIs. Mosaic structures are often evident in PAIs, showing that they undergo multiple recombination events in the course of the adaptation of bacterial pathogens to their hosts. The exact mechanisms of horizontal transfer of PAIs are not well understood. However, the presence of some bacteriophage integrase genes or their remnants in PAIs, as well as 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. Different levels of stability have been observed among the numerous PAIs known to date. Some PAIs are mobile and relatively plastic, whereas other have only nonfunctional remnants of mobility genes and seem to be subject to less recombination events. Integrase genes, transposase genes, and insertion sequences within PAIs as well as direct repeats at their ends are supposed to allow the 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. Some authors have also suggested that PAIs may stabilize over time in parallel to the adaptation of their functions and of their regulation to their host (Hacker and Kaper 2000). For instance, the chromosomal localization of PAIs may allow their stable replication and partition, thus fixing advantageous characteristics.

Pathogens have found many ways of optimizing the expression of virulence genes from PAIs. In the simplest case, the regulator genes responsible for the expression of virulence factors of a PAI may be located on the PAI. However, they may also sometimes be found at other chromosomal locations or on plasmids. Cross talk between different PAIs have even 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). As for many other horizontally transferred elements, the foreign origin of PAIs is ascertained, but their exact origins have usually not been clearly elucidated.

ILLUSTRATIONS OF VIRULENCE EVOLUTION

Clonal and Parallel Evolution of Virulence in *Salmonella* and *E. 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

Table 2.1. Common Characteristics of Pathogenicity Islands

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

their divergence from *Escherichia* some hundred million years ago, *Salmonella* began sequentially accumulating virulence characteristics through HGT (fig. 2.1). 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 the pathogenicity island SPI-1 and of other invasin and adhesin genes not located on pathogenicity islands). These genes are found in practically all *Salmonella* and were acquired before the two species *Salmonella enterica* and *Salmonella bongori* diverged (Bäumler 1997; Groisman and Ochman 1997; Porwollik et al. 2002). Another pathogenicity island (SPI-5) was acquired during the same period (Mirold et al. 2001) and possibly a third (SPI-3) or some of its constituents (Blanc-Potard et al. 1999).

The *Salmonella enterica* lineage has subsequently acquired virulence factors allowing for the development of systemic infections. 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 pathogenicity island SPI-2 with its factors involved in survival within macrophages seems to have been a major step in this latter direction (Bäumler 1997; Groisman and Ochman 1997; fig. 2.1). *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. This particular lineage further diversified into numerous serovars. Some serovars have developed an increased propensity to cause systemic infections in specific host species. This host adaptation is clearly multifactorial and current investigations show that it is again associated with repeated HGT and recombinations (Bäumler et al. 1998)

In contrast to *Salmonella*, where the successive acquisition of virulence genes and PAIs along a single evolutionary line led to the development of a group of relatively similar pathogens, the evolution of *E. coli* was 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

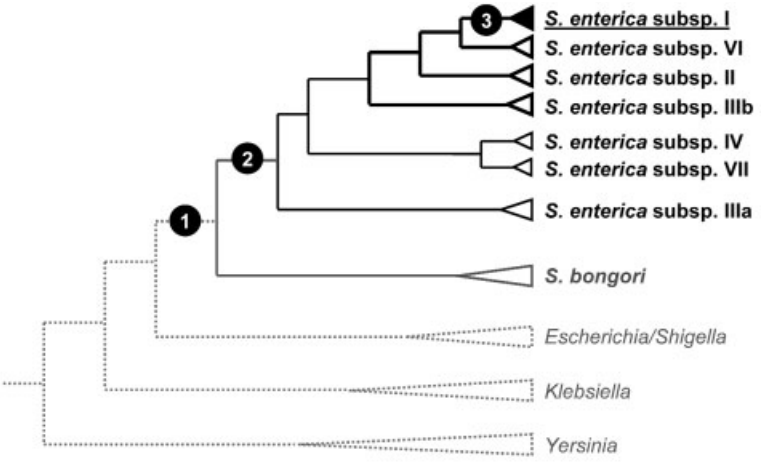


Figure 2.1. Stepwise evolution of virulence in *Salmonella* spp. Schematic representation of the *Salmonella* genus evolution with successive acquisition of virulence characteristics. The length of the branches is not proportional to time. (1) Acquisition of the pathogenicity island SPI-1 and other virulence genes associated with adherence and invasion of intestinal epithelial cells; (2) acquisition of the pathogenicity island SPI-2 and of the ability to survive in macrophages; (3) adaptation to warm-blooded animals. (Figure adapted from Bäumler 1997; Groisman and Ochman 1997; Porwollik et al. 2002)

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 the so-called attaching and effacing (AE) lesions (see chapter 16 and fig. 16.3). 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.

Despite the clonal nature of *E. coli* populations, phylogenetic analysis showed that the distribution of virulence factors of EPEC and EHEC did not entirely fit a simple clonal evolution (Wieler et al. 1997; Reid et al. 2000; Donnenberg and Whittam 2001; fig. 2.2). For instance, The LEE and its variants are found in phylogenetically unrelated *E. coli* lineages, and one suspects that 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, which is discussed later in this section**.** Furthermore, subsequent to the acquisition of the LEE, the EAF plasmid was acquired separately in several lineages, thus leading to the repeated emergence of at least two unrelated EPEC lineages (EPEC 1 and EPEC 2 in fig. 2.2). Finally, the same event seems to have happened to the EHEC plasmid and to 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 in fig. 2.2).

The mobility of the LEE is also supported by its presence in bacterial species other than *E. coli*. Similarly, the presence of plasmids related to the EHEC plasmid in numerous phylogenetically unrelated Shiga-toxin-producing *E. coli* 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 as well as the demonstration of their mobilization *in vitro* and their 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.

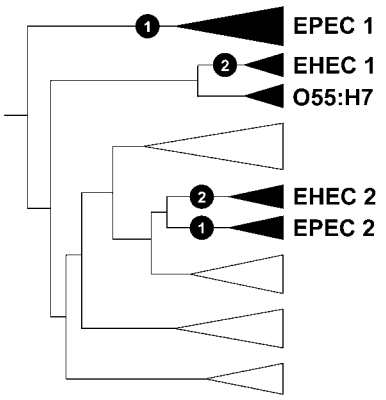


Figure 2.2. Parallel evolution of virulence in EHEC and EPEC. Phylogenetic tree representing the repeated acquisition of virulence factors in *E. coli* populations The length of the branches is not proportional to time. The black triangles represent lineages containing the LEE (the exact time sequence of acquisition of the LEE in the different lineages is unknown). The white triangles represent lineages of nonpathogenic *E. coli* and of pathotypes other than EPEC and EHEC. (1) Repeated independent acquisition of the EAF plasmid in different lineages; (2) repeated independent acquisition of the EHEC plasmid and Stx-phages in different lineages. (Figure adapted from Wieler 1997; Reid et al. 2000; Donneberg and Whittam 2001)

The study of the LEE in EPEC and EHEC is also a good example of the different effects of selection on microevolution of virulence factors and on the emergence of mosaic structures. Intimin, an adherence factor encoded by the LEE, can be divided into three different domains: The N-terminal periplasmic domain, the central transmembrane domain, and the C-terminal extracellular domain. The two former domains appear to be much more conserved than the C-terminus, and intimin clearly shows a mosaic structure (each piece of the mosaic having a different evolutionary history in terms of origin or speed of evolution). 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 the LEE as a whole has 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. A comparison of synonymous and nonsynonymous mutations in the different domains of the intimin gene shows that in order to maintain its functionality, a purifying selection (selection against amino acid substitutions and emergence of new variants) is at work on most of the intimin molecule. However, despite this general purifying selection, diversifying selection (selection in favor of substitutions and the emergence of new variants) seems to affect the C-terminal domain of intimin and to promote amino acid replacements associated with changes of charges in this exposed part of the molecule (Tarr and Whittam 2002). This variability is supposed 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 secreted or surface-exposed virulence factors.

Listeria and Bacilli: Clonal Evolution with a Different Outcome

Listeria are widespread saprophytic inhabitants of soil and decaying vegetation, but two of the six species of this genus (*Listeria monocytogenes* and *Listeria 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 also in the nonpathogenic species *Listeria seeligeri* (Vazquez-Boland et al. 2001). It's interesting that 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. A 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* (fig. 2.3). This distribution would, at a 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 the G + C content and codon usage of LIPI-1 are similar to the one of the core genome of *Listeria* species, thus 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 exact acquisition mechanism is not clear yet but may have involved either phage transduction or movement through

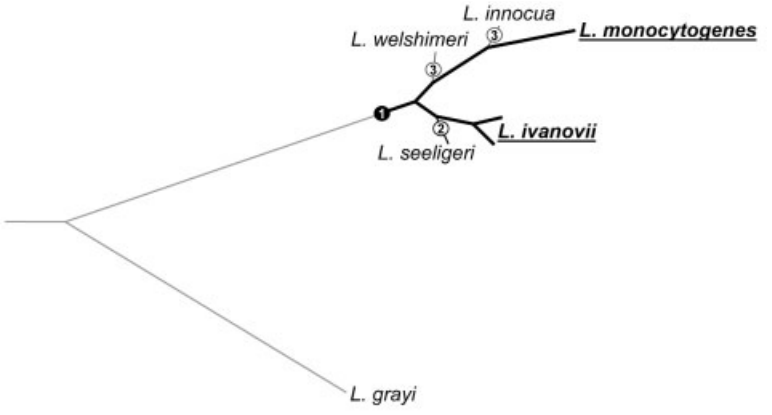


Figure 2.3. Evolution of the pathogenicity islet LIPI-1 in the genus *Listeria*. Phylogenetic tree of the evolution of the genus *Listeria* based on 16S rRNA gene sequences. Only *L. monocytogenes* and *L. ivanovii* are pathogens and carry an active LIPI-1 islet. (1) Acquisition of LIPI–1; (2) inactivation of LIPI-1 expression through insertions; (3) deletion of LIPI-1 in *L. welshimeri* and *L. innocua*. (Figure adapted from Vaneechoutte et al. 1998; Vazquez-Boland et al. 2001)

transposons (Cai and Wiedmann 2001).

LIPI-1 was probably of advantage as a defense against phagocytosis by soil inhabitants and for the colonization of the new ecological niche represented by vertebrates. It was subsequently lost and/or inactivated in the nonpathogenic species for which it was not of selective advantage (Vazquez-Boland et al. 2001). The genus *Listeria* therefore represents a nice 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*.

L. monocytogenes causes a variety of diseases in humans and animals, but all the *L. monocytogenes* strains are apparently equipped with the same arsenal of virulence genes, and no clear difference has been demonstrated to date in the propensity of some strains to cause one or the other kind of clinical disease. However, the frequency of recovery of some clonal lineages from clinical cases or from food or the environment varies drastically. Some clones are particularly prone to cause disease or outbreaks, whereas others seem to be better adapted to particular environments and food but less frequently associated with disease. Thus, similarly to what happened with host adaptation in *S. enterica* subspecies I, a whole range of clonal adaptation to specific ecological niches has developed within a single apparently relatively homogeneous bacterial species like *L. monocytogenes*. This certainly represents a common theme in the evolution of pathogens.

The *Bacillus cereus* complex is a more striking example of such a divergent evolution. This complex is composed of three species with a very different natural history. *B. cereus*, the archetype of the group, is a common environmental bacillus often associated with food poisoning and only 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 outside of its hosts under natural conditions.

Despite their very different behaviors and host ranges, recent phylogenetic investigations have demonstrated that these three bacilli are highly related and should be regarded as pathotypes of a single species (Helgason et al. 2000). These pathotypes are in fact so closely related that they are sometimes difficult to identify in the laboratory. The only major differences between them rely on their different sets of virulence factors and host adaptation mechanisms. Horizontal gene transfer seems to have again played a major role in the development of these behavioral differences. For instance, the virulence of *B. anthracis* is mainly due to factors encoded by its two large plasmids pXO1 and pXO2, which are not found in *B. cereus* and *B. thuringiensis*. The pXO2 structure is not well known, but analysis of the pXO1 plasmid shows the presence of a structure similar to a PAI specific for *B. anthracis* and carrying most of the known virulence genes of this plasmid (Okinaka et al. 1999). In *B. thuringiensis*, the toxin genes responsible for its pathogenicity in insects are apparently mobile and can be transferred from one strain to another. Finally, the emetic toxin and enterotoxin genes of *B. cereus* are found not only in a subset of *B. cereus* strains but also in other bacilli of the cereus group and even in other more distantly related *Bacillus* species. This widespread patchy distribution suggests again mobility of the corresponding virulence genes. Thus, HGT was able to shape three very different pathogens with completely different pathotypes and ecotypes without modifying much of their core genome, up to the point that they were considered until recently as completely different species.

Type III Secretion Systems: A Widespread Communication Tool Among Pathogens and Symbionts

Type III secretion systems (TTSS) 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, *Salmonella enterica*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Bordetella pertusis*, *Aeromonas salmonicida*, and diverse *Chlamydiaceae*) and from plants (*Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris*). TTSS were first thought to be strictly associated with virulence, and certainly represent one of the major and most fascinating virulence mechanisms discovered recently 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 emerging as a widespread communication system between prokaryotic and eukaryotic cells (Foultier et al. 2002).

TTSS generally consist of more than 20 different components forming a needlelike 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 practically similar in all species, but the translocated effector molecules vary drastically from one species to the other, thus leading to very different effects on the host cells and therefore to a broad variety of diseases. The genetic organization of TTSS genes is variable but some blocks of genes are relatively conserved between bacterial species and allow the delineation of phylogenetically based broad families of TTSS (to date at least five). The distribution of these TTSS families does not fit with the general phylogeny of the bacteria, and some species like *S. enterica* even simultaneously contain two TTSS systems of different families (Foultier et al. 2002).

TTSS are usually located on mobile elements like plasmids and phages, or within pathogenicity islands. 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, once again, one suspects that HGT has played a major role in the evolution and spread of TTSS in bacterial populations. TTSS show strong structural and genetic homologies with flagella and are supposed to have derived from these structures by divergent evolution (Hueck 1998).

The exact ancestral origin of TTSS 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 TTSS are found only in some species or even only in some clones within one species (a sign of recent acquisition), they were found in practically all the *Chlamydia* that were investigated. The TTSS 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 TTSS were not acquired recently by *Chlamydia* through one single HGT as in other bacterial species but were present for a very long time in *Chlamydia* and may find their origin in this phylogenetic lineage (Kim 2001).

Sharing: The Art of Saving Resources in Obligate Pathogens

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 to 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 that would be of advantage under other unlikely conditions. Generalist opportunistic pathogens, like *Pseudomonas aeruginosa*, confronted with many different environments will tend to present more variable characteristics and will therefore tend to have a larger genome than organisms restricted to a specific environment or host. Extremely specialized organisms like primary symbionts and obligate 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, and have consequently an even smaller genome. Examples of such genomic reductions have started to emerge with the availability of the first obligate pathogen genomes (for instance, *Mycoplasma* species, *Chlamydia trachomatis*, *Mycobacterium leprae*, *Rickettsia prowazekii*, and *Borrelia burgdorferi*).

Small genomes can be found sporadically in many unrelated bacterial phyla, thus confirming that they 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 microorganisms. This is not the case, and much less 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. 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 (period of small-size population) 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. Such examples are known, for

instance, in the transition of *Yersinia pseudotuberculosis* to *Yersinia pestis* or in the emergence of *Shigella* spp. and enteroinvasive *E. coli* from apathogenic *E. coli*. In both cases, the loss of some metabolic functions was associated with an increase in virulence of the pathogen and to a concomitant bottleneck in its evolution.

CONCLUSION

Despite their comparatively small genome, bacterial pathogens continuously 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 the environment also bring changes in the world of bacterial pathogens. For instance, more and more infectious diseases are becoming of multifactorial and polymicrobial nature, which will certainly lead to unexpected synergies and coevolution mechanisms between the agents involved in such infections. Similarly, the intensive and sometimes indiscriminate use of antimicrobial agents has very strong implications in terms of bacterial evolution.

Bacterial pathogens have already started to counter our defense strategies, for instance by the acquisition of antimicrobial resistance mechanisms. Some pathogens even show linked virulence and resistance determinants on genetically mobile elements. Imprudent use of antimicrobial agents is therefore expected not only to boost the development of antimicrobial resistance, but also to help the spread of virulence determinants in bacterial populations and to speed up the evolution of pathogens. Thus, a better understanding of the rules governing virulence and bacterial evolution may help us to develop additional therapeutic tools and preventive strategies in our constant fight against bacterial pathogens in animals.

REFERENCES

Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis, and F. Heffron. 1999. *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. Mol. Microbiol. 31:971–982.

Bäumler, A. J. 1997. The record of horizontal gene transfer in *Salmonella*. Trends Microbiol. 5:318–22.

Bäumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams. 1998. Evolution of host adaptation in *Salmonella enterica*. Infect. Immun. 66:4579–87.

Blanc-Potard, A. B., F. Solomon, J. Kayse, and E. A. Groisman. 1999. The SPI3 pathogenicity island of *Salmonella enterica*. J. Bacteriol. 181:998–1004.

Cai, S., and M. Wiedmann. 2001. Characterization of the *prfA* virulence gene cluster insertion site in non-hemolytic *Listeria* spp.: probing the evolution of the *Listeria* virulence gene island. Curr. Microbiol. 43:271–7.

Dale, C., G. R. Plague, B. Wang, H. Ochman, and N. A. Moran. 2002. Type III secretion systems and the evolution of mutualistic endosymbiosis. Proc. Natl. Acad. Sci. U S A 99:12397–402.

Dobrindt, U., and J. Hacker. 2001. Whole genome plasticity in pathogenic bacteria. Curr. Opin. Microbiol. 4:550–7.

Donnenberg, M. S., and T. S. Whittam. 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. J. Clin. Invest. 107:539–48.

Ebert, D., and J. J. Bull. 2003. Challenging the trade-off model for the evolution of virulence: is virulence management feasible? Trends Microbiol. 11:15–20.

Ewald, P. W. 1993. The evolution of virulence. Sci. Am. 268:86–93.

Ewald, P. W., J. B. Sussman, M. T. Distler, C. Libel, W. P. Chammas, V. J. Dirita, C. A. Salles, A. C. Vicente, I. Heitmann, and F. Cabello. 1998. Evolutionary control of infectious disease: prospects for vectorborne and waterborne pathogens. Mem. Inst. Oswaldo Cruz 93:567–76.

Fitzgerald, J. R., and J. M. Musser. 2001. Evolutionnary genomics of pathogenic bacteria. Trends Microbiol. 9:547–553.

Foultier, B., P. Troisfontaines, S. Muller, F. R. Opperdoes, and G. R. Cornelis. 2002. Characterization of the *ysa* pathogenicity locus in the chromosome of *Yersinia enterocolitica* and phylogeny analysis of type III secretion systems. J. Mol. Evol. 55:37–51.

Giraud, A., M. Radman, I. Matic, and F. Taddei. 2001. The rise and fall of mutator bacteria. Curr. Opin. Microbiol. 4:582–5.

Groisman, E. A., and H. Ochman. 1997. How *Salmonella* became a pathogen. Trends Microbiol. 5:343–9.

Hacker, J., and E. Carniel. 2001. Ecological fitness, genomic islands and bacterial pathogenicity. EMBO Rep. 2:376–381.

Hacker, J., and J. B. Kaper. 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. 54:641–79.

Helgason, E., O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and

Kolsto. 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66:2627–30.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62:379–433.

Kim, J. F. 2001. Revisiting the chlamydial type III protein secretion system: clues to the origin of type III protein secretion. Trends Genet. 17:65–9.

Lawrence, J. G. 1997. Selfish operons and speciation by gene transfer. Trends Microbiol. 5:355–9.

Lawrence, J. G., and H. Ochman. 1998. Molecular archeology of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. U S A. 95:9413–9417.

Levin, B. R. 1996. The evolution and maintenance of virulence in microparasites. Emerg. Infect. Dis. 2:93–102.

McGraw, E. A., J. Li., R. K. Selander, and T. S. Whittam. 1999. Molecular evolution and mosaic structure of α, β, and γ intimins of pathogenic *Escherichia coli*. Mol. Biol. Evol. 16:12–22

Mirold, S., K. Ehrbar, A. Weissmüller, R. Prager, H. Tschäpe, H. Rüssmann, and W. Hardt. 2001. *Salmonella* host cell invasion emerged by acquisition of a mosaic of separate genetic elements, including *Salmonella* pathogenicity island 1 (SPI1), SPI5, and *sopE2*. J. Bacteriol. 183:2348–2358.

Moran, N. A. 2002. Microbial minimalism: genome reduction in bacterial pathogens. Cell 108:583–586.

Moschhäuser, J., G. Köhler, W. Ziebuhr, G. Blum-Oehler, U. Dobrindt, and J. Hacker. 2000. Evolution of microbial pathogens. Phil. Trans. R. Soc. Lond. B. 355:695–704.

Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304.

Okinaka, R. T., K. Cloud, O. Hampton, A. R. Hoffmaster, K. K. Hill, P. Keim, T. M. Koehler, G. Lamke, S. Kumano, J. Mahillon, D. Manter, Y. Martinez, D. Ricke, R. Svensson, and P. J. Jackson. 1999. Sequence and organization of pOX1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin gene. J. Bacteriol. 181:6509–6515.

Pfennig, K. S. 2001. Evolution of pathogen virulence: the role of variation in host phenotype. Proc. R. Soc. Lond. B Biol. Sci. 268:755–760.

Porwollik, S., R. M. Wong, and M. McClelland. 2002. Evolutionary genomics of *Salmonella*: gene acquisitions revealed by microarray analysis. Proc. Natl. Acad. Sci. U.S.A. 99:8956–61.

Rainey, P. B. 1999. Evolutionary genetics: the economics of mutation. Curr. Biol. 9:R371–R373.

Reid, S. D., C. J. Herbelin, A. C. Bumbaugh, R. K. Selander, and T. S. Whittam. 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature 406:64–67.

Spratt, B. G., and M. C. Maiden. 1999. Bacterial population genetics, evolution and epidemiology. Philos. Trans. R. Soc. Lond. B Biol. Sci. 354:701–710.

Syvanen, M., and C. I. Kado. 2002. *Horizontal gene transfer*. San Diego: Academic Press.

Tarr, C. L., and T. S. Whittam. 2002. Molecular evolution of the intimin gene in O111 clones of pathogenic *Escherichia coli*. J. Bacteriol. 184:479–487.

Vaneechoutte, M., P. Boerlin, H. V. Tichy, E. Bannerman, B. Jäger, and J. Bille. 1998. Comparison of PCR-based DNA fingerprinting techniques for the identification of *Listeria* species and their use for atypical *Listeria* isolates. Intern. J. System. Bacteriol. 48:127–139

Vazquez-Boland, J. A., G. Dominguez-Bernal, B. Gonzalez-Zorn, J. Kreft, and W. Goebel. 2001. Pathogenicity islands and virulence evolution in *Listeria*. Microbes Infect. 3:571–584.

Wieler, L. H., T. K. McDaniel, T. S. Whittam, and J. B. Kaper. 1997. Insertion site of the locus of enterocyte effacement in enteropathogenic and enterohemorrhagic *Escherichia coli* differs in relation to the clonal phylogeny of the strains. FEMS Microbiol. Lett. 156:49–53.

Woolhouse, M. E., L. H. Taylor, and D. T. Haydon. 2001. Population biology of multihost pathogens. Science 292:1109–1112.

Ziehbuhr, W., K. Ohlsen, H. Karch, T. Korhonen, and J. Hacker. 1999. Evolution of bacterial pathogenesis. CMLS Cell. Mol. Life Sci. 56:719–728.

3
Streptococcus

J. F. Timoney

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 that may consist of 50 or more attached cells. Most streptococci are facultatively anaerobic, catalase negative, non-spore-forming, and nonmotile. Their nutritional requirements are complex and variable, reflecting adaptation as commensals or parasites of a wide variety of vertebrates. Identification is based on a combination of characteristics including hemolytic properties, carbohydrate and protein antigen composition, fermentation and other biochemical reactions, phenotypic and growth characteristics, multilocus enzyme electropherotype, and DNA sequence including 16S rRNA gene sequences (Facklam 2002).

The majority of the pathogenic streptococci possess a dominant serologically active carbohydrate that is antigenically different from one species or group of species to another. These cell wall antigens, the basis of the Lancefield grouping system, are widely used by clinical laboratories in a variety of methodologies for serogrouping an unknown isolate. These group-specific antigens, designated A–H and K–V, are readily extracted from cell walls by autoclaving, formamide treatment, or enzymic digestion. Groups B, C, D, E, G, L, U, and V contain the pyogenic animal streptococci that cause mastitis in cattle and other hosts; strangles in horses; meningoencephalitis, arthritis, and cervical lymphadenitis in swine; neonatal septicemias in kittens; and lymphadenitis in juvenile cats and laboratory rodents. Some pathogenic streptococci, notably *S. uberis*, *S. parauberis*, and *S. 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 3.1. With the exception of *S. pneumoniae* and *S. suis*, the pathogens listed are often termed the "pyogenic streptococci" because of their association with pus and purulence. In general, virulence of the pathogenic streptococci is based on surface structures that directly or indirectly impede phagocytosis or are involved in adhesion. The best-understood streptococcal virulence factors are the hyaluronic acid capsule and the antiphagocytic M proteins. However, other molecules, which include streptolysins, proteases, leukocidal toxins, streptokinase, and possibly plasmin receptors found on the surface or secreted, also contribute to lesion development. In addition, most pathogenic streptococci have the ability to bind components of the host's plasma, such as albumin, immunoglobulins, and fibrinogen. Organisms coated with one or more of these components may be able to evade host defenses either by escaping detection or by blocking deposition of opsonic components of complement.

STREPTOCOCCUS AGALACTIAE

Streptococcus agalactiae, the lone member of 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. In humans, it causes neonatal septicemia and meningitis. Human and bovine populations of *S. agalactiae* are not identical, and epidemiologic evidence does not support animal to human transmission. Salicin and lactose fermentation and bacteriocin and bacteriophage typing differentiate populations from humans and cattle (Finch and Martin 1984).

The polysaccharide capsule includes the specific antigenic types Ia, Ib, Ic, II, and III. Type Ic is often associated with a C protein antigen. Type Ia

Table 3.1. Pathogenic Streptococci of Animals

Species	Lancefield group	Virulence factors	Disease
S. agalactiae	B	Capsular polysaccharide; C, R and X proteins; CAMP factor; hyaluronidase; lipoteichoic acid; proteases; collagenase; β hemolysin; C5a peptidase	Mastitis
S. dysgalactiae subsp. *dysgalactiae*	C	Hyaluronidase; streptokinase; fibronectin-binding proteins fnb A and B; protein G; plasminogen receptor; streptodornase; M-like proteins; L2 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; streptolysin S; pyrogenic exotoxins SePE-H and I; peptidoglycan; fibronectin-binding protein; proteases; adhesins; streptokinase; SzPSe protein	Equine strangles
S. zooepidemicus	C	Capsular hyaluronic acid; streptokinase; proteases; streptolysin S; peptidoglycan; SzP protein; fibronectin binding protein; IgG binding protein	Opportunist pyogen; pneumonia; metritis; joint-ill
S. suis	D	Capsule; MRP and EF proteins; suilysin; adhesins.	Meningo encephalitis; 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. pneumoniae	—	Capsule; neuraminidases; NanA, B; peptidoglycan; teichoic acid; IgA protease; adhesins; fibronectin binding proteins	Equine respiratory infection

constitutes about 70% of bovine isolates in New York State, but different types may be numerous in other geographic areas (Norcross and Oliver 1976). About 25% of *S. agalactiae* strains are untypeable.

S. 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 mamma-

ry infection followed by treatment or culling of these animals. Calves that feed on mastitic milk may transmit infection by suckling penmates' immature teats. In humans, *S. agalactiae* is a commensal of the gastrointestinal tract.

Virulence Factors

Much of the information on potential virulence factors of *S. agalactiae* has been derived from studies on human isolates, and therefore must be cautiously interpreted in the context of bovine mastitis. The capsular polysaccharide, including the polysaccharide type-specific antigen, is antiphagocytic, and antibodies to these type-specific antigens are protective in mice (Lancefield et al. 1975). Type-specific antibody is also important in resistance of human infants to group B streptococcal infection.

Type III capsular polysaccharide has a terminal sialic acid residue that inhibits activation of the alternate complement pathway and prevents deposition of C3 on the bacterial surface. The capsule increases the affinity of factor H for C3b bound to the surface of the cell wall and thereby reduces both the activity of C3 convertase and further deposition of C3b on the cell (Marques et al. 1992).

Bovine strains express less capsular polysaccharide on their surfaces and thus are capable of activating the alternate pathway. Moreover, bovine antibodies to the group B polysaccharide antigen, although not opsonic by themselves, are able to fix complement and C3 via the classical pathway, and opsonization is thereby effected (Rainard and Boulard 1992).

A diverse and large number of proteins is expressed on the surface of *S. agalactiae,* which function in adhesion, invasion, binding lactoferrin, enzymic processes, and inhibition of phagocytosis. Some of these proteins (β) are trypsin resistant (e.g., R1, R3, R4), others (α) are not and include the C, X, BPS, and Sip proteins. The C or alpha C proteins cluster in molecular mass at about 100 kDa and contain a series of long tandem repeats. They probably function in an early stage of pathogenesis. The C protein found on all type Ib, 60% of type II, and occasional type III strains has also been shown to elicit protective, opsonophagocytic antibodies (Ferrieri 1988). The mode of action of this virulence factor is not known. The alpha component is responsible for the virulence effect of the C proteins.

R antigens are trypsin-resistant proteins found on filamentous protrusions from the cell surface of *S. agalactiae* (Wagner et al. 1982). Strains expressing R proteins do not express C protein and vice versa. Clinical studies and mouse protection experiments with type III strains of *S. agalactiae* indicate that R proteins contribute to virulence, possibly because they enhance colonization of epithelia, although not by promoting adhesion (Kurl et al. 1984).

Another surface protein antigen of about 100 kDa, termed X, that occurs on many untypeable bovine strains of *S. agalactiae* from cases of bovine mastitis, is of unknown significance in pathogenesis (Rainard et al. 1991). This antigen is opsonic and apparently different from the cell surface Sas 97/104 protein (Wanger and Dunny 1987; Rainard et al. 1994), which is immunodominant for the bovine and found on about 50% of bovine strains. Its presence or absence does not affect bacterial virulence in a guinea pig model.

A serine protease, EspA, 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 53 kDa Sip protein is exposed on the polar surfaces of all serovars of *S. agalactiae* (Brodeur et al. 2000). 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.

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 this protein 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 the mouse LD_{50} by a factor of 50 (Hollingshead et al. 1989). The 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 lipoteichoic acid.

S. agalactiae enters through the end of the teat, 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 host barrier to physical penetration of the epithelial lining. Multiplication of the invading streptococcus is controlled by a combination of the lactoperoxidase-thiocyanante-H_2O_2 system, by lysozyme, and by the flushing action of milk at milking. Adherence and multiplication of the organism on the epithelium of the teat and duct sinuses result 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 a 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 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 (Yokomizo and Norcross 1978). Serum antibodies appear to have little or no protective effect. The presence of agglutinins in milk of infected cows together with failure of the infection to be cleared naturally from the udder of most infected cattle suggest that active immune responses are ineffective in clearance of infection. However, immunoglobulins specific for the group B 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 antigenicity of group B polysaccharide is greatly increased by conjugation to ovalbumin. Immunization of cows with conjugate produced strong group B polysaccharide serum IgG1 and IgG2 responses (Rainard 1992). Other approaches to immunization of cattle include the use of surface dehydrogenase protein (plasma receptor, Gap C) 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 (Vierra et al. 1998; Facklam 2002), based on genotypic and phenotypic characterization establishes the subspecies *dysgalactiae* and *equisimilis*. Strains in subspecies *dysgalactiae* are group C only and alpha hemolytic, and are found mainly as a cause of acute and subclinical mastitis in cattle. Strains in subspecies *equisimilis* are beta hemolytic, 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, α2 macroglobulin receptor, plasminogen receptor and M-like proteins, which bind IgG, fibronectin, α2 macroglobulin, plasminogen, and fibrinogen, respectively (Vasi et al. 2000). Secreted proteins include streptokinase, streptolysin O or S, streptodornase (DNase), and hyaluronidase.

The roles of these proteins in 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. Release of hyaluronidase and fibrinolysin may be of value in tissue penetration and dissemination (Calvinho et al. 1998). Infections of the mammary gland are usually asso-

ciated 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.

S. dysgalactiae subsp. *equisimilis* include 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 the sow in which the organism is carried in the tonsil. The piglet is invaded via wounds, umbilicus, or tonsil, and develops a 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 and from aborted placentas. The streptokinases of subsp. *equisimilis* show strict species specific plasminogen activation, that is, porcine isolates activate porcine but not equine plasminogen (McCoy et al. 1991).

STREPTOCOCCUS EQUI

S. 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 appear to constitute a clone or biovar of the closely related *S. zooepidemicus*. This conclusion is supported by multienzyme electrophoresis and 16S sRNA interspacer sequence studies (Chanter et al. 1997), which confirm the genetic homogeneity of different isolates. Nevertheless, polymorphism in DNA restriction fragments suggests there is some limited genomic sequence variability (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 *S. equi* is possibly dependent on a specific phenotype that, when altered, results in loss of that variant. Recent sequence analysis has shown a much higher frequency of homologues of the IS*861* insertion sequence in *S. equi* than in *S. zooepidemicus,* which suggests that replication of this insertion element may have played a role in the emergence of the clonal *S. equi* from a putative ancestral *S. zooepidemicus*.

S. equi is highly host adapted so that its survival depends on the presence of horse, donkey, or mule populations. Survival in soil or on pasture is very brief, but may extend to a few days in water. In the absence of competing microflora, survival times of 7–9 weeks have been recorded on sterilized wood or glass. The usual source of *S. equi* is therefore a nasal discharge or pus from an abscess, or feed or water directly contaminated by a shedding animal. Clinically inapparent carrier animals, although infrequent, may be associated with persisting infection in resident horse populations as vehicles of transfer to previously uninfected premises. 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 onset of the typical disease is marked by fever, lassitude, nasal discharge, slight cough, difficulty in swallowing, and swelling of the intermandibular areas, with tenderness and swelling of the mandibular lymph node. Pressure of the enlarging retropharyngeal nodes on the airway may cause respiratory difficulty and is the source of the common name of the disease. Metastasis of organisms 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 an atypical or catarrhal form of the disease with slight nasal discharge, cough, slight fever, and abscessation of lymph nodes in some cases. The mild form of the disease is often seen in older animals with preexisting antibody and experiencing a second infection. Strains of *S. equi* expressing bacteriophage-encoded hyaluronidase have also been associated with a clinically mild form of strangles.

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

Virulence Factors

The virulence factors of *S. equi* include a nonantigenic hyaluronic acid capsule, hyaluronidase, streptolysin S, streptokinase, IgG Fc-receptor proteins, pyrogenic exotoxins including SePE-I and H, peptidoglycan, and the antiphagocytic M protein (SeM). There is also circumstantial evidence for the production of a leukocidal toxin.

The hyaluronic acid capsule is a high-molecular weight polymer consisting of alternating residues of

N-acetylglucosamine and glucuronic acid. Isolates of *S. equi* from cases of strangles are almost always highly encapsulated and produce very mucoid colonies. Nonencapsulated mutants of *S. equi* are much less avirulent for mice and horses (Timoney and Galán 1985; Anzai et al. 1999b). The antiphagocytic capsule greatly reduces the numbers of streptococci that become associated with the surface of neutrophils and are subsequently ingested and killed. Capsular hyaluronic acid increases the negative charge and hydrophilicity of the bacterial surface and produces a localized reducing environment that protects the activity of oxygen–labile proteases or toxins such as streptolysin S. Capsule is also required for the functionality of SeM and possibly other surface exposed hydrophobic proteins. In the absence of the hydrophilic capsule, these proteins aggregate, losing the three-dimensional conformation essential for functionality. Thus, nonencapsulated *S. equi* expressing normal amounts of the antiphagocytic SeM are efficiently phagocytosed.

Streptokinase released by *S. equi* interacts with the C-terminal serine protease domain of equine plasminogen to form active plasmin, which hydrolyses fibrin (McCoy et al. 1991). A role for plasmin in pathogenesis has not been proven but its lytic action on fibrin may aid in spread and dispersion of the bacteria in tissue. Other possible roles include in situ activation of complement and production of low-molecular-weight nitrogenous substrates for bacterial growth. A receptor for plasmin also occurs on the surface of *S. equi*.

The oxygen stable 36 amino acid oligopeptide, streptolysin S, is responsible for the beta hemolysis produced by *S. equi* (Flanagan et al. 1998). Production of this bacteriocin-like cytotoxin is encoded by a nine-gene locus, and biologic activity requires stabilization by association with a carrier molecule such as double-stranded RNA or albumin. Binding of the streptolysin S complex to erythrocytes results in formation of a transmembrane pore and irreversible osmotic lysis of the cell, a process similar to complement-mediated hemolysis (Carr et al. 2001). Damage to keratinocytes has also been noted. 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 action of the toxin appears to be on 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.

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 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 leukocytes.

As many as four pyrogenic mitogens (superantigens)—SePE-H, SePE-I, SePE-M, and SePE-G—are expressed by *S. equi*. Genes for these pyrogenic exotoxins were probably acquired by phage-mediated transfer, an event that may have been important in formation of the clonal more virulent *S. equi* from its putative *S. zooepidemicus* ancestor (Anzai et al. 1999a; Artiushin et al. 2002). Unlike conventional antigens, 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, proinflammatory 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* carries numerous proteins anchored by their carboxy-termini (LPXTG motif), by their N termini (LYXC motif), or by other physicochemical interactions. The functions and roles of most of these 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 marked tendency to be clustered in loci suggesting coregulation or enbloc acquisition. For instance, the gene for SzPSe, an M-like surface protein, is clustered with *Se73.9*, *Se51.9*, *Se46.8*, *Se44.2*, and *Se30.0*, genes

for surface-anchored proteins and a sortase (Qin et al. 2003).

Binding of host plasma proteins to the surface of the whole organism could be an effective mode of concealment from host cellular recognition mechanisms. The bound proteins might also block access of C3 or specific antibody to target sites on the organism. IgG-binding proteins have been implicated in molecular mimicry and environmental sensing activities (Cleary and Retnoningrum 1994).

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 is about 50–60 nm long, 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-terminus, which is anchored in the cell wall (fig. 3.1). The M protein (SeM) of *S. equi* has a molecular mass of about 58 kDa. The main fragments in acid extracts have molecular masses of 46 kDa, 41 kDa, and 29–30 kDa.

The antiphagocytic action is due to binding of fibrinogen to the N-terminal half of SeM and IgG to the central region (Boschwitz and Timoney 1994b; 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 *S. pyogenes* or *S. zooepidemicus*, the M protein of *S. equi* is highly conserved, and with the exception of isolates from persistent guttural pouch carriers shows no variation in size or antigenicity (Galán and Timoney 1987). 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). Isolates with truncated SeM proteins were more susceptible to phagocytosis, but their virulence in the horse 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.

S. equi enters via the mouth or nose and attaches to cells in the crypt of the tonsil and adjacent lymphoid nodules (fig. 3.2). The mechanism and ligands responsible for binding are unknown. Several fibronectin-binding proteins have been characterized on *S. pyogenes* that mediate attachment and invasion of host cells in concert with αβ integrins. FNZ, a fibronectin-binding protein produced by *S. zooepidemicus*, is also produced by *S. equi* but without a C-terminal anchor, so may not be functional (Lindmark et al. 1996). After a few hours, the organism is difficult to detect on the mucosal surface because it is translocated below the mucosa into the local lymphatics where it may be found in one or more of the lymph nodes that drain the pharyngeal/tonsillar region. Complement-derived chemotactic factors generated after interaction of C1 with bacterial peptidoglycan attract large numbers of PMNs (Mukhtar and Timoney 1988). The lack of efficacy of PMNs in phagocytosing and killing the

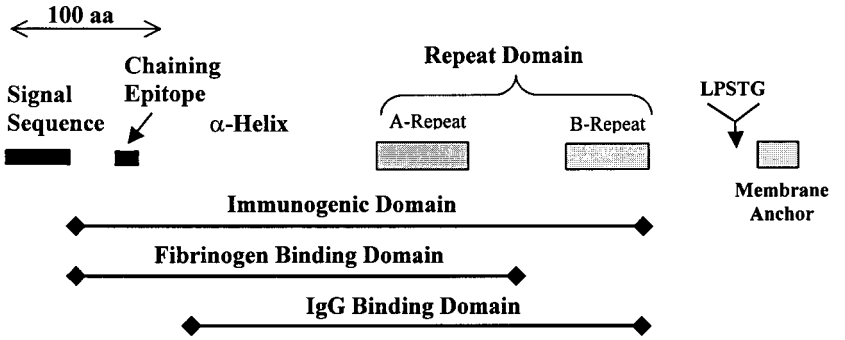


Figure 3.1. Diagram showing the structural and functional features of the antiphagocytic SeM protein of *Streptococcus equi*. The chaining epitope is the binding site for antibody that inhibits separation of dividing cells. Organisms replicating in the presence of this antibody form very long chains.

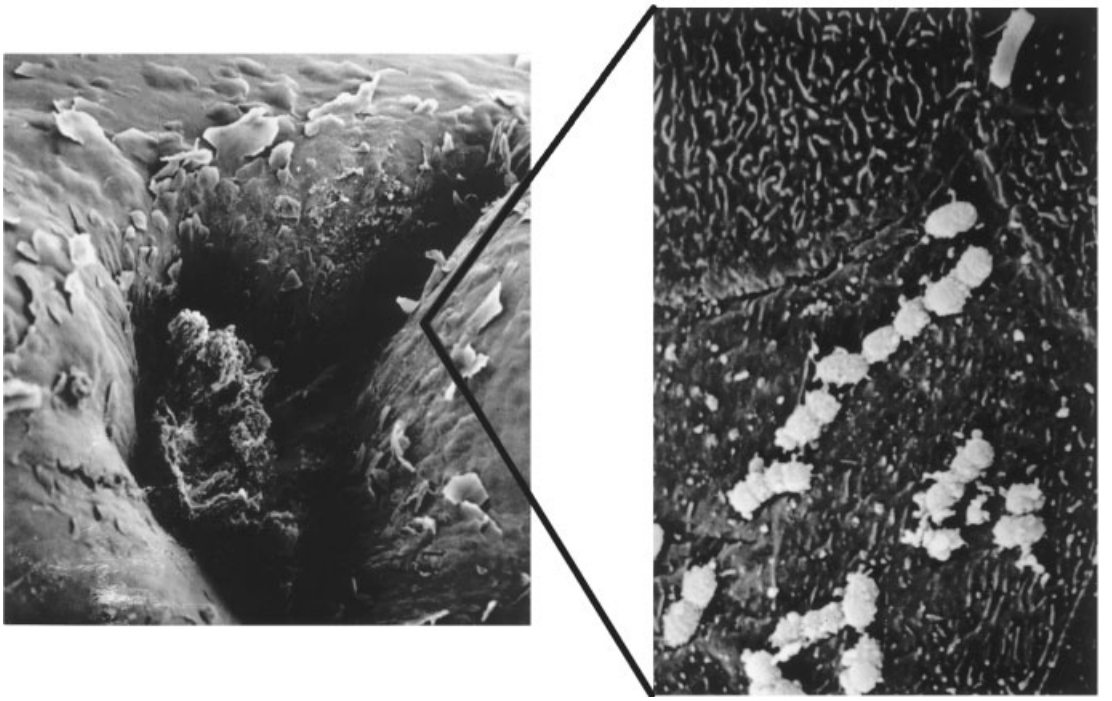


Figure 3.2. This scanning electron micrograph shows a typical crypt in the palatine tonsil of a horse and (inset) short chains of *Streptococcus equi* subsp. *equi* adherent to a crypt epithelial cell.

streptococci appears to be the result of a combination of the hyaluronic acid capsule, antiphagocytic M protein, and a leukocidal toxin released by the organism. This culminates in accumulation of many extracellular streptococci in the form of long chains and large numbers of degenerating PMNs (fig. 3.3). Final disposal of these organisms is dependent on lysis of the abscess capsule and evacuation of its contents.

Streptolysin S and streptokinase may also contribute to abscess development and lysis by 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 in horses inoculated intranasally with *S. equi* and in noninoculated contact horses that became infected. Blood cultures were more likely to be positive on days 6 to 12 following inoculation. These interesting findings have not been confirmed but clearly show the potential for localization of *S. equi* in body sites other than the lymph nodes of the head and neck and for the formation of circulating immune complexes.

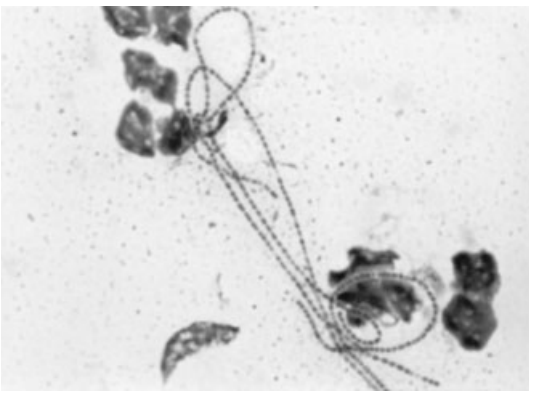


Figure 3.3. These streptococci show long chains and large numbers of degenerating PMNs typical of extracellular growth in the absence of opsonizing antibodies.

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 for months after clinical recovery and be a source of contagion for other susceptible horses (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). Horses in the immediate postconvalescent 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 surface-exposed proteins including SeM, Se44.2, Se46.8, Se45.5, and Se42.0 are produced during convalescence. Opsonophagocytic serum IgGb specific for the highly immunogenic SeM appears late in convalescence in some but not in all horses (Timoney and Eggers 1985). In addition, SeM-specific IgGa is induced during and shortly after *S. equi* infection. There are strong SeM-specific mucosal IgA and IgGb responses during the acute and convalescent phases but not following intramuscular vaccination (Sheoran et al. 1997).

Mucosal and systemic (serum) antibody responses are independent and local mucosal responses require local stimulation. Anamnestic mucosal responses are elicited following reinfection and contribute to protection against a second or further occurrence of disease. The mode of action of locally produced SeM-specific antibody is not known. Mucosal antibodies may prevent adhesion to receptors on tonsillar cells or prevent interiorization of the organism after it adheres.

Milk from mares that have recovered from strangles contains IgGb and IgA with specificities similar to those found in nasopharyngeal mucus of convalescent horses. Suckling foals therefore benefit from the protective effects of this antibody until weaned. Colostral antibodies ingested during the first 24 hours of life have also been shown to recirculate to the nasopharyngeal mucosa, thus providing an additional source of protection to the foal during its first weeks. Foals that suckle immune mares are usually resistant to *S. equi* infection until weaning.

The antiphagocytic SeM is widely believed to be a major protective antigen of *S. equi*. This heat- and acid-resistant protein antigen is protective for mice (Timoney and Trachman 1985) and is an important component of commercial strangles vaccines. However, a SeM-negative mutant of *S. equi* protected horses against experimental challenge following intranasal vaccination (Timoney et al. 2000). Thus, antigens other than SeM are involved in protective responses.

Vaccines containing either the acid-extracted or enzymatically extracted M protein are very effective in stimulating serum opsonic bactericidal antibodies when administered in a course of two inoculations, but have given a disappointing performance 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. Since this study was terminated 16 days after the third vaccination, conclusions as to longer term protection cannot be made.

Substantial bactericidal (opsonic) activity has been noted in sera of vaccinated yearlings that subsequently developed strangles within a month or two of the date of collection of the sera. This suggests that opsonic activity must be complemented by antibodies to other protective antigens (Timoney and Eggers 1985) and is consistent with Bazely's observations many years ago that a heat labile component is an essential component of an effective vaccine.

Given the complex multiphasic pathogenesis of strangles involving adhesion and penetration of tonsillar epithelium followed by unrestricted bacterial extracellular multiplication in lymph nodes despite the presence of massive numbers of neutrophils, it is hardly surprising that protective immunity should be correspondingly complex.

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 following strangles, exhibited a high level of protective efficacy in experimental ponies (Timoney and Galán 1985). Safety issues recognized since use of the live intranasal vaccine has become widespread include residual virulence for some vaccinates and abscesses at injection sites of other vaccines.

STREPTOCOCCUS ZOOEPIDEMICUS

Although *S. zooepidemicus* shares very high DNA homology with its clonal derivative *S. equi*, the

species differ greatly in their 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 (Hoffman et al. 1993), and uterus. In situations of concurrent influenza virus infection, high summer temperature, or transport stress, it can be a devastating and rapidly fatal pathogen in the respiratory tract.

S. zooepidemicus is also a serious zoonotic pathogen of humans who may become infected by exposure to contaminated milk products or by contact with horses.

Virulence Factors

S. zooepidemicus produces many of the virulence factors described for *S. equi*. As expected from the greater than 98% DNA homology shared by these species, their protein profiles are almost identical including expression of immunoglobulins and fibronectin-binding proteins (Jonsson et al. 1995; Lindmark and Guss 1999). Notable differences are the lack of the antiphagocytic SeM and the pyrogenic exotoxins SePE-I and H as well as homologues of two or three other surface-exposed or secreted proteins. Capsule synthesis is highly variable in *S. zooepidemicus* and is usually quickly lost following primary culture. Therefore, synthesis is highly regulated, unlike *S. equi* in which expression is constitutive. Isolates of *S. zooepidemicus* from the tonsil and other mucosal sites of healthy animals are almost always unencapsulated. Toxigenic strains have been implicated in outbreaks of septicemia in greyhounds but have not been tested for pyrogenic exotoxins (Sundberg et al. 1981). Paradoxically, hyaluronidase is produced by some strains. It is questionable whether hyaluronidase should be regarded as a virulence factor since it should render the organism more sensitive to phagocytosis by removing its capsule.

The M-like 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 and opsonogenic, and are found on isolates from different animal hosts. They bind fibrinogen but less so 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 (Timoney et al. 1997).

The severe necrotizing lesions seen in transit pneumonia in adult horses are suggestive of protease release by the invading clone (Oikawa et al. 1994).

Immunity

Since serum antibodies to the SzP proteins of *S. zooepidemicus* are opsonic (Causey et al. 1995) and mouse protective, it is likely they contribute to protection in the horse and other animals. The SzP-specific IgA and IgG in the nasopharyngeal secretions of horses may have a role in controlling numbers of *S. zooepidemicus* in the tonsil. 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 and vaccination regimens will be difficult to develop. An added concern is the risk of glomerulonephritis associated with complexes of streptococcal proteins such as SzP and specific antibody (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, beta-hemolytic streptococci from animals that react with group G typing antiserum and produce beta-galactosidase but not hyaluronidase. They are thereby distinguished from strains of *S. dysgalactiae* subsp. *equisimilis* of human origin that react with group G antiserum. Thus, group G streptococci from humans and animals represent genetically distinct and separate populations (Simpson et al. 1987), although animal strains are occasionally isolated from lesions in humans.

The anal mucosa is the main carrier site of *S. canis* in the dog. The organism is also found on the genitals, groin, chin, tonsil, mouth, and nose of both dogs and cats. Infections in animals are usually sporadic and opportunistic and found in wounds, rectum, mammary gland, prostate, lymph nodes, 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, suggesting that the virulent clone involved did not persist. 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, suggesting that the outbreak was not the result of emergence of a virulent clone (DeWinter and Prescott 1999). Possible explanations of the Ontario epizootic include induction of superantigen expression by concurrent exposure to fluoroquinolones. Subsequent work identified the bacteriophage-inducing effect of a fluoroquinolone for an isolate of *S. canis* and the presence of a homologue of pokeweed mitogen on the lytic bacteriophage induced (Ingrey et al. 2003). Expression of the mitogen was demonstrated, and it was speculated that this might lead to superantigen-like effects leading to toxic shock and necrotizing fasciitis (Ingrey et al. 2003).

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).

Virulence Factors

Very little has been reported on the virulence factors of *S. canis*. Although hyaluronidase, streptokinase, or hyaluronic acid have not been detected, streptolysin O and M protein are produced in large quantity by recent clinical isolates. In contrast, strains isolated from normal mucosal sites express very little detectable M protein. The M protein of a virulent *S. canis* clone from an epizootic of neonatal septicemia and mandibular lymphadenitis of juvenile cats had 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 phagocytosed equally well by preimmune and hyperimmune serum to the M protein, suggesting either that an antigenically different M protein was present or that the M protein was absent or 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 (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 in a week or so after 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). Opsonic antibodies that neutralize the antiphagocytic effects of the M protein are protective. These antibodies develop as a result of exposure to *S. canis* and so older cats tend to have higher opsonic titers. Older queens show much lower vaginal colonization rates and organisms are fewer in those queens that are colonized. The role of locally produced mucosal antibodies in colonization resistance has not been elucidated.

STREPTOCOCCUS SUIS

S. suis of Lancefield group D is associated with a wide range of clinical syndromes in swine and other domestic animals and is a serious zoonotic infection of humans. The principal carrier site in pigs is the palatine tonsil and transmission is by the respiratory and oral routes. Although many pigs are tonsillar carriers, most do not develop clinical disease. Tonsillar infection may occur shortly after birth and

a range of serotypes based on capsular antigens are found. The well-studied 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.

Association with, and entry to, lingual and palatine tonsil are followed by an innate immune response involving neutrophils, macrophages, and epithelial cells together with responses of B and CD4+ and CD8+ lymphocytes. These responses usually limit the infection to the tonsil where the organism persists.

The events that lead to invasion of the blood stream have not been worked out but are often associated with stress, which putatively compromises local immune-mediated control of multiplication of *S. suis* or results in expression of virulence factors that enhance the organism's ability to evade intracellular killing and invade the mandibular lymph nodes. 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 development of meningoencephalitis. It is unclear whether during the bacteremic phase *S. suis* is attached to or within phagocytes or is free in the plasma. Earlier studies (Williams 1990) indicated that an essential feature of pathogenesis was the ability of *S. suis* to survive in circulating mononuclear cells, which carried it "Trojan-horse style" to joints and the meninges. Recent studies using murine macrophages indicated the association includes surface attachment (Segura and Gottschalk 2002). Since it is likely that some surface-associated bacteria become internalized, Williams's conclusion may still be valid. Once the ependyma is breached, entry to the central nervous system via the choroid plexus with spread to the cerebrospinal fluid ensues. Recent observations indicate that *S. suis* adheres to but does not invade endothelial cells of the brain microvasculature (Gottschalk and Segura 2000). The hemolytic toxin suilysin release may then damage these cells thereby facilitating translocation into adjacent tissue. In addition, proinflammatory 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

S. 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 the route of entry and immune status of the host 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 best-studied virulence factors are the capsular polysaccharide, the muramidase-released protein (MRP), the extracellular protein factor (EF), the cytotoxic suilysin, and adhesins.

Capsular Polysaccharide

The antiphagocytic sialic acid-rich capsular polysaccharide probably functions 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, it 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 North America. Antibody to capsule is opsonic and partially protective and is elicited at low levels during convalescence. Higher levels of antibody 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 by a "multi-hit" mechanism of action. Suilysin is thermolable and either secreted or loosely cell bound. The 44% of isolates from the lung express the toxin compared to 80 to 90% of isolates from other sites in pigs (Staats et al. 1999). Thus, the toxin is not an essential virulence factor in the pig although suilysin-specific antibody protects pigs against challenge by a homologous toxin-producing strain (Jacobs et al. 1996).

Muramidase Released Protein (MRP)

MRP is released by muramidase digestion of the cell wall and is a 136 kDa surface-exposed protein 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 (Smith et al. 1993). The function of MRP is unknown. Deletion of the gene does not result in loss of virulence for newborn piglets (Smith et al. 1996) although antibody to the protein does contribute to protection against strains expressing the protein.

Extracellular Protein (EF)

EF and MRP proteins 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 on 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. Genetic studies suggest that these isolates constitute a clone (Staats et al. 1999).

Adhesins

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 (Haataja et al. 1996). It is highly immunogenic and induces bactericidal antibody in mice.

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 also been shown to have protective efficacy albeit limited to challenge with homologous strains that express these factors (Jacobs et al. 1996).

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.

Accumulating evidence favors the conclusion that effective subunit vaccines must contain proteins including MRP and EP combined with the common capsular antigens. In addition, the 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 disappointing efficacy of formalin-killed vaccines 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 PORCINUS

S. porcinus, of Lancefield group E, U, V, or P, is the cause of a contagious cervical lymphadenitis (porcine strangles) of young 8- to 10-week-old swine. Although *S. porcinus* has been isolated from a variety of opportunist infections in horses, cats, and humans, the pig is the normal host. As is the case for many 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 disease in the United States.

In experimental infections, enlargement of the mandibular, parotid, and retropharyngeal lymph nodes is evident about 2 weeks after infection. Abscessed nodes drain during the following week or become encapsulated.

Virulence Factors

S. 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, both of which are attributes of M protein antibody. However, complete characterization and purification of the antiphagocytic factor has not been reported. The phagocytic resistance of the organism is increased during growth in 10% porcine serum or 2% bovine serum albumin.

STREPTOCOCCUS UBERIS

S. uberis is a tonsillar, intestinal, mucosal, and epithelial commensal of cattle that is responsible for about 20 to 30% of cases of clinical mastitis in dairy herds in North America and Europe. It may be distinguished from the phenotypically similar but rarely isolated *S. parauberis* by immunoblot fingerprint, absence of growth at 10°C, and by PCR (Groschup et al. 1991; Hassan et al. 2001). Isolates vary in genotype and protein phenotype between and within infected herds although persisting infections in individual cows are usually caused by a single genotype (Wang et al. 1999). Many infections appear to opportunistically invade the mammary gland of older cows under conditions of heavy environmental soiling with feces.

Following entry through the teat canal, the organism attaches, proliferates, and induces an influx of neutrophils into the secretory acini that is evident at 24 hours. 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 macrophages in the alveolar lumina, but are infrequent in neutrophils (Thomas et al. 1994) and are also present in lymphatic vessels, lymph nodes, and attached 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

A hyaluronic acid capsule that protects against phagocytosis and intracellular killing is expressed on a small percentage of isolates on solid media. The majority of isolates of *S. uberis* do not produce mucoid colonies indicating either that capsule expression is up-regulated only *in vivo* or is not an essential virulence factor. Mutants in which the *hasA* or *C* genes were disrupted by insertional mutagenesis lose their resistance to phagocytosis by bovine neutrophils (Ward et al. 2001). Binding of casein to the bacterial surface also increases resistance to phagocytosis (Leigh and Field 1994). Other potential virulence factors of *S. uberis* include hyaluronidase, a 28 kDa in uberis factor similar to the CAMP factor of *S. agalactiae*, an adhesin specific for cubic mammary gland cells (Lammers et al. 2001), and a plasminogen activator, PauA (Rosey et al. 1999). Activation of plasmin may be important in generation of essential amino acids from casein, and hydrolysis of host matrix proteins may uncover target sites for adhesins on the *S. suis* surface.

S. uberis enters and survives within mammary epithelial cells for extended periods (Oliver et al. 1998). Lactoferrin-binding molecules on the bacterial surface may contribute to attachment to cells by serving as bridges between epithelial receptors and the bacterium (Fang et al. 1998).

Immunity

Specific immune responses have been shown to be involved in clearance of *S. uberis* from the udder of cows experimentally challenged following recovery 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 begin to appear at 3 days after challenge but the role of these antibodies in clearance is uncertain. Earlier studies indicated that milk becomes opsonic for *S. uberis* after mammary infection. Although the specificity of the opsonin was not determined, a trypsin-resistant protein of 65 kDa has been shown to stimulate antibodies in guinea pigs that function opsonically with bovine neutrophils (Groschup and Timoney 1992). Milk of previously infected mammary glands has a significant inhibitory effect on growth of *S. uberis* (Fang et al. 1998). However, protection against infection is not dependent on opsonic activity and neutrophils. Vaccination with culture filtrates rich in PauA, the plasminogen activator from *S. uberis*, induced protection in about 50% of challenged quarters of immunized cows. Protection was correlated with levels of PauA-specific inhibitory antibodies (Leigh et al. 1999). 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, these observations together suggest that effective immunity against *S. uberis* is multifactorial and involves a combination of immunogens.

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 is found in the respiratory tract of normal horses and has been associated with lower airway disease 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 is 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 (Blunden et al. 1994). 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, 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.

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 been noted, however, 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). Toxin involvement in pneumococcal pneumonia in humans is suggested by the acute fulminating and toxic clinical character of the disease. Neuraminidase may act both to decrease the viscosity of mucus and to alter oligosaccharides of mucosal cells by removing N-acetyl neuraminic acid residues and thus expose receptors for bacterial attachment.

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 that proliferate in the highly cellular exudate will generate highly inflammatory streptococcal cell-wall products.

The significance in lesion development of the large numbers of *S. zooepidemicus* often found with *S. pneumoniae* in tracheal aspirates is unknown. It is possible that IgA protease produced by *S. pneumoniae* may destroy protective antibodies that control proliferation of *S. zooepidemicus*.

Immunity

Much of the information on protective immunity to *S. pneumoniae* must be interpreted with caution since it is based on mouse models. Type-specific capsular

antibody produced during convalescence is opsonizing and protective. However, capsular polysaccharide is often poorly immunogenic. Protein antigens including PspA, pneumolysin, PsaA, autolysin, the neuraminidases, NanA and B, and at least six other surface proteins reactive with human convalescent serum and mouse protective (Wizeman et al. 2001) may have potential as vaccine components.

CONCLUSIONS

The breadth and diversity of host and tissue tropisms of the pathogenic veterinary streptococci suggest multiple paths of evolution arising from acquisition of new genes, modifications or loss of existing genes, and changes in regulatory networks. Genetic events such as these have resulted in pathogens uniquely fitted for invasion of the equine tonsil and associated lymphatic tissue (*S. equi*) or ruminant mammary gland (*S. agalactiae*). In contrast, other successful streptococcal pathogens including *S. zooepidemicus* and *S. dysgalactiae* subsp. *equisimilis* have evolved with almost no host adaptation and capable of causing disease only as opportunists in situations of preexisting damage to mucosal barriers or impaired immune defenses.

Which streptococcal determinants are involved in host adhesion and penetration? Much research effort has been invested in streptococcal proteins that bind promiscuously to abundant host molecules such as fibronectin, immunoglobulins, and fibrinogen but that are unlikely to serve as determinants of host specificity. Exciting new and more rational approaches to the identification of molecules involved in penetration and protective immunity have been provided by the genomic sequences of *S. equi*, *S. agalactiae*, *S. uberis*, *S. pneumoniae,* and *S. suis*. Genomic sequence provides access to the entire gene repertoire of the pathogen, which in turn allows prediction of proteins that are surface exposed or secreted and have the potential to interact directly with host tissue. Moreover, these proteins are much more likely to be involved in protective immune responses and so are logical candidates for inclusion in new-generation vaccines. DNA from each gene presented in a micro-array of complete or defined sets of genes can be used to hybridize to labeled RNA from the host at different stages of infection to identify virulence genes expressed at these times. Specific gene function can be predicted or determined by comparison with sequences of genes of known function in the databases or by infecting the natural host with a strain of the pathogen in which the gene in question has been inactivated. Another approach of value in identifying immunogens expressed only during infection is to screen an expression gene library with convalescent serum. Reactive clones can quickly be identified using partial DNA sequence to locate the corresponding open reading frames in the database.

These technological advances are grounds for optimism that much new information on streptococcal virulence factors, including their contribution to each stage of pathogenesis and protective immune responses, will soon be forthcoming. Defined pools of candidate genes and their proteins can be used in immunization–challenge studies to ultimately reveal the most effective combination and route of presentation of a new-generation vaccine.

REFERENCES

Anzai, T., A. S. Sheoran, Y. Kuwamoto, T. Kondo, R. Wada, T. Inoue, and J. F. Timoney. 1999a. *Streptococcus equi* but not *Streptococcus zooepidemicus* produces potent mitogenic responses from equine peripheral blood mononuclear cells. Vet. Immunol. Immunopathol. 67:235–246.

Anzai, T., J. A. Walker, M. B. Blair, T. M. Chambers, and J. F. Timoney. 2000. Comparison of the phenotypes of *Streptococcus zooepidemicus* isolated from tonsils of healthy horses and specimens obtained from foals and donkeys with pneumonia. Am. J. Vet. Res. 61:162–166.

Anzai, T., J. F. Timoney, T. Kuwamoto, Y. Fujita, R. Wada, and T. Inoue. 1999b. *In vivo* pathogenicity and resistance to phagocytosis of *Streptococcus equi* strains with different levels of capsule expression. Vet. Microbiol. 67:277–286.

Artiushin, S. C., J. F. Timoney, A. S. Sheoran, and S. K. Muthupalani. 2002. Characterization and immunogenicity of pyrogenic mitogens SePE-H and SePE-I of *Streptococcus equi*. Microbiol. Pathogenesis 32:71–85.

Blanchard, P. C. 1987. Group G streptococcal infections in kittens: pathogenesis, immune response and maternal carrier state. PhD. Diss. University of California, Davis.

Blunden, A. S., D. Hannant, D. Livesay, and J. A. Mumford. 1994. Susceptibility of ponies to infection with *Streptococcus pneumoniae* (capsular type 3). Equine Vet. J. 26:22–28.

Boschwitz, J. S., and J. F. Timoney. 1994a. Inhibition of C3 deposition on *Streptococcus equi* subsp. *equi* by M protein: a mechanism for survival in equine blood. Infect. Immun. 62:3515–3520.

———. 1994b. Characterization of the antiphagocytic properties of fibrinogen for *Streptococcus equi* subsp. *equi*. Microbiol. Pathogenesis 17:121–129.

Brodeur, B. R., M. Bozer, I. Charlebois, J. Hamel, F. Couture, C. R. Rioux, and D. Martin. 2000. Identification of group B streptococcal Sip protein, which elicits cross-protective immunity. Infect. Immun. 68:5610–5618.

Calvinho, C. F., R. A. Almeida, and S. P. Oliver. 1998. Potential virulence factors of *Streptococcus dysgalactiae* associated with bovine mastitis. Vet. Microbiol. 61:93–110.

Carr, A., D. D. Sledjiski, A. Podbielski, M. D. P. Boyle, and B. Kreikemeyer. 2001. Similarities between complement-mediated and streptolysin S-mediated hemolysis. J. Biol. Chem. 276:41790–41796.

Causey, R. C., D. L. Paccamonti, and M. J. Todd. 1995. Antiphagocytic properties of uterine isolates of *Streptococcus zooepidemicus* and mechanisms of killing in freshly obtained blood of horses. Am. J. Vet. Res. 56:321–328.

Chanter, N., N. C. Talbot, J. R. Newton, D. Hewson, and K. Verhezen. 2000. *Streptococcus equi* with truncated M-proteins isolated from outwardly healthy horses. Microbiol. 146:1361–1369.

Chanter, N., N. Collin, and N. Holmes. 1997. Characterization of the Lancefield group C streptococcus 16S-23S RNA gene intergenic spacer and its potential for identification and sub-specific typing. Epidemiol. Infect. 118:125–135.

Chapman, P. S., C. Green, J. P. Main, P. M. Taylor, F. M. Cunningham, A. J. Cook, and C. M. Marr. 2000. Retrospective study of the relationships between age, inflammation and the isolation of bacteria from the lower respiratory tract of thoroughbred horses. Vet. Rec. 146:91–95.

Cleary, P., and D. Retnoningrum. 1994. Group A streptococcal immunoglobulin-binding proteins: adhesins, molecular mimicry or sensory proteins? Trend. Microbiol. 2:131–136.

Coming, B. F., J. C. Murphy, and J. G. Fox. 1991. Group G streptococcal lymphadenitis in rats. J. Clin. Microbiol. 29:2720–2723.

DeWinter, L. M., and J. F. Prescott. 1999. Relatedness of *Streptococcus canis* from canine streptococcal toxic shock syndrome and necrotizing fasciitis. Can. J. Vet. Res. 63:90–95.

Deynes, R. A., and C. H. Armstrong. 1973. An antiphagocytic factor associated with group E streptococci. Infect. Immun. 7:298–304.

Divers, T. J., J. F. Timoney, R. M. Lewis, and C. A. Smith. 1992. Equine glomerulonephritis and renal failure associated with complexes of group-C streptococcal antigen and IgG antibody. Vet. Immunol. Immunopathol. 32:93–102.

Ellis, R. P., and C. H. Armstrong. 1971. Production of capsules, streptokinase and streptodornase by streptococcus group E. Am. J. Vet. Res. 32:349–356.

Erdogan, S., P. K. Fagan, S. R. Talay, M. Rohde, P. Ferrieri, A. Flores, C. Guzman, M. Walker, and G. S. Chhatwal. 2002. Molecular analysis of group B protective surface protein; a new cell surface protective antigen of group B streptococci. Infect. Immun. 70:803–811.

Evers, W. O. 1968. Effect of furaltadone on strangles in horses. J. Am. Vet. Med. Assoc. 152:1394–1398.

Facklam, R. 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin. Microbiol. Rev. 15:613–630.

Fang, W., D. A. Luther, R. A. Almeida, and S. P. Oliver. 1998. Decreased growth of *Streptococcus uberis* in milk from mammary glands of cows challenged with the same mastitis pathogen. Zentralbl-Veterinarmed-[B]. 45:539–549.

Ferrieri, P. 1988. Surface–localized protein antigens of group B streptococci. Rev. Infect. Dis. 10 (Suppl. 2):S363–366.

Finch, L., and D. R. Martin. 1984. Human and bovine group B streptococci—two distinct populations. J. Appl. Bacteriol. 57:273–278.

Flanagan, J., N. Collin, J. Timoney, T. Mitchell, J. A. Mumford, and N. Chanter. 1998. Characterization of the haemolytic activity of *Streptococcus equi*. Microbiol. Pathogen. 24:211–221.

Fontaine, M. C., J. Perez-Casal, X. M. Song, J. Shelford, P. J. Willson, and A. A. Potter. 2002. Immunization of dairy cattle with recombinant *Streptococcus uberis* Gap C or a chimeric CAMP antigen confers protection against heterologous bacterial challenge. Vaccine 20:2278–2286.

Frost, A. I., D. D. Wanasinghe, and J. B. Woolcock. 1977. Some factors affecting selective adherence of microorganisms in the bovine mammary gland. Infect. Immun. 15:245–253.

Galán, J. E., and J. F. Timoney. 1985. Mucosal nasopharyngeal immune response of the horse to protein antigens of *Streptococcus equi*. Infect. Immun. 47:623–628.

———. 1987. Molecular analysis of the M protein of *Streptococcus equi* and cloning and expression of the M protein gene in *Escherichia coli*. Infect. Immun. 55:3181–3187.

———. 1988. Immunologic and genetic comparison of *Streptococcus equi* isolates from the United States and Europe. J. Clin. Microbiol. 26:1142–1146.

Gottschalk, M., and M. Segura. 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. 76:259–272.

Gottschalk, M., R. Higgins, and S. Quessy. 1999. Dilemma of the virulence of *Streptococcus suis* strains. J. Clin. Microbiol. 37:4202–4203.

Groschup, M. H., and J. F. Timoney. 1992. An R-like protein of *Streptococcus uberis* stimulates opsonic antibodies. Res. Vet. Sci. 54:124–126.

Groschup, M. H., G. Hahn, and J. F. Timoney. 1991. Antigenic and genetic homogeneity of *Streptococcus uberis* strains from the bovine udder. Epidemiol. Infect. 107:297–310.

Haataja, S., K. Tikkanen, J. Hytonen, and J. Finne. 1996. The Gal alpha 1-4 Gal-binding adhesin of *Streptococcus suis*, a gram-positive meningitis-associated bacterium. Adv. Exp. Med. Biol. 408:25–34.

Hamlen, H. J., J. F. Timoney, and R. J. Bell. 1994. Epidemiologic and immunologic characteristics of *Streptococcus equi* infection in foals. J. Am. Vet. Med. Assoc. 204:768–775.

Harris, T. O., D. W. Shelner, J. F. Bohnsack, and C. E. Rubens. 2003. A novel streptococcal surface protease promotes virulence, resistance to opsonophagocytosis, and cleavage of human fibrinogen. J. Clin. Invest. 111:61–70.

Hassan, A. A., I. U. Khan, A. Abdulmawjood, A., and C. Lammler. 2001. Evaluation of PCR methods for rapid identification and differentiation of *Streptococcus uberis* and *Streptococcus parauberis*. J. Clin. Microbiol. 39:1618–1621.

Hill, A. W. 1988. Protective effect of previous intramammary infection with *Streptococcus uberis* against subsequent clinical mastitis in the cow. Res. Vet. Sci. 44:386–387.

Hoffman, A. M., H. R. Staempfli, J. F. Prescott, and L. Viel. 1991. Field evaluation of a commercial M protein vaccine against *Streptococcus equi* infection in foals. Am. J. Vet. Res. 52:589–595.

Hoffman, A. M., L. Viel, J. F. Prescott, S. Rosendal, and J. Thorsen. 1993. Association of microbiologic flora with clinical endoscopic and pulmonary cytologic findings in foals with distal respiratory tract infection. Am. J. Vet. Res. 54:1615–1622.

Hollingshead, S. K., P. W. Canfield, and D. G. Pritchard. 1989. Insertional mutagenesis of CAMP factor in group B streptococci. Am. Soc. Microbiol. Annual Mtg. Abstr. B-28.

Holt, M., M. R. Enright, and T. J. L. Alexander. 1988. Immunization of pigs with live cultures of *Streptococcus suis* type 2. Res. Vet. Sci. 45:349–352.

Ingrey, K., J. Ren, and J. F. Prescott. 2003. A fluoroquinolone induces a novel mitogen-encoding bacteriophage in *Streptococcus canis*. Infect. Immun. 71:3028–33.

Jacobs, A. A. C., A. J. G. Van den Berg, and P. L. W. Loeffen. 1996. Protection of experimentally infected pigs by suilysin, the thiol-activated haemolysin of *Streptococcus suis*. Vet. Rec. 139:225–228.

Jonsson, H., H. Lindmark, and B. Guss. 1995. A protein G related cell surface protein in *Streptococcus zooepidemicus*. Infect. Immun. 63:2968–2975.

Kurl, D. N., K. K. Christensen, and P. Christensen. 1984. Colonization of the upper respiratory tract of mice with group B streptococci type III with reference to the R protein. J. Med. Microbiol. 17:347–351.

Lammers, A., C. J. van Vorstenbosch, J. H. Erkens, and H. E. Smith. 2001. The major bovine mastitis pathogens have different cell tropisms in cultures of bovine mammary gland cells. Vet. Microbiol. 80:255–265.

Lancefield, R. C., M. McCarty, and W. M. Everly. 1975. Multiple mouse protective antibodies directed against group B streptococci. J. Exp. Med. 142:165–179.

Leigh, J. A., and T. R. Field. 1994. *Streptococcus uberis* resists the bactericidal action of bovine neutrophils despite the presence of bound immunoglobulins. 62:1854–1859.

Leigh, J. A., J. M. Finch, T. R. Field, N. C. Real, A. Winter, A. W. Walton, and S. M. Hodgkinson. 1999. Vaccination with the plasminogen activator from *Streptococcus uberis* induces an Inhibitory response and protects against experimental infection in the dairy cow. Vaccine 17:851–857.

Lindmark, H., and B. Guss. 1999. SFS, a novel fibronectin-binding protein from *Streptococcus equi* inhibits the binding between fibronectin and collagen. Infect. Immun. 67:2383–2388.

Lindmark, H., K. Jacobson, L. Frykberg, and B. Guss. 1996. Fibronectin-binding protein of *Streptococcus equi* subsp. *zooepidemicus*. Infect. Immun. 64:3993–3999.

MacKintosh, M. E., S. T. Grant, and M. H. Buffell. 1988. Evidence for *Streptococcus pneumoniae* as a cause of respiratory disease in young thoroughbred horses in training. In Equine Infectious Diseases V, Ed. D.G. Powell. pp. 41–46. Lexington: University of Kentucky Press.

Marques, M. B., D. L. Kasper, M. K. Pangbum, and M. R. Wessels. 1992. Presentation of C3 deposition by capsular polysaccharide is a virulence mechanism of type III streptococci. Infect. Immun. 62:3986–3993.

McCoy, H. E., C. C. Broder, and R. Lottenberg. 1991. Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. J. Infect. Dis. 164:515–521.

Meehan, M., S. M. Kelly, N. C. Price, and P. Owen. 2002. The C-terminal portion of the fibrinogen-binding protein of *Streptococcus equi* subsp. *equi* contains extensive α-helical coiled coil structure and contributes to thermal stability. FEMS 206:81–86.

Meyer, J. C., A. Koterba, G. Lester, and B. L. Purich. 1992. Bacteremia and pneumonia in a neonatal foal caused by *Streptococcus pneumoniae* type 3. Equine Vet. J. 24:407–410.

Mukhtar, M. M., and J. F. Timoney. 1988. Chemotactic response of equine polymorphonuclear leucocytes to *Streptococcus equi*. Res. Vet. Sci. 45:225–229.

Newton, J. R., J. L. N. Wood, K. A. Dunn, M. N. De Brauwere, and N. Chanter. 1997. Naturally occurring persistent and asymptomatic infection of the guttural pouches of horses with *Streptococcus equi*. Vet. Rec. 140:84–90.

Norcross, N. L., and N. Oliver. 1976. The distribution and characterization of group B streptococci in New York State. Cornell Vet. 66:240–248.

Norcross, N. L., K. Dodd, and D. M. Stark. 1968. Use of the mouse protection test to assay streptococcal antibodies in bovine serum and milk. Am J. Vet. Res. 29:1201–1205.

Oikawa, M., M. Kamada, Y. Yoshikawa, and T. Yoshikawa. 1994. Pathology of equine pneumonia associated with transport and isolation of *Streptococcus equi* subsp. *zooepidemicus.* J. Comp. Pathol. 111:205–212.

Oliver, S. P., R. A. Almeida, and L. F. Calvinho. 1998. Virulence factors of *Streptococcus uberis* isolated from cows with mastitis. Zblatt. Vet. Med. B. 45:461–471.

Prescott, J. F., and L. DeWinter. 1997. Canine streptococcal toxic shock syndrome and necrotizing fasciitis. Vet. Rec. 140:263.

Qin, A., S. Artiushin, and J. F. Timoney. 2003. Identification and genomic associations of genes or novel immunogenic proteins of *Streptococcus equi*. Submitted for review.

Rainard, P. 1992. Isotype antibody response in cows to *Streptococcus agalactiae* group B polysaccharide-ovalbumin conjugate. J. Clin. Microbiol. 30:1856–1862.

Rainard, P., and C. Boulard. 1992. Opsonization of *Streptococcus agalactiae* of bovine origin by complement and antibodies against group B polysaccharide. Infect. Immun. 60:4801–4808.

Rainard, P., P. Sarradin, and B. Poutrel. 1994. Phenotyptic variability of x-protein expression by mastitis-causing *Streptococcus agalactiae* of serotype NT/x and opsonic activities of specific antibodies. Microb. Pathogen. 16:359–372.

Rainard, P., Y. Lautrou, P. Sarradin, and B. Poutrel. 1991. Protein X of *Streptococcus agalactiae* induces opsonic antibodies in cows. J. Clin. Microbiol. 29:1842–1846.

Rosey, E. L., R. A. Lincoln, P. N. Ward, R. J. Yancey, and J. A. Leigh. 1999. PanA: a novel plasminogen activator from *Streptococcus uberis*. FEMS Microbiol. Lett. 178:27–33.

Segura, M., and M. Gottschalk. 2002. *Streptococcus suis* interactions with the murine macrophage cell live J774: adhesion and cytotoxicity. Infect. Immun. 70:4312–4322.

Sheoran, A. S., B. T. Sponseller, M. A. Holmes, and J. F. Timoney. 1997. Serum and mucosal antibody isotype responses to M-like protein (SeM) of *Streptococcus equi* in convalescent and vaccinated horses. Vet. Immunol. Immunopathol. 59:239–251.

Simpson, W. J., J. C. Robbins, and P. P. Cleary. 1987. Evidence for group A-related M protein genes in human but not animal associated group G streptococcal pathogens. Microb. Pathogen. 3:339–350.

Smith, H. E., F. H. Reek, U. Vecht, A. L. Gielkens, and M. A. Smits. 1993. Repeats in an extracellular protein of weakly pathogenic strains of *Streptococcus suis* type 2 are absent in pathogenic strains. Infect. Immun. 61:3318–3326.

Smith, H. E., U. Vecht, A. Gielkens, and M. A. Smits. 1992. Cloning and nucleotide sequence of the gene encoding the 136-kilodalton surface protein (muramidase-released protein) of *Streptococcus suis* type 2. Infect. Immun. 60:2361–2367.

Smith, H. E., U. Vecht, H. J. Wisselink, N. Stockhope-Zurweiden, Y. Bierman, and M. A. Smits. 1996. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase released protein and extracellular protein induced disease in newborn germ-free pigs. Infect. Immun. 64:4409–4412.

Staats, J. J., B. L. Plattner, G. C. Stewart, and M. M. Chengappa. 1999. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet. Microbiol. 70:201–211.

Sundberg, J. P., D. Hill, D. S. Wyand, M. I. Ryan, and C. H. Baldwin. 1981. *Streptococcus zooepidemicus* as the cause of septicemia in racing Greyhounds. Vet. Med. Small Anim. Clin. 177:839–842.

Swindle, M. M., O. Narayan, M. Luzarraga, and D. L. Bobbie. 1981. Contagious streptococcal lymphadenitis in cats. J. Am. Vet. Med. Assoc. 177:829–830.

Takai, S., T. Anzai, H. Yashiro, C. Ishii, S. Tsubaki, R. Wada, and J. F. Timoney. 2000. Detection of DNA restriction fragment polymorphism in *Streptococcus equi*. Vet. Rec. 146:159–161.

Thomas, L. H., W. Haider, A. W. Hill, and R. S. Cook. 1994. Pathologic findings of experimentally induced *Streptococcus uberis* infection in the mammary gland of cows. Am. J. Vet. Res. 55:1723–1728.

Timoney, J. F., and D. E. Eggers. 1985. Serum bactericidal responses to *Streptococcus equi* of horses

following infection or vaccination. Equine Vet. J. 17:306–310.

Timoney, J. F., and J. E. Galán. 1985. The immune response of the horse to an avirulent strain of *Streptococcus equi*. In Recent advances in streptococci and streptococcal diseases. Y. Kimura, S. Kotami, and Y. Shiokawa (eds.). Bracknell, UK: Reedbooks, pp. 294–95.

Timoney, J. F., and J. Trachman. 1985. Immunologically reactive proteins of *Streptococcus equi*. Infect. Immun. 48:29–34.

Timoney, J. F., and P. C. Blanchard. 1986. Unpublished data. School of Veterinary Medicine, University of California, Davis.

Timoney, J. F., J. Wang, S. Artiushin, A. Sheoran, J. Nally, and P. Kumar. 2000. Virulence and immunogenicity of clone of *Streptococcus equi* in which expression of SeM is blocked by Tn916 mutagenesis. In Streptococci and streptococcal diseases, XIV Lancefield International Symposium on Streptococci and Streptococcal Diseases. D. R. Martin and J. R. Tagg (eds.). Porirua, New Zealand, pp 699–702.

Timoney, J. F., S. C. Artiushin, and J. S. Boschwitz. 1997. Comparison of the sequences and functions of *Streptococcus equi* M-like proteins SeM and SzPSe. Infect. Immun. 65:3600–3605.

Todd, T. G. 1910. Strangles. J. Comp. Path. Therap. 23:212–229.

Vasi, J., L. Frykberg, C. E. Carlsson, M. Lindberg, and B. Guss. 2000. M-like proteins of *Streptococcus dysgalactiae*. Infect. Immun. 68:294–302.

Vecht, U., H. J. Wisselink, J. E. van Dijk, and H. E. Smith. 1992. Virulence of *Streptococcus suis* type 2 strains in newborn germ-free pigs depends on phenotype. Infect. Immun. 60:550–556.

Vierra, V. V., L. M. Teixeira, V. Zahner, H. Momen, R. R. Facklam, A. G. Steigerwalt, D. J. Brenner, and A. C. D. Castro. 1998. Genetic relationships among the different phenotypes of *Streptococcus dysgalactiae* strains. Int. J. Syst. Bact. 48:1231–1243.

Wagner, M., V. Kubin, and E. Gunther. 1982. Immunoelectron microscopic analysis of polysaccharide and protein surface antigens in wild strains of group B streptococci. Zblatt. Bakt. Hyg. Immunol. (Abt 1 Orig.) 253:331–343.

Walker, J. A., and J. F. Timoney. 1998. Molecular basis of variation in protective SzP proteins of *Streptococcus zooepidemicus*. Am. J. Vet. Res. 59:1129–1133.

Wang, S. M., M. A. Deighton, J. A. Capstick, and N. Gerraty. 1999. Epidemiological typing of bovine streptococci by pulsed-field gel electrophoresis. Epidemiol. Infect. 123:317–324.

Wanger, A. R., and G. M. Dunny. 1987. Identification of a *Streptococcus agalactiae* protein antigen associated with bovine mastitis isolates. Infect. Immun. 55:1170–1175.

Ward, P. N., T. R. Field, W. G. F. Ditcham, E. Maguin, and J. A. Leigh. 2001. Identification and disruption of two discrete loci encoding hyaluronic acid capsule biosynthesis genes *hasA*, *hasB* and *hasC* in *Streptococcus uberis*. Infect. Immun. 69:392–399.

Whatmore, A. M., S. J. King, N. C. Doherty, D. Sturgeon, N. Chanter, and C. Dowson. 1999. Molecular characterization of equine isolates of *Streptococcus pneumoniae*: natural disruption of genes encoding the virulence factors pneumolysin and autolysin. Infect. Immun. 67:2776–2782.

Widders, P. R., S. Warner, and P. J. Huntington. 1995. Immunization of mares to control endometritis caused by *Streptococcus zooepidemicus*. Res. Vet. Sci. 58:75–81.

Williams, A. E. 1990. Relationship between intracellular survival in macrophages and pathogenicity of *Streptococcus suis* type 2 isolates. Microb. Pathog. 8:189–196.

Wisselink, H. J., N. Stockhope-Zurwieden, L. A. T. Hilgers, and H. E. Smith. 2002. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of *Streptococcus suis* serotype 2. Vet. Microbiol. 84:155–168.

Wizeman, T. M., J. H. Heinrichs, and J. E. Adomou. 2001. Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. Infect. Immun. 69:1593–1598.

Wood, R. D., and R. F. Ross. 1975. Purification and serological characterization of type-specific antigen of *Streptococcus equisimilis*. Infect. Immun. 12:881–887.

Yokomizo, Y., and N. L. Norcross. 1978. Bovine antibody against *Streptococcus agalactiae*, type 1A, produced by preparturient intramammary and systemic vaccination. Am. J. Vet. Res. 39:511–516.

4
Staphylococcus

K. Hermans, L. A. Devriese, and F. Haesebrouck

Staphylococci are gram-positive cocci (0.5 to 1.5 μm in diameter) that occur singly, in pairs, tetrads, short chains (three or four cells), and irregular grapelike clusters. Nearly 50 species and subspecies were known by the beginning of the twenty-first century, and new taxons and names continue to be described.

CHARACTERISTICS OF THE ORGANISM

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 to 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. Micrococci with a G + C content of 65–75 mol % are not related to the staphylococci but to the corynebacteria and more particularly to *Arthrobacter*. Phylogenetic studies have shown that the inclusion of these two genera in a common family, the Micrococcaceae, is obsolete.

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 a different staphylococcal flora. This can be seen at species level and at strain level. At the infraspecies level, genotypic studies have characterized staphylococcal species in epidemiological (fingerprinting) studies at different levels up to strain level. From a veterinary and zoonotic perspective, typing methods distinguishing between host-associated groups of strains (ecovars) can be particularly useful.

Phenotypic Criteria

The catalase reaction is positive for all *Staphylococcus* species, except for *S. aureus* subsp. *anaerobius* and *S. 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 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. Coagulase production, that is, the ability to clot rabbit plasma, continues to be the most widely used and generally accepted criterion for the identification of pathogenic staphylococci associated with acute infections. 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, three staphylococcal species are of major pathogenic importance: *S. aureus*, *S. intermedius,* and *S. hyicus* (Devriese 1990). The former two species 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: *S. delphini* and *S. schleiferi*. *S. delphini* has been described in dolphins in 1988 (Varaldo et al. 1988). *S. schleiferi* has long been regarded as a coagulase-negative species until, in 1990, a new subspecies was described after isolation from otitis externa from dogs, namely *S. schleiferi* subsp. *coagulans*, which was 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. intermedius,* and *S. hyicus,* as mentioned above, while all others are to be considered as minor pathogens. In veterinary medicine, the latter are only 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 becomes more common in companion animal medicine.

PATHOGENIC *STAPHYLOCOCCUS* SPECIES

MAJOR PATHOGENIC *STAPHYLOCOCCUS* SPECIES

S. aureus is a well-documented human opportunistic pathogen. It may cause skin infections and also septicemic infections and is important as the cause of toxic shock syndrome. *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 its antimicrobial resistance. Methicillin-resistant *S. aureus* (MRSA) is a major clinical and epidemiological problem in human hospitals. MRSAs have a tendency to accumulate, besides 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 antibiotics. These strains are rare in animal medicine. From the original description of MRSA in animals (Devriese and Hommez 1975) to the end of the twentieth century, only MRSA strains showing characteristics commonly seen in human epidemic strains were isolated. Animals apparently are infected by their human attendants.

Typical animal strains of *S. aureus* are important pathogens in veterinary medicine, causing disease in cattle, small ruminants, poultry, rabbits, pigs, horses (Devriese 1990), and many other 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, exudative dermatitis, subcutaneous abscesses, and pododermatitis (Okerman et al. 1984). Pigs may sporadically suffer from septicemia due to a *S. aureus* infection (Devriese 1990), and in horses, the bacterium may cause dermatitis and cellulitis (Devriese 1984).

S. intermedius is the most important non-*aureus* species. It is the predominant coagulase-positive *Staphylococcus* causing skin infections in dogs. It 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 in pigs are specifically caused by *S. hyicus* (Wegener and Skov-Jensen 1992). 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 in 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, supplemented sometimes 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 fingerprinting methods are probably better suited for veterinary strains.

MAJOR PATHOGENIC *STAPHYLOCOCCUS* SPECIES

The other *Staphylococcus* 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 the human and animal body surfaces and constitute a major component of the 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 have also been shown to protect to some extent against infections with other udder pathogens, such as *S. aureus* (Lam et al. 1997). Species described in subclinical as well as clinical mastitis of dairy cattle are *S. simulans*, *S. chromogenes*, *S. haemolyticus*, *S. xylosus*, *S. carnosus*, *S. warneri*, *S. hominis*, *S. epidermidis*, *S. sciuri,* and *S. cohnii* (Jarp 1991; Devriese et al. 1994). There is a negligible difference in pathogenicity between these species (Jarp 1991). Coagulase-negative staphylococci 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*, *S. lentus*, *S. equorum*, *S. capitis*, *S. arlettae*, *S. saprophyticus,* and *S. lugdunensis* (Deinhofer and Pernthaner 1993; Fthenakis et al. 1994). These identifications should be viewed with caution, since different methods of species identification may not yield the same results (Burriel and Scott 1998).

The animal species in which the different staphylococcal species are found are summarized in table 4.1.

SOURCES OF THE BACTERIUM

In general, staphylococci are facultative pathogenic organisms that are part of the normal skin flora of most animal species. 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 are carriers of 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 flora differ from the flora associated with the 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 the apparently normal birds in poultry flocks. Two or more strains can

Table 4.1. Farm and Pet Animal Hosts and Staphylococcal Species with Well-Established Pathogenicity

Animal species	*S. aureus*	*S. intermedius*	*S. hyicus*	Other (in mastitis)
Cattle	X		X	X
Horse	X	X	X	
Pig	X		X	
Sheep	X			X
Goat	X			X
Dog		X		
Rabbit	X			
Mink		X		
Chicken	X		X	
Turkey	X		X	
Pigeon		X		
Canary	X		X	
Psittacines	X			

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 1980). 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 1980).

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 littermates, and between stablemates (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 forms a potential risk of infection by high-virulence *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 and in the nasal cavities or external ears of healthy pigs (Takeuchi et al. 1985). Healthy breeding sows may transmit the bacterium from their vaginal flora to the skin of their offspring during birth. The vaginal strains of the sow thus become part of the skin flora of the piglets (Wegener and Skov-Jensen 1992).

Dogs may be carrier of *S. intermedius* 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 *S. intermedius* and in other species. The virulence factors can be divided into cell-associated components, exoenzymes, and exotoxins. These factors 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 will 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, but 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, which 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.

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. Evidence that coagulase is a virulence factor is limited, although probably the bacteria could 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 and thus disrupting 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 supposed to have a negative effect on immune function. Furthermore, one of the roles of lipase is probably also to harvest 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 V8 protease. Proteases have been proposed to function in blocking the action of antibodies by cleaving and inactivating them, since 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 protein, thus inducing the bacterial spread after the initial adherence step. Another possible role for these proteases is obtaining nutrients from the environment (McGavin et al. 1997). Minor pathogenic staphylococci involved in mastitis often secrete to different degrees a 34–36-kDa protein with cell-rounding cytotoxic activity causing cell detachment in several cell lines and expressing cytotoxic activity (Zhang and Maddox 2000).

Exotoxins

Enterotoxins and Toxic-shock Syndrome Toxin

S. aureus secretes enterotoxins and toxic shock syndrome toxin (TSST-1), which are two types of toxin with superantigen activity. Diseases in which these toxins play a predominant role have mainly been described in humans, but have also been seen in cattle, goats, and sheep due to *S. aureus* (Ho et al. 1989). In dogs, enterotoxins produced by *S. intermedius* 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 toxic shock syndrome (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).

Superantigens stimulate T cells nonspecifically without normal antigenic recognition. Cytokines are released in large amounts, causing the symptoms of TSS (Fraser et al. 2000).

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 has been shown to be due to toxins resembling *S. aureus* exfoliative toxins in their activity, but they are not closely related immunologically (Tanabe et al. 1993). The exfoliative toxins of *S. aureus* and *S. hyicus* also have different species specificity. 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 1992).

Haemolysins (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 lysis of bovine or ovine erythrocytes (Cifrian et al. 1996). 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. intermedius* from dogs on ox or sheep blood agar. The pathogenic role of this toxin is largely unknown, however.

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 been described to have direct and indirect effects on the activity of neutrophils and monocytes and thus to have a proinflammatory 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 is regulated through three global regulatory loci, namely *agr*, *xpr,* and *sar* (McNamara and Iandolo 1998). 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.

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). Furthermore, *S. aureus* bacteria are 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 causes an 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 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 plates of the bones, where they bind to proteins and collagen. Partial occlusion of these channels by bacterial colonies and infiltration of heterophils leads to failure of the local blood supply, which results in micro-abscess 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 rabbits it 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 1992). Skin trauma is considered to be the most 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 to *S. aureus* (Lämmler et al. 1985). The first active line of the pig's 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

Exposure of extracellular matrix proteins due to underlying conditions favors colonization and adhesion by certain *S. intermedius* 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 suggest that these toxins are important for staphylococcal survival and pathogenesis. Generally speaking, studies on characteristics of strains isolated from lesions and from carrier sites failed to demonstrate more or less 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 only *S. intermedius* is associated with these conditions. Other species, including highly virulent *S. aureus* strains, 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

S. 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 loss, particularly milk loss, varying from 10% to 25% of total yield according to the intensity of inflammation and the stage of lactation. Treatment failures (not due to acquired antibiotic 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 highly economic impact in 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 to be 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 is also produced. 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 has been shown to be one of the major causes of leg weakness in broiler flocks.

Staphylococcus aureus Infections in Rabbits

At rabbit flock level, two types of *S. aureus* infection can be distinguished: a first type, caused by low-virulence strains, affecting only a limited number of rabbits in the flock, and a second type, due to high-virulence strains, causing epidemic spread of disease in the rabbitry. 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

S. intermedius rarely ever causes systemic disease in dogs or other animals. Pyoderma is a frequently occurring condition starting with papular eruptions progressing into pustule formation with 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 to prevent 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 (PMN). 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 onto 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 PMN, ingestion and intracellular destruction of these bacteria take place. For *S. aureus,* however, it is proven that PMN 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, masking 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 yet exactly been defined. On the contrary, a massive release of cytokines leading to severe disease may be induced by expression of staphylococcal superantigens, such as enterotoxins and toxic shock syndrome 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 at 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 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. In a study by McCullagh et al. (1998), pulsed field gel electrophoresis revealed 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. It has also been proposed that 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 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. It is known that 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 is 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 1992).

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

IMMUNITY AND ITS IMPACT ON PATHOGENESIS

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 of the frequency and the 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; Mamo et al. 2000). Tests with these vaccines in cows have not yet been described. However, whatever the antigenic preparations used, complete protection against infections in cows has not yet been achieved.

Vaccination has also been suggested as a possible method to control rabbit staphylococcosis. The ability of immunization with purified staphylococcal alpha toxin to reduce the so-called blue breast or gangrenous mastitis caused by *S. aureus* to a less-severe form of mastitis has been described (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 the cases of rabbit staphylococcal mastitis. Studies have been described in rabbits with bacterins against staphylococcosis. These vaccines may have a certain effect on the rabbit's immunity against *S. aureus* under experimental circumstances. However, in practice, their usefulness has not been proven.

Vaccination of sows during pregnancy with autogenous vaccines prepared from both bacterial cells and culture supernatant that contains exfoliative toxins of *S. hyicus* may be of value in protecting their piglets against exudative epidermitis (Wegener and Skov-Jensen 1992).

Autogenous staphylococcal bacterins have been administered using various protocols for treatment of dogs with pyoderma. Controlled studies for

evaluation of efficacy are, however, lacking, and clinical impressions of effectiveness have ranged from 0 to 80%.

CONCLUSIONS: NEW DEVELOPMENTS

The immunological response to staphylococci is a complicated matter. 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. Recently, a study making use of whole-genome sequence information of *S. aureus* and of individual human sera was able to identify 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). Further identification of the role of these antigens in pathogenesis and of their presence in staphylococci infecting animals could possibly enable formulation of new vaccines, based on specific antigens that are not only important in pathogenesis but also highly antigenic. Another possible route for prevention and treatment of staphylococcal infections includes the development of peptides inhibiting staphylococcal virulence-gene regulator systems, such as *agr* (Dufour 2000). Considerable further research is required before effective preventive measures against staphylococcal infections will be achieved.

REFERENCES

Adlam, C., Ward, P. D., McCartney, A. C., Arbuthnott, J. P., and C. M. Thorley. 1977. Effect of immunization with highly purified alpha- and beta-toxins on staphylococcal mastitis in rabbits. Infect. Immun. 17:259–266.

Almeida, R. A., Matthews, K. R., Cifrian, E., Guidry, A. J., and S. P. Oliver. 1996. *Staphylococcus aureus* invasion of bovine mammary epithelial cells. J. Dairy Sci. 79:1021–1026.

Bergdoll, M. S. 1983. Enterotoxins. In C. S. Easmon and C. Adlam. Staphylococci and staphylococcal infections. London: Academic Press Inc., pp. 559–598.

Beveridge, T. J. 2000. The gram-positive cel wall. In V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (eds.). Gram-positive pathogens. Washington, DC: ASM Press, pp. 3–10.

Bohach, G. A., and T. J. Foster. 2000. *Staphylococcus aureus* exotoxins. In V. A. Fischetti, R. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (eds.). Gram-positive pathogens. Washington, DC: ASM Press, pp. 367–378.

Burriel, A. R., and M. Scott. 1998. A comparison of methods used in species identification of coagulase-negative staphylococci isolated from the milk of sheep. Vet. J. 155:83–188.

Butterworth, A. 1999. Infectious components of broiler lameness: a review. World Poult. Sci. J. 55:327–352.

Cifrian, E., A. J. Guidry, A. J. Bramley, N. L. Norcross, F. D. Bastida Corcuera and W. W. Marquardt. 1996. Effect of staphylococcal beta toxin on the cytotoxicity, proliferation and adherence of *Staphylococcus aureus* to bovine mammary epithelial cells. Vet. Microbiol. 48:187–198.

Cifrian, E., A. J. Guidry, C. N. O'Brien, S. C. Nickerson, and W. W. Marquardt. 1994. Adherence of *Staphylococcus aureus* to cultured bovine mammary epithelial cells. J. Dairy Sci. 77:970–983.

Cree, R. G. A., and W. C. Noble. 1995. In vitro indices of tissue adherence in *Staphylococcus intermedius*. Lett. Appl. Microbiol. 20:168–170.

Deinhofer, M., and A. Pernthaner. 1993. Differentiation of staphylococci from ewe and goat milk samples. Deutsche Tierartzliche Wochenschrift 100:234–236.

Devriese, L. A. 1980. Pathogenic staphylococci in poultry. J. Appl. Bact. 50:357.

———. 1984. Identification and characteristics of staphylococci isolated from lesions and normal skin of horses. Vet. Microbiol. 10:269–277.

——— 1990. Staphylococci in healthy and diseased animals. J. Appl. Bact. Symposium Supplement, 71S–80S.

Devriese, L. A., and J. Hommez. 1975. Epidemiology of methicillin-resistant *Staphylococcus aureus* in dairy herds. Res. Vet. Sci. 19:23–27.

Devriese, L. A., C. Godard, L. Okerman, and L. Renault. 1981. Characteristics of *Staphylococcus aureus* strains from rabbits. Ann. Rech. Vét. 12:327–332.

Devriese, L. A., H. Laevens, F. Haesebrouck, and J. Hommez. 1994. A simple identification scheme for coagulase negative staphylococci from bovine mastitis. Res. Vet. Sci. 57:240–244.

Dufour, P., S. Jarraud, M. Bes, F. Vandenesch, J. Etienne, and G. Lina. 2000. A polymorphic regulation system in *Staphylococcus aureus*. Med. Maladies Infect. 30:739–744.

Edwards, V. M., J. R. Deringer, S. D. Callantine, C. F. Deobald, P. H. Berger, V. Kapur, C. V. Stauffacher, and G. A. Bohach. 1997. Characterization of the canine type C enterotoxin produced by *Staphylococcus intermedius* pyoderma isolates. Infect. Immun. 65:2346–2352.

Etz, H., D. B. Minh, T. Henics, A. Dryla, B. Winkler, C. Triska, A. P. Boyd, J. Sollner, W. Schmidt, U.

von Ahsen, M. Buschle, S. R. Gill, J. Kolonay, H. Khalak, C. M. Fraser, A. von Gabain, E. Nagy, and A. Meinke. 2002. Identification of in vivo expressed vaccine candidate antigens from *Staphylococcus aureus*. P. Natl. Acad. Sci. U S A 99:6573–6578.

Farrell, A. M., Taylor, D., and K. T. Holland. 1995. Cloning, nucleotide sequence determination and expression of the *Staphylococcus aureus* hyaluronate lyase gene. FEMS Microbiol. Lett. 130:81–85.

Fraser, J., Arcus, V., Kong, P., Baker, E., and T. Proft. 2000. Superantigens—powerful modifiers of the immune system. Mol. Med. Today 6:125–132.

Frebourg, N. B., S. Lefebvre, S. Baert, and J. F. Lemeland. 2000. PCR-based assay for discrimination between invasive and contaminating *Staphylococcus epidermidis* strains. J. Clin. Microbiol. 38:877–880.

Freney, J., W. E. Kloos, V. Hajek, and J. A. Webster. 1999. Recommended minimal standards for description of new staphylococcal species. Int. J. Syst. Bacteriol. 49:489–502.

Frost, A. J. 1975. Selective adhesion of microorganisms to the ductular epithelium of the bovine mammary gland. Infect. Immun. 12:1154–1156.

Fthenakis, G. C., R. R. Marples, J. F. Richardson, and J. E. T. Jones. 1994. Some properties of coagulase-negative staphylococci isolated from cases of ovine mastitis. Epidemiol. Infect. 112:171–176.

Gillaspy, A. F., C. Y. Lee, S. Sau, A. L. Cheung, and M. S. Smeltzer. 1998. Factors affecting the collagen binding capacity of *Staphylococcus aureus*. Infect. Immun. 66:3170–3178.

Grov, A. 1973. Studies on the interaction between staphylococcal protein A and the Fc region of immunoglobulin G. Acta Path. Microbiol. Scand. Sect. A. Suppl. 236:77–83.

Hagen, K. W. 1963. Disseminated staphylococcic infection in young domestic rabbits. J. Am. Vet. Med. Assoc. 142:1421–1422.

Harvey, R. G., and W. C. Noble. 1998. Aspects of nasal, oropharyngeal and anal carriage of *Staphylococcus intermedius* in normal dogs and dogs with pyoderma. Vet. Dermatol. 9:99–104.

Harvey, R. G., R. R. Marples, and W. C. Noble. 1994. Nasal carriage of *Staphylococcus intermedius* in humans in contact with dogs. Microb. Ecol. Health. D. 7:225–227.

Hermans, K., P. De Herdt, L. A. Devriese, W. Hendrickx, C. Godard, and F. Haesebrouck. 1999. Colonization of rabbits with *Staphylococcus aureus* in flocks with and without chronic problems of staphylococcosis. Vet. Microbiol. 67:37–46.

Hesselbarth, J., and S. Schwarz. 1995. Comparative ribotyping of *Staphylococccus intermedius* from dogs, pigeons, horses and mink. Vet. Microbiol. 45:11–17.

Ho, G., W. H. Campbell, M. S. Bergdoll, and E. Carlson. 1989. Production of a toxic shock syndrome variant by *Staphylococcus aureus* strains associated with sheep, goats and cows. J. Clin. Microbiol. 27:1946–1948.

Huebner, J., and D. A. Goldmann. 1999. Coagulase-negative staphylococci: role as pathogens. Annu Rev. Med. 50:223–236.

Igimi, S., E. Takahashi, and T. Mitsuoka. 1990. *Staphylococcus schleiferi* subsp *coagulans* subspnov, isolated from the external auditory meatus of dogs with external ear otitis. Int. J. Syst. Bacteriol. 40:409–411.

Jarp, J. 1991. Classification of coagulase–negative staphylococci isolated from bovine clinical and subclinical mastitis. Vet. Microbiol. 27:151–158.

Kloos, W. E., and K. H. Schleifer. 1986. Genus IV. *Staphylococcus*. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and, J. G. Holt (eds.). Bergey's manual of systematic bacteriology.. Baltimore: Willliams and Wilkins, pp. 1013–1035.

Kuusela, P. 1978. Fibronectin binds to *Staphylococcus aureus*. Nature 276:718–720.

Lam, T. J. G. M., Y. H. Schukken, J. H. Van Vliet, F. J. Grommers, M. J. M. Tielen, and A. Brand. 1997. Effect of natural infection with minor pathogens on susceptibility to natural infection with major pathogens in the bovine mammary gland. Am. J. Vet. Res. 58:17–22.

Lammers, A., P. J. M. Nuijten, E. Kruijt, N. Stockhofe-Zurwieden, U. Vecht, H. E. Smith, and F. G. van Zijderveld. 1999. Cell tropism of *Staphylococcus aureus* in bovine mammary cell cultures. Vet. Microbiol. 67:77–89.

Lämmler, C., J. C. Defreitas, G. S. Chatwal, and H. Blobel. 1985. Interactions of immunoglobulin G, fibrinogen and fibronectin with *Staphylococcus hyicus* and *Staphylococcus intermedius*. Zbl. Bakt.—Int. J. Med. M. 260:232–237.

Mamo, W., G. Froman, and H. P. Muller. 2000. Protection induced in mice vaccinated with recombinant collagen-binding protein (CnBP) and alpha-toxoid against intramammary infection with *Staphylococcus aureus*. Microbiol. Immunol. 44:381–384.

Mamo, W., G. Froman, and T. Wadstrom. 1988. Interaction of sub-epithelial connective tissue components with *Staphylococcus aureus* and coagulase-negative staphylococci from bovine mastitis. Vet. Microbiol. 18:163–176.

Mamo, W., M. Boden, and J. I. Flock. 1994a. Vaccination with *Staphylococcus aureus* fibrinogen

binding proteins (FgBPs) reduces colonisation of *S. aureus* in a mouse mastitis model. FEMS Immunol. Med. Microbiol. 10:47–53.

Mamo, W., P. Jonsson, J. I. Flock, M. Lindberg, H. P. Muller, , T. Wadstrom, and L. Nelson. 1994b. Vaccination against *Staphylococcus aureus* mastitis: immunological response of mice vaccinated with fibronectin-binding protein (FnBP-A) to challenge with *S. aureus*. Vaccine 12:988–992.

Matthes, S. 1995. Staphylokokkeninfektionen bei Kaninchen und Pelztieren. 9. Arbeitstagung über Haltung und Krankheiten der Kaninchen, Pelztiere und Heimtiere, 242–250.

McCullagh, J. J., P. T. McNamee, J. A. Smyth, and H. J. Ball. 1998. The use of pulsed field gel electrophoresis to investigate the epidemiology of *Staphylococcus aureus* infection in commercial broiler flocks. Vet. Microbiol. 63:275–281.

McGavin, M. J., C. Zahradka, K. Rice, and J. E. Scott. 1997. Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. Infect. Immun. 65:2621–2628.

McNamara, P. J., and J. J. Iandolo. 1998. Genetic instability of the global regulator *agr* explains the phenotype of the *xpr* mutation in *Staphylococcus aureus* KSI9051. J. Bacteriol. 180:2609–2615.

McNamee, P. T., and J. A. Smyth. 2000. Bacterial chondronecrosis with osteomyelitis ("femoral head necrosis") of broiler chickens: a review. Avian Pathol. 29:253–270.

McNamee, P. T., J. J. McCullagh, J. D. Rodgers, B. H. Thorp, H. J. Ball, T. J. Connor, D. McConaghy, and J. A. Smyth. 1999. Development of an experimental model of bacterial chondronecrosis with osteomyelitis in broilers following exposure to *Staphylococcus aureus* by aerosol, and inoculation with chicken anaemia and infectious bursal disease viruses. Avian Pathol. 28:26–35.

Mirt, D. 1999. Lesions of so-called flank biting and necrotic ear syndrome in pigs. Vet. Rec. 144:92–96.

Nelson, L., J. I. Flock, M. Hook, M. Lindberg, H. P. Muller, and T. Wadstrom. 1991. Adhesins in staphylococcal mastitis as vaccine components. In, C. Burvenich, G. Vandeputte-Van Messom, and A. W. Hill (eds.). New insights into the pathogenesis of mastitis. Gent, Flem. Vet. J., 111–125.

Nilsson, I. M., O. Hartford, T. Foster, and A. Tarkowski. 1999. Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. Infect. Immun. 67:1045–1049.

Noble, W. C., and D. H. Lloyd. 1997. Pathogenesis and management of wound infections in domestic animals. Vet. Dermatol. 8:243–248.

Okerman, L., L. A. Devriese, L. Maertens, F. Okerman, and C. Godard. 1984. Cutaneous staphylococcosis in rabbits. Vet. Rec. 114:313–315.

Projan, S. J., and R. P. Novick. 1997. The molecular basis of pathogenicity. Crossley, K. B., and G. L. Archer, The Staphylococci in Human Disease. Pp. 55–81. New York: Churchill Livingstone Inc.

Rossi, G., C. Stanzel, and W. Witte. 1995. *Staphylococcus aureus* infections in the rabbit and the transmission of the pathogens with the sperma. 9. Arbeitstagung über Haltung und Krankheiten der Kaninchen, Pelztiere und Heimtiere, 251–257.

Schleifer, K. H., W. P. Hammes, and O. Kandler. 1976. Effect of endogenous and exogenous factors on the primary structures of bacterial peptidoglycan. Adv. Microb. Physiol. 13:245–292.

Schmitz, F. J., K. E. Veldkamp, K. P. M. Van Kessel, J. Verhoef, and J. A. G. Van Strijp. 1997. Delta-toxin from *Staphylococcus aureus* as a costimulator of human neutrophil oxidative burst. J. Infect. Dis. 176:1531–1537.

Skeeles, J. K. 1991. Staphylococcosis. In B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder (eds.). Diseases of poultry. Ames: Iowa State University Press, pp.293–299

Soell, M., M. Diab, A. G. Haan, A. Beretz, C. Herbelin, B. Poutrel, and J. P. Klein. 1995. Capsular polysaccharide types 5 and 8 of *Staphylococcus aureus* bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect. Immun. 63:1380–1386.

Staali, L., H. Monteil, and D. A. Colin. 1998. The staphylococcal pore-forming leukotoxins open Ca2+ channels in the membrane of human polymorphonuclear neutrophils. J. Membr. Biol. 162:209–216.

Takahashi, T., I. Satoh, and N. Kikuchi. 1999. Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. Int. J. Syst. Bacteriol. 49:725–728.

Takeuchi, S., Y. Kobayashi, T. Morozumi, and S. Niibori. 1985. Isolation and some properties of *Staphylococcus hyicus* subsp. *hyicus* from pigs, chickens and cows. Jpn. J. Vet. Sci. 47:841–843.

Tanabe, T., H. Sato, M. Kuramoto, and H. Saito. 1993. Purification of exfoliative toxin produced by *Staphylococcus hyicus* and its antigenicity. Infect. Immun. 61:2973–2977.

Varaldo, P. E., R. Kilpperbalz, F. Biavasco, G. Satta, and K. H. Schleifer. 1988. *Staphylococcus delphini* sp-nov, a coagulase-positive species isolated from dolphins. Int. J. Syst. Bacteriol. 38:436–439.

Wanasinghe, D. D. 1981. In vitro adherence of *Staphylococcus aureus* to bovine mammary gland epithelial cells. Acta Vet. Scand. 22:99–108.

Wegener, H. C., and E. W. Skov-Jensen. 1992. Exudative epidermitis. In B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (eds.). Disease of swine, 8th ed. Ames: Iowa State University Press, pp. 469–474.

Zhang, S. L., and C. W. Maddox. 2000. Cytotoxic activity of coagulase-negative staphylococci in bovine mastitis. Infect. Immun. 68:1102–1108.

Zhu, X. Y., and P. Y. Hester. 2000. The etiology of staphylococcosis. Avian Poult. Biol. Rev. 11:97–112.

5
Bacillus anthracis

M. A. Weiner and T. C. Dixon

Anthrax is a disease that occurs when *Bacillus anthracis* endospores enter a host through inhalation, ingestion, or abrasions in the skin. *B. anthracis* most commonly infects grazing herbivores, but can infect humans and a wide range of animal species. Anthrax manifests quickly in most infected hosts and may result in deadly infections that are difficult to treat because of the sudden onset of disease, the first symptom of which is often death in animals. Systemic infections, which mainly develop from ingested or inhaled endospores, have a mortality rate approaching 100%. In infection of animals with *B. anthracis,* death may occur with no signs of illness, unlike human cases of anthrax where symptoms typically precede death by several days. Whatever the portal of entry, systemic anthrax is a massive bacteremia and toxemia with nondescript initial symptoms until the onset of hypotension, shock, and sudden death. Manifestations of advanced disease including shock and death are believed to be the result of lethal toxin (LeTx) secreted by anthrax bacilli (Hanna 1998; Hanna et al. 1993).

EPIDEMIOLOGY

B. anthracis is a highly monomorphic species with little genetic variance between strains and is geographically ubiquitous in soil. Outside of the laboratory, vegetative bacilli exist only in host organisms. There has never been a documented report of vegetative bacilli outside of a host in the environment. There are six major strains of *B. anthracis,* which can be divided into types A and B, both of which can cause lethal infections (Keim et al. 1997, 1999). *B. anthracis* Sterne, Vollum, and Ames are all type A. *B. anthracis* Vollum and Ames are fully virulent strains, whereas *B. anthracis* Sterne is a vaccine strain lacking the virulence plasmid pXO2. Type A strains of *B. anthracis* have been identified throughout the world, while type B strains have been isolated only in Southern Africa (Keim et al. 1997; Smith et al. 1999, 2000). The increased diversity of *B. anthracis* in sub-Saharan Africa has led Keim et al. (1997, 1999) to suggest that *B. anthracis* may have originated there.

Naturally occurring anthrax enzootics are ongoing throughout the world with recent attention being paid to outbreaks in North Dakota, the Northwest Territory of Canada, France, Australia, Kruger National Park in South Africa, Etosha National Park in Namibia, and southern India (Lindeque and Turnbull 1994; Patra et al. 1998; Turnbull et al. 1998; Dragon et al. 1999; Smith et al. 1999; Turner et al. 1999a, 1999b**;** Vijaikumar et al. 2002). Human anthrax is related to the occurrence of disease in animals in an area.

Studies performed in Etosha National Park in Namibia on the airborne movement of endospores at the sites of infections demonstrated that environmental spores are associated with heavy particles and are not likely to be airborne, despite rain, wind, or slight soil agitation (Turnbull et al. 1998). It is therefore unlikely that animals in proximity to the site of an infected carcass would contract anthrax via inhalation (Turnbull et al. 1998). Naturally occurring cases of inhalation anthrax among animal populations in confined areas have been suggested to occur when large quantities of dust are stirred into the air. During the late summer rut, sexually mature male bison stomp violently and throw themselves into the ground repeatedly, aerosolizing clouds of contaminated dust. It is interesting that

outbreaks of disease in Canada show a disproportionate number of sexually mature bulls that die of anthrax, although confirmation of inhalation anthrax versus gastrointestinal or oropharyngeal anthrax was not possible (Dragon et al. 1999), and inhalation anthrax has not been demonstrated to occur naturally in animals.

Outbreaks of anthrax usually occur during dry months in open plains (Smith et al. 1999, 2000). The Kruger National Park (KNP) outbreak occurred after a wet spring during the dry months of summer when the temperature was consistently above average for that time of year. Likewise, the conditions under which anthrax outbreaks occur in the American Southwest and the Canadian Northwest are dependent on hot and dry weather. One theory suggests that a wet spring that involves flooding may cause spore-laden soil to drain into a central area that, upon drying out, becomes the infectious focus of an outbreak (Turner et al. 1999b). The location of cases of anthrax in animals in Etosha National Park in Namibia correlated with the geographic area where the floodwater drained (Turnbull et al. 1998). A second way in which infectious foci can be created is by major geologic processes that bring spores up to the soil surface where they can then infect animals.

There is renewed public interest in anthrax after the recent bioterrorist attack in the United States. Between October 4 and November 20, 2001, 11 cases of inhalation anthrax, 7 cases of cutaneous anthrax, and 5 suspected cases of cutaneous anthrax in humans were reported to the Centers for Disease Control and Prevention (CDC) (Bush et al. 2001; Lane and Fauci 2001; Barakat et al. 2002; Freedman et al. 2002; Mina et al. 2002).

The devastating effects of aerosol dispersion of anthrax spores were demonstrated on Gruinard Island, UK (Manchee 1981; Manchee et al. 1982; Sterne 1982; Wynn 1982), and in Sverdlovsk (former USSR) (Abramova et al. 1993; Meselson et al. 1994; Jackson et al. 1998). In 1979, *B. anthracis* spores released from a Soviet bioweapons facility in Sverdlovsk caused human and animal fatalities (Meselson et al. 1994). Identification and analysis of anthrax-infected cattle killed in the Sverdlovsk outbreak determined the range and the pattern of the spore dispersal. These findings combined with meteorological data from Sverdlovsk at the time of the outbreak serve as the most current model for the aerosol dispersal of anthrax spores into the environment (Meselson et al. 1994). The area of the outbreak was narrow in width and extended more than 50 km downwind from the suspected site of spore dispersal (Meselson et al. 1994). There are also references to past use of anthrax as a biological weapon during the Second World War by the Japanese (Harris 1994). Because anthrax is endemic in several parts of the world including parts of the United States and Canada (Fox et al. 1977; Shireley et al. 2001; Dragon et al. 1999), and because of its potential use as a biological weapon, it is imperative that veterinarians and physicians be familiar with the clinical manifestations of this disease in humans and animals.

BACTERIOLOGY

Bacillus anthracis is a nonmotile, gram-positive, aerobic rod measuring 1.2–10 μm x 0.5–2.5 μm that is capable of forming central or terminal endospores (Claus and Berkeley 1986) (fig. 5.1). *B. anthracis* is part of the *B. cereus* group of *Bacillus* spp. consisting of *B. cereus*, *B. thuringiensis*, and *B. mycoides* (Turnbull et al. 1992). Except for *B. anthracis*, all members of this group are resistant to penicillin, a phenotype attributed to the production of chromosomally encoded β-lactamases (Penn and Klotz 1998a). The unique colony morphology of *B. anthracis* on blood agar aids in differentiation from other members of the *B. cereus* group. Colonies of *B. anthracis* are nonhemolytic and white to gray, often having a ground-glass appearance (Parry et al. 1983). Microscopic examination of the irregular edges of the colony shows a "medusa head" appearance with long chains of rods growing out and curving back into the colony. The colonies are unusually tenacious and retain their shape when manipulated.

B. anthracis forms a characteristic poly-D-glutamic acid capsule on nutrient agar containing 0.7% bicarbonate and incubated overnight at 37°C in the presence of 5–20% CO_2 (Green et al. 1985). These colonies are mucoid, and the capsule can be observed microscopically in a smear stained with McFadyean's polychrome methylene blue, or Giemsa or Wright's stain (Parry et al. 1983). These stains show a blue organism surrounded by a pink to purplish capsule. Blood samples obtained from patients late in the course of infection and stained in the same manner show large numbers of encapsulated bacilli arranged singly, in pairs, or occasionally in short chains. *B. anthracis* can also be observed in, and cultured from, ascites fluid, pleural effusions, and cerebrospinal fluid (CSF) (in cases of meningitis) (Penn and Klotz 1998b).

If samples are contaminated with other *Bacillus* spp., polymyxin-lysozyme-EDTA-thallous acetate

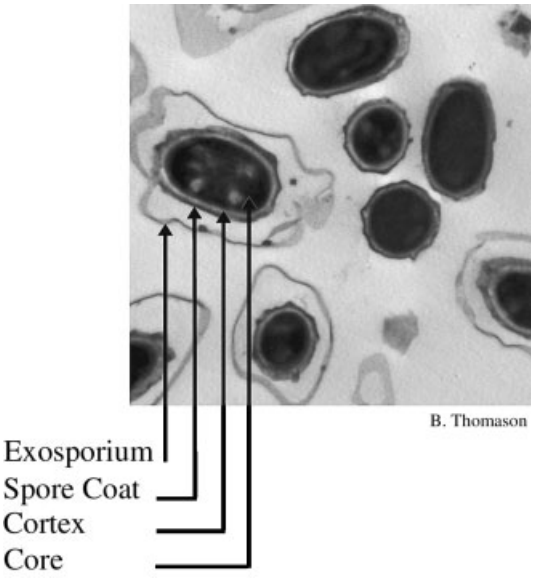


Figure 5.1. Transmission electron micrograph of *B. anthracis* endospores 19,000X. Exosporia can be seen surrounding some but not all of the endospores. Exosporia were likely sheared off of samples during their preparation.

(PLET) agar may be used as a selective agar for *B. anthracis* (Knisely 1966). The API 50 CH test strip (API Laboratory Products, Inc., Plainview, NY) can be used in conjunction with the API 20E test strip to identify a number of *Bacillus* spp., including *B. anthracis* (Logan et al. 1985). Confirmation of *B. anthracis* can also be achieved by using gamma phage lysis and by detection of capsule and cell-wall antigens by direct fluorescent antibody assays (Barakat et al. 2002). Additionally, PCR can identify *B. anthracis*-specific DNA. Multiple locus variable number tandem repeat (VNTR) analysis can also be performed to type unknown isolates against different strains of *B. anthracis* (Barakat et al. 2002). Blood cultures, in cases of systemic anthrax infections, are almost always positive because of the large numbers ($>10^8$ bacterial cells/ml) in circulation (Hanna 1998). Cultures from skin lesions, however, are positive in only 60–65% of cases, perhaps because of the microbicidal activity of local antagonistic skin flora (Shlyakhov and Rubinstein 1996). Relatively few endospores are required to cause cutaneous anthrax, and the titer of vegetative bacilli that grow at the site of infection may be low. Due to the potential of deliberately engineered new strains of *B. anthracis*, antibiotic susceptibilities should be performed on all isolated cultures.

Sporulation of *B. anthracis* occurs in an opened carcass under conditions of nutrient deprivation in a decomposing host and results in the formation of mature endospores that are stable and resistant to harsh environmental conditions. Sporulation requires aerobic growth conditions, and in the unopened carcass the vegetative forms do not survive putrefaction. Sporulation begins with an asymmetric septation of a vegetative bacillus (mother cell). The mother cell and the forespore compartment are both involved in the sporulation process during which the forespore compartment matures and the mother cell surrounding the spore dies, producing one endospore per mother cell.

Endospores are made of a dehydrated core containing protein, DNA, calcium, and dipicolinic acid (DPA). Calcium and DPA, together, stabilize the core in the absence of water by mimicking hydrogen bonding. Immediately surrounding the core is a paracrystaline lipid bilayer that is believed to contain germination receptors. The spore cortex surrounding the membrane is composed of a thick cross-linked peptidoglycan. Around the cortex are the spore coats and the exosporangium, respectively, which are deposited by the mother cell during sporulation. It is believed that the cortex and the spore coats are, in part, responsible for excluding water from the interior of the spore.

Endospore germination requires Ger proteins encoded by *ger* operons. In *B. anthracis*, there are five chromosomal and one pXO1 encoded *ger*-like operons, each of which facilitates the germination of spores in the presence of specific nutrients termed germinants. The *ger* operons encode three products, two of which have been implicated in the recognition of germinant molecules (Moir et al. 1994; Paidhungat and Setlow 1999). Deletion of specific *ger* operons abrogates endospore germination in the presence of the appropriate germinants. The germinants recognized by *B. anthracis* that are sufficient to trigger germination include L-alanine alone, and specific binary combinations of L-alanine (low concentrations that are subgerminal) with several other amino acids or with purine nucleosides (Ireland and Hanna 2002; Weiner et al. 2003).

Endospores utilize different germinant sensors to respond to different environments. *B. anthracis* endospores likely utilize multiple germinant sensors to distinguish between the complex environment of the host and the *ex vivo* environment. Germination of endospores is characterized by the degradation of the cortex and the hydration of the core. As water floods the core, calcium and dipicolinic acid are

expelled into the extracellular environment. Expulsion of dipicolinic acid from the core activates cortex lytic enzymes that degrade the spore cortex, a modified cross-linked peptidoglycan.

Endospores have no measurable metabolism, are completely inert, and do not replicate. *B. anthracis* endospores, like endospores from other species, are quite robust and resistant to drying, heat, UV light, and many disinfectants. Endospores have no ATP production, no macromolecular synthesis, no active enzymology, and little or no water. Endospores can remain dormant in the environment for decades, if not centuries, until they encounter a host. All important *B. anthracis* virulence genes, indeed all genes, are expressed *de novo* upon entry into the body.

PATHOGENESIS

Anthrax is one of the most ancient diseases observed by humans. It is believed to have been one of the Egyptian plagues during the time of Moses, and cases were clearly recorded by the ancient Greeks (Dirckx 1981). The anthrax bacillus was the model first used in the development of Koch's postulates and was the first "germ" (Koch 1877). Pasteur later generated a toxin-null anthrax strain that was used as the first live, attenuated bacterial vaccine (Pasteur 1881).

Upon entry into a host, endospores are rapidly phagocytosed by regional macrophages, which then migrate to regional lymph nodes. Evidence suggests that endospore*s* can germinate inside the macrophage, where they outgrow into vegetative bacteria (Ross 1957). Vegetative bacteria are released from the macrophage and grow to high titers in the blood, up to 10^7–10^8 per ml of blood, causing a massive bacteremia. *B. anthracis* expresses virulence factors, including toxins, which facilitate both the escape of bacilli from phagosomes and their release from the macrophage (Dixon et al. 2000). The resulting toxemia is intimately associated with systemic symptoms of anthrax. New clinical reports obtained during the 2001 terrorist attacks in the United States describe a two-stage cycle of disease in which there is initial mediastinal andenopathy caused by vegetative bacilli and subsequent progression involving widespread toxin release, septicemia, and death (Mayer et al. 2001).

The major virulence factors of *B. anthracis*, capsule and toxins, are encoded on two large virulence plasmids, pXO1 and pXO2, which are both required for full virulence. Vaccine strains against anthrax are attenuated for virulence and contain either pXO1 or pXO2, but not both. *B. anthracis* Sterne is an attenuated strain that was isolated in the 1930s in Africa; it carries only pXO1 and can synthesize both lethal toxin (LeTx) and edema toxin (EdTx), but lacks a capsule. The Pasteur strain of *B. anthracis* carries only pXO2 and is encapsulated but does not express either LeTx or EdTx.

The plasmid pXO1 is 184.5 kilo-basepairs (kbp) in size and encodes the genes that make up the tripartite toxin. The toxin gene complex is made up of protective antigen (*pag*, PA), lethal factor (*lef*, LF), and edema factor (*cya*, EF) (Leppla 1991). The three exotoxin components combine to form two binary toxins that act as potent enzymes in the cytoplasm of target host cells. EdTx consists of edema factor, a calmodulin-dependent adenylate cyclase (Leppla 1982, 1984), and protective antigen, the host-cell-binding moiety that facilitates entry of edema factor into the target cell cytoplasm. An increase in cellular cAMP disrupts water homeostasis and is believed to be responsible for the massive edema seen in cutaneous anthrax.

LeTx consists of lethal factor and protective antigen. Lethal factor is a zinc-metalloprotease (Hanna et al. 1994; Klimpel et al. 1994; Hammond and Hanna 1998) that *in vitro* cleaves a variety of substrates and inactivates MAPKK (Duesberry et al. 1998; Vitale et al. 1998). LF also uses protective antigen as its means to enter cells. Recent studies have provided valuable new information on the interaction of LF with host cells (Chaudry et al. 2002; Abrami et al. 2003). The 83 kDa PA binds to a 368 amino acid cell surface protein (anthrax toxin receptor, ATR) and is then cleaved by furin, leaving a 63 kDa fragment (PA63) bound to ATR. LF binds to heptamerized PA63 and is internalized by a specific endocytic pathway in a process that involves both lipid rafts and clathrin. ATR is considered a potential target for ameliorating the effects of the toxin in the host.

Lethal toxin triggers both the oxidative burst pathway in macrophages and concomitant release of shock-inducing cytokines TNF-α and Il-1β**,** which are believed to be responsible for the shock and sudden death seen in systemic anthrax (Hanna et al. 1992, 1993, 1994). Mice depleted of their macrophages were immune to challenge with LeTx, while mice containing macrophages showed symptoms of anthrax including shock, which resulted in their deaths after challenge with LeTx (Hanna et al. 1993).

The plasmid pXO2 is 95.3 kbp and encodes genes involved in the synthesis of the poly-D-glutamic acid capsule (*capB*, *capC*, and *capA*) (Makino et al. 1989; Dixon et al. 1999). The negatively charged

capsule inhibits the phagocytosis of vegetative cells by macrophages. Beneath the capsule is an S-layer, which is made up of two proteins and is not required for capsulation (Fouet et al. 1999). The S-layer proteins are produced *in vivo,* but their contribution to virulence is not known. Antibodies against the S-layer proteins failed to penetrate the capsule (Fouet et al. 1999), but the toxins are able to pass from the cell through the capsule to the exterior.

Expression of the virulence factors (capsule and toxins) is regulated by host-specific signals including increased temperature (37°C), CO_2 concentration (5%), and the presence of serum components (Makino et al. 1988; Dai et al. 1995).

Anthrax is unique in that it rapidly progresses from a mild disease with nondescript symptoms to death. It is very difficult to distinguish superficially between gastrointestinal and inhalation cases in animals because the disease is not often detected until after the death of the animal. A recent study of outbreaks in northern Canada discussed the pathology of anthrax in bison (Dragon et al. 1999). Features of the disease include edema of the ventral abdomen, sloughing of the cape hair, and a bloody discharge from the mouth and anus. At necropsy, the researchers noted hemorrhagic, yellow, gelatinous subcutaneous exudates; enlarged hemorrhagic lymph nodes; dark blood that clotted poorly; and a dark enlarged spleen. Except for the sloughing of the hair, similar observations have been reported for other species in which septicemic disease develops.

There is considerable variation in the susceptibility of various animal species to *B. anthracis*. Among the domestic animals, cattle, sheep, and goats appear to be among the most susceptible, with horses being intermediate, and pigs and dogs being resistant. Although carrion appear to be resistant, domestic ostriches have developed acute disease following infection. The disease in ostriches was important in earlier times but is now rare (Verwoerd 2000). Disease also occurs from time to time in bison, white-tailed deer, mink, and moose. Guinea pigs are highly susceptible and are used for experimental determination of pathogenicity and virulence. Rats are resistant and are used to follow the course of infection in resistant species. In the resistant species, the organisms are observed to multiply briefly then suffer dispersal of their capsules followed by rapid extracellular destruction. Some strains of mice are susceptible whereas others are resistant.

In cattle, sheep, and goats, the disease usually runs an acute course, with high fever, followed by death in 1 to 2 days. Dead animals may have blood exuding from the rectum, mouth, and nose. Sometimes there is sudden death, and in other cases the disease may last up to a week. Vaccinated animals with incomplete protection may show mild signs and may recover. Horses commonly develop an acute disease with death in 1 to 3 days. Biting flies may initiate disease in horses, in which subcutaneous edema is a prominent feature of the clinical picture.

Pigs are resistant to infection but sporadic disease and outbreaks have been reported, often after consumption of spore-contaminated feeds (Edginton 1990; Williams et al. 1992). Affected animals may suffer primarily digestive disturbances, or disease may be characterized by swelling of the throat, with lesions in the pharyngeal and laryngeal mucosa. Following experimental infection of pigs by contamination of feed with anthrax spores, 2 of 50 pigs died within 8 days and the others showed mild signs of disease before they recovered (Redmond et al. 1997).

Cutaneous Anthrax

In the United States, 224 cases of cutaneous anthrax in humans were reported between 1944 and 2000, of which only five were reported between 1984 and 2000 (Freedman et al. 2002). Patients often give an occupational history of contact with animals or animal products. The most common areas that are affected are the head, neck, and extremities, although any area of the body can be involved. Endospores are introduced subcutaneously via cut or abrasion, though infection may be transmitted by insect bites, presumably after the insect fed on an infected carcass (Turell and Knudson 1987; Bradaric and Punda-Polic 1992).

The name of the disease is derived from the Greek word *anthrakos* meaning coal, referring to the typical black eschar that is seen on affected areas. The early skin lesion is usually a nondescript pruritic papule appearing 3–5 days after introduction of endospores. In 24–36 hours, the lesion forms a vesicle that undergoes central necrosis and drying, leaving a characteristic black eschar surrounded by massive edema and a number of purplish vesicles (Dixon et al. 1999). The edema is usually more extensive on the head or neck than on the trunk or extremities (Smego et al. 1998). The common description "malignant pustule" is a misnomer, as the cutaneous lesion is characteristically painless and not purulent. If the eschar is painful and pustular, and the patient is febrile, it indicates

that a secondary infection, most often with *Staphylococcus* or *Streptococcus* spp., is likely present (Edwards 1992). Cutaneous anthrax can be self-limiting, but antibiotic treatment is recommended. Lesions resolve without complications or scarring in 80–90% of cases. Malignant edema is a rare complication characterized by severe edema, induration, multiple bullae, and symptoms of shock (Doganay et al. 1987; Kutluk et al. 1987). A few cases of temporal arteritis associated with cutaneous anthrax infection, as well as corneal scarring from cases of palpebral (eyelid) cutaneous anthrax have been reported (Yorston and Foster 1989; Doganay et al. 1994).

Histological examination of *B. anthracis* lesions shows necrosis and massive edema with lymphocytic infiltrates within the dermis. There is no evidence of liquefaction or abscess formation indicating that the lesions are not suppurative. Focal points of hemorrhage are observed with some instances of thrombosis (Mallon and McKee 1997). Gram-staining of subcutaneous tissues reveals numerous gram-positive bacilli (Mallon and McKee 1997).

Gastrointestinal Anthrax

Gastrointestinal anthrax is a highly fatal form of the disease that usually presents 2 to 5 days after the ingestion of meat contaminated with endospores from diseased animals. Contaminated meats can be directly consumed or can be dispersed in animal feed containing infected animal products. No human cases have ever been reported in the United States (Freedman et al. 2002). Among grazing herbivores, spores can enter a host through cuts or abrasions in the lining of the mouth and digestive tract that result from the chewing of contaminated grasses, plant material, or feed. Evidence in bovines suggests that spores enter the host through preexisting ulcerations of the gut (Patra et al. 1998). It is unclear exactly where the spores germinate within the gastrointestinal tract, but it is presumed that bacterial inoculation can occur at a breach in the mucosal lining. Pathologic examination has identified ulceration in patients with this disease. It is not clear whether ulceration is caused by the primary infection or by the action of anthrax toxins similar to that seen in cutaneous anthrax (Dutz et al. 1970, 1971, 1981). Other gastrointestinal symptoms in humans include fever and diffuse abdominal pain with rebound tenderness. There are reports of constipation and diarrhea where the stools are either melanic or blood tinged (Nalin et al. 1977; Alizad et al. 1995). Patients often vomit blood-tinged material, which is secondary to ulceration of the gastrointestinal mucosa.

Ascites develops with a concomitant reduction in abdominal pain 2–4 days after the onset of symptoms. The ascites fluid ranges from clear to purulent and often yields colonies of *B. anthracis* when cultured (LaForce 1994; Nalin et al. 1977). Morbidity is attributed to blood loss, fluid/electrolyte imbalances, and subsequent shock. Fatalities result from intestinal perforation, anthrax toxemia, or septicemia. If the patient survives, most of the symptoms subside in 10–14 days (Alizad et al. 1995). Microscopic examination of affected tissues reveals massive edema and mucosal necrosis at the site of infection (Dutz et al. 1981). Inflammatory infiltrates are similar to those seen in cutaneous anthrax.

Oropharygeal anthrax is less common than the classic gastrointestinal form in humans and is also associated with the ingestion of contaminated meat, or vegetation in the case of herbivores. Initial symptoms include cervical edema and local lymphadenopathy causing dysphagia and respiratory difficulties. Lesions can be seen in the oropharynx and usually have the appearance of pseudomembranous ulcerations. This is a moderate form of the disease and has a more favorable prognosis when treated (Edwards 1992; Alizad et al. 1995).

Inhalation Anthrax

Inhalation anthrax is the most threatening form of *B. anthracis* infection in humans. Without treatment, death usually occurs 1–7 days after the appearance of systemic symptoms. Endospores can remain intact in the lungs for more than 60 days before initiating an infection, which creates an incubation time for the disease that is dependent on the size of the inoculum (Barakat et al. 2002). Natural incidences of inhalation anthrax are rare, occurring after the inhalation of pathogenic endospores by workers handling contaminated animal hides or products ("Woolsorters' disease"). There were only 18 reported cases of inhalation anthrax in humans in the United States during the twentieth century. In the terrorist attacks in the United States between October 4 and November 20, 2001, 11 cases of inhalation anthrax were reported resulting in five deaths (Lane and Fauci 2001; Barakat et al. 2002). Previous reports of inhalation anthrax describe a mortality approaching 100% in untreated humans and animals. The reduced mortality rate among victims of the 2001 attacks was attributed to rapid diagnosis and aggressive antibiotic and supportive therapies.

B. anthracis spores are ideal for inhalation and deposition in alveolar spaces because of their size (Brachman et al. 1966; Brachman 1980; Penn and Klotz 1997). Endospores are phagocytosed by alveolar macrophage and are transported within the phagocytes to the mediastinal and parabronchial lymph nodes. Endospores germinate and outgrow in macrophage en route to the mediastinal lymph nodes, where the bacilli cause a hemorrhagic mediastinitis, and subsequently spread throughout the body in the blood (Albrink et al. 1960; Dutz et al. 1971; Bush et al. 2001; Lane and Fauci 2001; Mayer et al. 2001; Barakat et al. 2002; Mina et al. 2002).

The initial symptoms in humans can include cough (with or without blood-tinged sputum), fever, myalgias, and malaise that resemble a viral upper-respiratory infection (Bush et al. 2001; Mayer et al. 2001; Mina et al. 2002; Barakat et al. 2002). Early in the course of disease, chest radiographs show a widened mediastinum, evidence of hemorrhagic mediastinitis, and marked pleural effusions (Bush et al. 2001; Mina et al. 2002; Barakat et al. 2002; Mayer et al. 2001). Chest CTs reveal hyperdense enlarged mediastinal adenopathy and diffuse mediastinal edema, and non-contrast-enhanced CT of the chest shows much more dramatic evidence of mediastinal lymph node enlargement (Mayer et al. 2001). Hemorrhagic mediastinal lymph nodes represent the primary lesion.

In humans, the disease takes a fulminant course 1–3 days after the onset of severe symptoms with dyspnea, seizures, and subsequent respiratory failure, culminating in death (Edwards 1992; Penn and Klotz 1997; Bush et al. 2001). Inhalation anthrax does not cause clinical pneumonia (Bush et al. 2001; Mayer et al. 2001; Lane and Fauci 2001; Barakat et al. 2002; Mina et al. 2002), but autopsy series show foci of necrotizing pneumonia, presumably at the portal of infection (Abramova et al. 1993). In one recent case, partial collapse of the lung was noted with areas of subpleural and perivascular hemorrhage (Bush et al. 2001). Severe cases of pulmonary anthrax are also accompanied by *B. anthracis*-mediated hemorrhagic meningitis.

Meningitis

Involvement of the meninges by *B. anthracis* is a rare but life-threatening complication of primary anthrax (Tahernia and Hashemi 1972). The most common portal of entry is the skin, where organisms can germinate, grow, and spread to the central nervous system by hematogenous or lymphatic routes. Anthrax meningitis also occurs in inhalation and gastrointestinal anthrax (Abramova et al. 1993; Tabatabaie and Syadati 1993). This manifestation is potentially fatal with death occurring 1–6 days after the onset of illness, often despite intensive antibiotic therapy. In cases of survival, antibiotic therapy was combined with administration of antitoxin, prednisone, or both (Tahernia 1967; Tahernia and Hashemi 1972). In addition to common meningeal symptoms and nuchal rigidity, the patient experiences fever, fatigue, myalgias, headache, nausea, vomiting, and sometimes agitation, seizures, and symptoms of delirium. Initial signs are followed by rapid neurological degeneration and death. Pathological findings are consistent with hemorrhagic meningitis where there is extensive edema, inflammatory infiltrates, and numerous gram-positive bacilli in the leptomeninges (Dutz and Kohout 1971; Rangel and Gonzalez 1975). The cerebrospinal fluid is often bloody, revealing gram-positive bacilli on Gram staining (Pluot et al. 1976). At autopsy the leptomeninges exhibit massive hemorrhage giving an appearance described as “Cardinal’s Cap” (Abramova et al. 1993).

THERAPEUTICS

B. anthracis is uniformly susceptible to ciprofloxacin, doxycycline, chloramphenicol, clindamycin, rifampin, vancomycin, clarithromycin, penicillin, and amoxicillin (Lane and Fauci 2001). Ciprofloxacin is recommended as first-line treatment for inhalation anthrax (Inglesby et al. 2002). Intervention shortly after the onset of symptoms with antibiotics (including ciprofloxacin, rifampin, and clindamycin) and supportive therapies can slow the progression of the disease and result in patient survival (Mayer et al. 2001). Rifampin was chosen as a supplemental antibiotic because of its primarily intracellular site of action (Mayer et al. 2001). In cutaneous anthrax, treatment with oral penicillin renders lesions sterile after 24 hours, although they progress to eschar formation despite antibiotic therapy, perhaps because existing toxins are not affected by antibiotics. Supportive therapy should be initiated to prevent symptoms of septic shock or fluid/electrolyte imbalances, and to maintain airway patency.

In animals, treatment of individuals with acute disease is usually unrewarding. However, treatment with antibiotics is often effective in the resistant species such as pigs and dogs in which the disease is mild and runs a slow course. A high dose of penicillin is usually administered, and antibiotic treatment is

maintained for 6 weeks. Tetracycline in the feed has been used effectively to curtail an outbreak in pigs (Edginton 1990).

IMMUNITY AND CONTROL

The Sterne vaccine, which is a live spore vaccine attenuated by loss of the ability to produce a capsule, is the vaccine in common use for stimulating immunity to anthrax in animals. A dose consists of 2–10 million viable spores (Salmon and Ferrier 1992). Immunity appears to be largely due to anti-PA antibodies, but the vaccine also stimulates antispore activities, which appear to contribute to immunity (Welkos et al. 2001; Brossier et al. 2002). There is considerable evidence that anti-PA antibodies play a major role in protection, presumably by preventing binding of PA to host cells, thereby preventing the effects of EF and LF. PA-neutralizing titers are commonly used as an indicator of protective immunity. Immunization of mice with spores of a PA-deficient strain of *B. anthracis* induced protection against experimental challenge in guinea pigs and mice, leading to the conclusion that antispore antibodies may play an important role in protection (Brossier et al. 2002).

The Sterne vaccine has been effective in protecting animals against disease for over 60 years (Sterne 1937, 1939) and has been a valuable adjunct to the control of outbreaks and the eradication of the disease in many areas (Turnbull 1991; Salmon and Ferrier 1992; Hugh-Jones 1999; Turner et al. 1999b). Two doses of the vaccine induce immunity in cattle for a period of about 12 months. The vaccine may cause deaths in horses and goats and side effects in cattle. The side effects are attributable to the action of the toxin and include an elevation in temperature and a dependent edema.

Control of anthrax in domestic animals can be achieved by rapid identification of infected animals, vaccination, quarantine, enhanced surveillance, disposal of carcasses by burning and burial, and thorough disinfection of premises (Williams et al. 1992; Turner et al. 1999a, 1999b). In animal species, such as pigs, in which the disease may be mild or inapparent and organisms may not be detectable in traditional samples, rapid identification may present a problem. If the disease develops in an intensive pig rearing operation, the problem of satisfactory disposal of massive volumes of slurry is quite challenging (Williams et al. 1992). Eradication of the disease in wildlife is costly and difficult.

In humans, the current vaccine used in the United States is the anthrax vaccine adsorbed (AVA), which contains PA from a cell-free culture supernatant of the capsule-negative toxin-producing strain *B. anthracis* V770-NP1-R adsorbed to aluminum hydroxide (Bioport, Lansing, MI). AVA is used by military and civilian personnel who are at risk for *B. anthracis* infection (Lane and Fauci 2001). In the UK a similar vaccine is produced from a Sterne strain culture. These vaccines often cause injection site reaction and induce a short-lived immunity. A live attenuated strain of *B. anthracis* containing the pXO1 plasmid, but not pXO2, was produced and used in the former Soviet Union (Inglesby et al. 2002). The attenuated vaccine is not used in the United States because of safety concerns.

REFERENCES

Abrami, L., S. Liu, P. Cosson, S. H. Leppla, and F. G. van der Goot. 2003. Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. J. Cell Biol. 160:321–328.

Abramova, F. A., L. M. Grinberg, O. V. Yampolskaya, and D. H. Walker. 1993. Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979. Proc. Natl. Acad. Sci. U. S. A. 90:2291–2294.

Albrink, W. S., S. M. Brooks, R. E. Biron, and M. Kopel. 1960. Human inhalation anthrax: A report of three fatal cases. Am. J. Pathol. 36:457–471.

Alizad, A., E. M. Ayoub, and N. Makki. 1995. Intestinal anthrax in a two-year-old child. Pediatr. Infect. Dis. J. 14:394–395.

Barakat, L. A., H. L. Quentzel, J. A. Jernigan, D. L. Kirschke, K. Griffith, S. M. Spear, K. Kelley, D. Barden, D. Mayo, D. S. Stephens, T. Popovic, C. Marston, S. R. Zaki, J. Guarner, W-J. Shieh, H. W. Carver, R. F. Meyer, D. L. Swerdlow, E. E. Mast, and J. L. Hadler. 2002. Inhalational anthrax in a 94-year-old Connecticut woman. J.A.M.A. 287:863–878.

Brachman, P. S. 1980. Inhalation anthrax. Ann. N. Y. Acad. Sci. 353:83–93.

Brachman, P. S., A. F. Kaufman, and F. G. Dalldorf. 1966. Industrial inhalation anthrax. Bacteriol. Rev. 30:646–659.

Bradaric, N., and V. Punda-Polic. 1992. Cutaneous anthrax due to penicillin-resistant *Bacillus anthracis* transmitted by an insect bite [letter]. Lancet 340:306–307.

Brossier, F., M. Levy, and M. Mock. 2002. Anthrax spores make an essential contribution to vaccine efficacy. Infect. Immun. 70:661–664.

Bush, L. M., B. H. Abrams, A. Beall, and C. C. Johnson. 2001. Index case of fatal inhalational

anthrax due to bioterrorism in the United States. N. Engl. J. Med. 345:1607–1610.

Chaudry, G. J., M. Moayeri, S. Liu, and S. H. Leppla. 2002. Quickening the pace of anthrax research: three advances point towards possible therapies. Trends Microbiol. 10:58–62.

Claus, D., and R. C. W. Berkeley. 1986. Genus *Bacillus*. In Bergey's manual of systematic bacteriology, vol. 2. P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (eds.). Baltimore: Williams and Wilkins, pp. 1105–1139.

Dai, Z., J-C. Sirard, M. Mock, and T. M. Koehler. 1995. The *atxA* gene product activates transcription of the anthrax toxin genes and is essential for virulence. Mol. Microbiol. 16:1171–1181.

Dirckx, J. H. 1981. Virgil on anthrax. Am. J. Dermatopathol. 3:191–195.

Dixon, T. C., A. A. Fadl, T. M. Koehler, J. A. Swanson, and P. C. Hanna. 2000. Early *Bacillus anthracis*-macrophage interactions: intracellular survival and escape. Cell. Microbiol. 2:453–463.

Dixon, T. C., M. Meselson, J. Guillemin, and P. C. Hanna. 1999. Anthrax. New Engl. J. Med. 341:815–826.

Doganay, M., B. Aygen, M. Inan, O. Kandemir, and P. Turnbull. 1994. Temporal artery inflammation as a complication of anthrax. J. Infect. 28:311–314.

Doganay, M., M. Bakir, and I. Dokmetas. 1987. A case of cutaneous anthrax with toxaemic shock. Br. J. Dermatol. 117:659–662.

Dragon, D. C., B. T. Elkin, J. S. Nishi, and T. R. Ellsworth. 1999. A review of anthrax in Canada and implications for research on the disease in northern bison. J. Appl. Microbiol. 87:208–213.

Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel, T. D. Copeland, N. G. Ahn, M. K. Oskarsson, K. Fukasawa, K. D. Paull, and G. F. Vande Woude. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280:734–737.

Dutz, W., and E. Kohout. 1971. Anthrax. Pathol. Annu. 6:209–248.

Dutz, W., and E. Kohout-Dutz. 1981. Anthrax. Int. J. Dermatol. 20:203–206.

Dutz, W., F. Saidi, and E. Kohout. 1970. Gastric anthrax with massive ascites. Gut 11:352–354.

Edginton, A. B. 1990. An outbreak of anthrax in pigs: a practitioner's account. Vet. Rec. 127:321–324.

Edwards, M. S. Anthrax. In Textbook of pediatric infectious diseases, vol. 1. R. D. Feigin and J. D. Cherry (eds.). Philadelphia: W. B. Saunders, pp. 1053–1056.

Fouet, A., S. Mesnage, E. Tosi-Couture, P. Gounon, and M. Mock. 1999. *Bacillus anthracis* surface: capsule and S-layer. J. Appl. Microbiol. 87:251–255.

Fox, M. D., J. M. Boyce, A. F. Kaufmann, J. B. Young, and H. W. Whitford.1977. An epizootiologic study of anthrax in Falls County, Texas. J. Am. Vet. Med. Assoc. 130:327–333.

Freedman, A., O. Afonja, M. Wu Chang, F. Mostashari, M. Blaser, G. Perez-Perez, H. Lazarus, R. Schacht, J. Guttenberg, M. Traister, and W. Borkowsky. 2002. Cutaneous anthrax associated with microangiopathic hemolytic anemia and coagulopathy in a 7-month-old infant. JAMA 287:869–874.

Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins. 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. 49:291–297.

Hammond, S. E., and P. C. Hanna. 1998. Lethal factor active-site mutations affect catalytic activity in vitro. Infect. Immun. 66:2374–2378.

Hanna, P. 1998. Anthrax pathogenesis and host response. Curr. Top. Microbiol. Immunol. 225:13–35.

Hanna, P. C., B. A. Kruskal, R. A. Ezekowitz, B. R. Bloom, and R. J. Collier. 1994. Role of macrophage oxidative burst in the action of anthrax lethal toxin. Mol. Med. 1:7–18.

Hanna, P. C., D. Acosta, and R. J. Collier. 1993. On the role of macrophages in anthrax. Proc. Natl. Acad. Sci. U S A 90:10198–10201.

Hanna, P. C., S. Kochi, and R. J. Collier. 1992. Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells. Mol. Biol. Cell 3:1269–1277.

Harris, S. H. 1994. Factories of death: Japanese secret biological warfare, 1932–1945, and the American cover-up. London: Routledge.

Hugh-Jones, M. 1999. 1996–1997 global anthrax report. J. Appl. Microbiol. 87:189–191.

Inglesby, T. V., T. O'Toole, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. M. Friedlander, J. Gerberding, J. Hauer, J. Hughes, J. McDade, M. T. Osterholm, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat. 2002. Anthrax as a biological weapon, 2002 updated recommendations for management. JAMA 288:2236–2252.

Ireland, J. A., and P. C. Hanna. 2002. Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* ΔSterne endospores: *gerS* mediates responses to aromatic ring structures. J. Bacteriol. 184:1296–1303.

Jackson, P. J., M. E. Hugh-Jones, D. M. Adair, G. Green, K. K. Hill, C. R. Kuske, L. M. Grinberg, F. A. Abramova, and P. Keim. 1998. PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax

victims: the presence of multiple *Bacillus anthracis* strains in different victims. Proc. Natl. Acad. Sci. U S A 95:1224-1229.

Keim, P., A. Kalif, J. Schupp, K. Hill, S. E. Travis, K. Richmond, D. M. Adair, M. Hugh-Jones, C. R. Kuske, and P. Jackson. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. 179:818–824.

Keim, P., A. M. Klevytska, L. B. Price, J. M. Schupp, G. Zinser, K. L. Smith, M. E. Hugh-Jones, R. Okinaka, K. K. Hill, and P. J. Jackson. 1999. Molecular diversity in *Bacillus anthracis*. J. Appl. Microbiol. 87:215–217.

Klimpel, K. R., N. Arora, and S. H. Leppla. 1994. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol. Microbiol. 13:1093–1100.

Knisely, R. F. 1966. Selective medium for *Bacillus anthracis*. J. Bacteriol.92:784–786.

Koch, R. 1877. The aetiology of anthrax based on the otogeny of the anthrax bacillus. Beitr. Biol. Pflanz. 2:277–282.

Kutluk, M. T., G. Secmeer, G. Kanra, A. Celiker, and H. Aksoyek. 1987. Cutaneous anthrax. Cutis 40:117–118.

LaForce, F. M. 1994. Anthrax. Clin. Infect. Dis. 19:1009–1013.

Lane, H. C., and A. S. Fauci. 2001. Bioterrorism on the home front. JAMA 286:2595–2596.

Leppla, S. H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. U S A. 79:3162–3166.

———. *Bacillus anthracis* calmodulin-dependent adenylate cyclase: chemical and enzymatic properties and interactions with eucaryotic cells. Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17:189–198.

———. 1991. The anthrax toxin complex. In Sourcebook of bacterial protein toxins. J. Alouf (ed.). New York: Academic Press, pp.277–302.

Lindeque, P. M., and P. C. Turnbull. 1994. Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. Onderstepoort J. Vet. Res. 61:71–73.

Logan, N. A., J. A. Carman, J. Melling, and R. C. W. Berkeley. 1985. Identification of *Bacillus anthracis* by API tests. J. Med. Microbiol. 20:75–85.

Makino, S., C. Sasakawa, I. Uchida, N. Terakado, and M. Yoshikawa. 1988. Cloning and CO_2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. Mol. Microbiol. 2:371–376.

Makino, S-I., I. Uchida, N. Terakado, C. Sasakawa, and M. Yoshikawa. 1989. Molecular characterization and protein analysis of the cap region which is essential for encapsulation in *Bacillus anthracis*. J. Bact.171:722–730.

Mallon, E., and P. H. McKee. 1997. Extraordinary case report: cutaneous anthrax. Am. J. Dermatopathol.19:79–82.

Manchee, R. J. 1981. *Bacillus anthracis* on Gruinard Island. Nature 294:254–255.

Manchee, R. J., M. G. Broster, R. M. Henstridge, A. J. Stagg, and J. Melling. 1982. Anthrax island [letter]. Nature 296:598.

Mayer, T. A., S. Bersoff-Matcha, C. Murphy, J. Earls, S. Harper, D. Pauze, M. Nguyen, J. Rosenthal, D. Cerva, G. Druckenbrod, D. Hanfling, N. Fatteh, A. Napoli, A. Nayyar, and E. L. Berman. 2001. Clinical presentation of inhalational anthrax following bioterrorism exposure. JAMA 286:2549–2553.

Meselson, M., J. Guillemin, M. Hugh-Jones, A. Langmuir, I. Popova, A. Shelokov, and O. Yampolskaya. 1994. The Sverdlovsk anthrax outbreak of 1979. Science 266:1202–1208.

Mina, B., J. P. Dym, F. Kuepper, R. So, C. Arrastia, I. Kaplounova, H. Faraj, A. Kwapniewski, C. M. Krol, M. Grosser, J. Glick, S. Fochios, A. Remolina, L. Vasovic, J. Moses, T. Robin, M. DeVita, and M. L. Tapper. 2002. Fatal inhalational anthrax with unknown source of exposure in a 61-year-old woman in New York City. JAMA 287:858–862.

Moir, A., E. H. Kemp, C. Robinson, and B. M. Corfe. 1994. The genetic analysis of bacterial spore germination. J. Appl. Bact. 177:9S–16S.

Nalin, D. R., et al. 1977. Survival of a patient with intestinal anthrax. Am. J. Med. 162:130–132.

Paidhungat, M., and P. Setlow. 1999. Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. J. Bacteriol. 181:3341–3350.

Parry, J. M., P. C. B. Turnbull, and J. R. Gibson. 1983. A color atlas of *Bacillus* species, p. 272. London: Wolfe Medical Publications, Ltd.

Pasteur, L. 1881. De l'attenuation des virus et de leur retour a la virulence. CR Acad. Sci. Agric. Bulg. 92:429–435.

Patra, G., J. Vaissaire, M. Weber-Levy**,** C. Le Doujet, and M. Mock. 1998. Molecular characterization of *Bacillus* strains involved in outbreaks of anthrax in France in 1997. J. Clin. Microbiol. 36:3412–3414.

Penn, C. C., and S. A. Klotz. 1997. Anthrax pneumonia. Semin. Respir. Infect. 12:28–30.

———. 1998a. *Bacillus anthracis* and other aerobic spore-formers. In Infectious diseases. S. L.

Gorbach, J. G. Bartlett, and N. R. Blacklow (eds.). Philadelphia: W. B. Saunders, pp. 1747–1750.

———. 1998b. Anthrax. In Infectious diseases. S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (eds.). Philadelphia: W. B. Saunders, pp. 1575–1578.

Pluot, M., C. Vital, J. Aubertin, J. C. Croix, J. C. Pire, and D. Poisot. 1976. Anthrax meningitis. Report of two cases with autopsies. Acta Neuropathol. (Berl) 36:339–345.

Rangel, R. A., and D. A. Gonzalez. 1975. Bacillus anthracic meningitis. Neurology 25:525–530.

Redmond, C., G. A. Hall, P. C. Turnbull, and J. S. Gillgan. 1997. Experimentally assessed public health risks associated with pigs from farms experiencing anthrax. Vet. Rec. 141:244–247.

Ross, J. M. 1957. Pathogenesis of anthrax following administration of spores by the respiratory route. J. Pathol. Bacteriol. 73:485–494.

Salmon, D. D., and G. R. Ferrier. 1992. Post vaccination occurrence of anthrax in cattle. Vet. Rec. 130:140–141.

Shireley, L., T. Dwelle, D. Streitz, and L. Schuler. 2001. Human anthrax associated with an epizootic among livestock—North Dakota, 2000. MMWR 50:677–680.

Shlyakhov, E., and E. Rubinstein. 1996. Evaluation of the anthraxin skin test for diagnosis of acute and past human anthrax. Eur. J. Clin. Microbiol. Infect. Dis. 15:242–245.

Smego, R. A, Jr., B. Gebrian, and G. Desmangels. 1998. Cutaneous manifestations of anthrax in rural Haiti. Clin. Infect. Dis. 26:97–102.

Smith, K. L., V. De Vos, H. B. Bryden, L. B. Price, M. E. Hugh-Jones, and P. Keim. 2000. *Bacillus anthracis* diversity in Kruger National Park. J. Clin. Microbiol. 38:3780–3784.

Smith, K. L., V. De Vos, H. B. Bryden, M. E. Hugh-Jones, A. Klevytska, L. B. Price, P. Keim, and D. T. Scholl. 1999. Meso-scale ecology of anthrax in southern Africa: a pilot study of diversity and clustering. J. Appl. Microbiol. 87:204–207.

Sterne, M. 1937. Variation in *Bacillus anthracis*. Ondersteport J. Vet. Res. 8:271–349.

———. 1939. The use of anthrax vaccines prepared from avirulent (unencapsulated) variants of *Bacillus anthracis*. Ondersteport J. Vet. Res. 13:307–312.

———. 1982. Anthrax island [letter]. Nature 295:362.

Tabatabaie, P., and A. Syadati. 1993. *Bacillus anthracis* as a cause of bacterial meningitis. Pediatr. Infect. Dis. J. 12:1035–1037.

Tahernia, A. C. 1967. Treatment of anthrax in children. Arch. Dis. Child. 42:181–182.

Tahernia, A. C., and G. Hashemi. 1972. Survival in anthrax meningitis. Pediatrics 50:329–333.

Turell, M. J., and G. B. Knudson. 1987. Mechanical transmission of *Bacillus anthracis* by stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*). Infect. Immun. 55:1859–1561.

Turnbull, P. C. 1991. Anthrax vaccines: past, present and future. Vaccine. 9:533–539.

Turnbull, P. C., P. M. Lindeque, J. Le Roux, A. M. Bennett, and S. R. Parks. 1998. Airborne movement of anthrax spores from carcass sites in the Etosha National Park, Namibia. J. Appl. Microbiol. 84:667–676.

Turnbull, P. C., R. A. Hutson, J. J. Ward, M. N. Jones, C. P. Quinn, N. J. Finnie, C. J. Duggleby, J. M. Kramer, and J. Melling. 1992. *Bacillus anthracis* but not always anthrax. J. Appl. Bacteriol. 72:21–28.

Turner, A. J., J. W. Galvin, R. J. Rubria, and G. T. Miller. 1999a. Anthrax explodes in an Australian summer. J. Appl. Microbiol. 87:196–199.

Turner, A. J., J. W. Galvin, R. J. Rubria, R. J. Condron, and T. Bradley. 1999b. Experiences with vaccination and epidemiological investigations on an anthrax outbreak in Australia in 1997. J. Appl. Microbiol. 87:294–297.

Verwoerd, D. J. 2000. Ostrich diseases. Rev. Sci. Tech. 19:638–661.

Vijaikumar, M., D. M. Thappa, and K. Karthikeyan. 2002. Cutaneous anthrax: an endemic outbreak in south India. J. Trop. Pediatr. 48:225–226.

Vitale, G., R. Pellizzari, C. Recchi, G. Napolitani, M. Mock, and C. Montecucco. 1998. Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. Biochem. Biophys. Res. Commun. 248:706–711.

Weiner, M. A., T. Read, and P. C. Hanna. 2003. Identification and characterization of the *gerH* operon of *Bacillus anthracis* endospores: a differential role for nucleosides in germination. J. Bacteriol. 185:1462–1464.

Welkos, S., S. Little, A. Friedlander, D. Fritz, and P. Fellows. 2001. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology 147:1677–1685.

Williams, D. R., G. B. Rees, and M. E. Rogers. 1992. Observations on an outbreak of anthrax in pigs in north Wales. Vet. Rec. 131:363–366.

Wynn, J. 1982. Anthrax island. Why worry? [news]. Nature 298:506–507.

Yorston, D., and A. Foster. 1989. Cutaneous anthrax leading to corneal scarring from cicatricial ectropion. Br. J. Ophthalmol. 73:809–11.

6
Mycobacterium

C. O. Thoen and R. G. Barletta

Mycobacteria are rod-shaped, acid-fast bacilli with a high lipid content in their cell wall, which has been the focus of much attention. Although numerous cell-wall components of virulent mycobacteria have been isolated and identified, there is little definitive information on the role of these components in the pathogenesis of disease. The genomes of two strains of *Mycobacterium tuberculosis* and *M. leprae* have been sequenced and annotated (Cole et al. 1998, 2001; Fleischmann et al. 2002), while the sequencing of the genomes of *M. avium* subsp. *paratuberculosis* and *M. bovis* have just been completed (http://wit.integratedgenomics.com/GOLD/). Therefore, it is anticipated that new information on the role of factors associated with virulence will be available in the future.

Pathogenic mycobacteria produce granulomatous lesions in tissues of human beings and a wide range of domestic and wild animal species (Thoen and Williams 1994; Kaneene and Thoen 2004). Unexplained differences in susceptibility of different animals to various acid-fast bacilli occur (Thoen 1994). *Mycobacterium bovis*, a slow-growing nonphotochromogenic organism, is the etiologic agent of bovine tuberculosis and causes disease in other domestic and wild animals (Thoen et al. 1992; Thoen and Chiodini 1993). *M. tuberculosis*, the human tubercle bacillus, produces progressive generalized disease in nonhuman primates, dogs, swine, and certain exotic animals; cats, rabbits, and cattle are quite resistant. *M. tuberculosis* may induce tuberculin skin sensitivity in cattle and other animals. Guinea pigs are susceptible to *M. bovis* and *M. tuberculosis* and develop progressive lesions following infection (Thoen and Karlson 1984). Reliable biochemical tests are available for differentiating bacteria of the *M. tuberculosis* complex; and molecular techniques are also now widely used (Noordhoek et al. 1996).

M. avium ss *avium* and the taxonomically closely related *M. intracellulare* (with both microorganisms referred to as the *M. avium complex*) have the widest host range among mycobacteria. *M. avium* serovars 1, 2, and 3 are typical avian tubercle bacilli isolated from tuberculous lesions in avian species (Thoen 1997), whereas other serovars of *M. avium* produce only minimal disease (microscopic foci in liver and spleen) in chickens inoculated intravenously or intraperitoneally. These organisms are most often associated with granulomatous lesions in swine and cold-blooded animals (Thoen and Schliesser 1984; Thoen and Williams 1994). In birds, disease is usually progressive, with lesions in the liver and spleen; in nonhuman primates, cattle, and swine, infection due to *M. avium* ss *avium* is usually confined to lymph nodes associated with the intestinal tract. Rabbits are highly susceptible to experimental infection with *M. avium* serovars 1 and 2, but relatively resistant to other serovars of *M. avium.* It is interesting that birds are susceptible to *M. avium* serovars 1–3, but are very resistant to infection with *M. bovis* or *M. tuberculosis*. *M. avium* ss *paratuberculosis* is the cause of a transmissible intestinal disorder of ruminants commonly known as Johne's disease (paratuberculosis) (Thoen and Haagsma 1996; Harris and Barletta 2001). Cattle, sheep, goats, and certain exotic ruminants are susceptible to *M. avium* ss *paratuberculosis*, but horses and swine fail to develop clinical disease following experimental exposure. A characteristic that is useful in differentiating this species is dependency on the iron-chelating agent mycobactin for *in vitro*

growth. Mycobactin was initially extracted from *M. phlei*, but later, mycobactin J and certain extracellular iron-binding compounds were isolated from *M. avium* sp (Barclay and Ratledge 1983). Since mycobactin dependence has also been reported for certain strains of *M. avium* ss *avium*, more definitive methods such as polymerase chain reaction and restriction endonuclease analysis have been developed for identifying *M. avium* ss *paratuberculosis* (Labidi and Thoen 1989; Harris and Barletta 2001).

Other species of mycobacteria have been isolated from various animals. *M. fortuitum*, a rapid-growing, nonchromogenic organism has been isolated from dogs with lung lesions, cattle with mastitis, and from lymph nodes of slaughter cattle and swine. 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. *M. lepraemurium* has been isolated from leprosy-like lesions in cats, rats, and mice, whereas *M. leprae*, the cause of leprosy in humans, has been isolated from armadillos in the United States. *M. marinum*, *M. nonchromogenicum*, *M. chelonei*, and certain other opportunistic mycobacteria have been isolated from granulomatous lesions in cold-blooded animals (Thoen and Schliesser 1984).

VIRULENCE

Development of mycobacterial disease in a host depends on the ability of the mycobacteria to survive and multiply within the macrophages of the host. Pathogenicity of mycobacteria is a multifactorial phenomenon, requiring the participation and cumulative effects of several components (Cole et al. 1998). Available information suggests that viable *M. bovis* BCG has the ability to facilitate transport of molecules between the cytosol and phagosome of infected cells (Teitelbaum et al. 1999). This could allow the tubercle bacillus access to nutrients in the cytoplasm of the host cell and thereby contribute to pathogenesis. The structure and biologic functions of the glycolipid-containing cell wall of mycobacteria have been extensively investigated; however, an understanding of the functions of each cell-wall component in pathogenesis is unclear (McNeil and Brennan 1991). The mycobacterial cell wall is composed of peptidoglycan, arabinogalactan, and mycolic acids. Mycolic acids, such as cord factor (trehalose dimycolate) or other mycolic acid-containing glycolipids have been implicated in the pathogenesis of mycobacterial disease (Barry et al. 1998). Glycolipids and sulfur-containing glycolipids (sulfatides) appear to promote the survival of virulent tubercle bacilli within macrophages by inhibiting phagolysosome formation and avoiding exposure to hydrolytic enzymes present in the lysosomes. Sulfolipids induce changes in phagocytic cell function that may be important in decreasing the ability of phagocytes to respond efficiently to *M. tuberculosis* (Zhang et al. 1988).

Certain cell-wall components of tubercle bacilli including immunoreactive proteins, lipoarabinomannan, arabinogalactan, mycolic acids, and mycocersic acid may contribute to pathogenesis by promoting survival of the organism in macrophages. Of particular interest are the secreted proteins in the antigen 85 complex, since they may play a role in development of cell-mediated responses and disease in the host (Andersen et al. 1991), as well as in cell-wall biogenesis as evidenced by their mycolyltransferase activity (Belisle et al. 1997). Fibronectin binds to antigen 85 components, and the release of large amounts of this antigen could inhibit binding of fibronectin to tubercle bacilli (Abou-Zeid et al. 1991, 1998; Wiker and Harboe 1992). Other reports indicate that *M. tuberculosis* has ligands that bind to extracellular proteins such as fibronectin (Ratliff et al. 1993; Zhao et al. 1999). Mycobacterial PE_PGRS proteins, a family of highly related proteins present on the surface of mycobacteria, may influence interactions with other cells including macrophages (Brennan et al. 2001). Moreover, genes of *M. tuberculosis* (i.e., *mce, erp*) have been identified that code for proteins that enhance uptake of the bacillus and survival in phagocytic cells (Arruda et al. 1993; Berthet et al. 1995; Wei et al. 2000).

HOST RESPONSE

Cell-mediated immune (CMI) responses, including specific T lymphocytes and activated mononuclear macrophages, are important in host resistance to virulent acid-fast bacilli (Rook 1988). The acquired cell-mediated immune response to *M. tuberculosis* infections is complex and involves T cells. CD4 T cells secrete gamma interferon, which limits bacterial growth and may enhance clearance of the bacillus (Orme et al. 1993). CD8 T cells directly destroy intracellular bacteria by releasing the antimicrobial protein granulysin (Orme 1993; Orme and Cooper 1999; Stenger et al. 1999). Gamma delta T cells may influence the cellular response by promoting an influx of monocytes and lymphocytes and limit-

ing inflammatory cells that may cause tissue damage (D'Souza et al. 1997). Various cytokines (i.e., gamma interferon) activate tuberculostatic macrophage functions and limit the replication of mycobacteria; however, the mechanism(s) involved in killing of mycobacteria is unclear (Flesch and Kaufmann 1990; Denis 1991). The importance of active oxygen metabolites, such as superoxide anion or singlet oxygen, has been investigated, but convincing evidence is not available regarding the significance of these components in protection of the host. Reactive nitrogen intermediates (i.e., nitrogen oxide) produced by peritoneal macrophages have been proposed to be important in killing *M. bovis* BCG and virulent *M. tuberculosis* (Flesch and Kaufmann 1990; Chan et al. 1992; Cheville et al. 2001).

Numerous host mechanisms have been associated with susceptibility and development of disease in animals exposed to virulent mycobacteria. Phagocytosis is influenced by the presence of complement and complement receptors on monocytes (Schlesinger et al. 1990). Mature macrophages have receptors for both C3b and the cell-binding (Fc) portion of immunoglobulin G (IgG). Although neutrophils initially enter the infection site, activities of mononuclear macrophages are considered to be more important in development of protection of the host against pathogenic mycobacteria (Rook 1988). Macrophages may be activated after an encounter with a specific acid-fast organism or as a result of stimulation by cytokines, such as IL-2 and IFN-γ. Even within activated macrophages, *M. tuberculosis* is able to enhance its survival and thus its persistence in the host by up-regulating the expression of isocitrate lyase, an enzyme involved in fatty acid metabolism (McKinney et al. 2000). Macrophages are also involved in processing of mycobacterial antigens and in presenting antigens to T lymphocytes, which are an important recognition unit in the immune response to mycobacteria. The interaction of lymphocytes with specific antigens stimulates the release of lymphokines that attract, activate, and increase the number of mononuclear cells at the infection site (Jeevan and Asherson 1988). Mononuclear macrophages are long lived and may reenter the circulation from a lesion, thereby acting as vehicles for the dissemination of ingested viable mycobacteria.

The key functional parameters determining the outcome of immune responses to infectious agents is the nature of the cytokines (cellular signals) produced locally by immune and other cell types. Mycobacterial lipoproteins are potent stimulators of IL-12, a cytokine produced by macrophages that is a potent stimulator of T-helper type responses (Brightbill et al. 1999). T-helper cells can be divided into two distinct populations, based on their cytokine profiles, which dictate their functional role (Romagnani 1992). T-helper type-1 cells are involved in cellular immunity, while T-helper type-2 cells regulate antibody production. Type-1 and type-2 cells operate in a reciprocal fashion, whereby cellular and humoral immune responses are in opposition. Thus, while type-2 cells are associated with exacerbation and rapid lesion formation in several models of chronic infectious disease, type-1 cells are associated with resistance in the same model system (Salgame et al. 1991). Therefore, the existence or absence of an immune response does not predict resistance; this depends on the balance between the various types of immune responses of the host and the virulence of the bacterial strain for that host.

The macrophage acts as the principal effector cell in regulating mycobacterial infection in the host. Mycobacteria survive in nonactivated macrophages by inhibition of fusion of phagosomes and lysosomes (Lowrie and Andrew 1988). Mycobacteria also synthesize enzymes involved in the defense against reactive oxygen and nitrogen intermediates (St. John et al. 2001). Mononuclear macrophages activated by lymphokines (IFN-γ and TNF-α) exhibit marked changes, including an elevation of antimycobacterial mechanisms. Macrophage functions such as chemotaxis, phagocytosis, enzyme secretion, and cytotoxicity are influenced by intracellular nucleotide levels (Verghese and Snyderman 1983). Studies on the activation of adenylate cyclase in macrophage membranes suggest that certain nucleotides, such as cyclic guanosine-5'-monophosphate, present in the macrophage membrane, regulate adenylate cyclase activation. Moreover, prostaglandins can exert their effect on cyclic adenosine-5'-monophosphate (cAMP) production by stimulating membrane-bound adenylate cyclase (Orme and Cooper 1999). The importance of cAMP in phagolysosome formation in macrophages infected with *M. bovis* BCG and certain other mycobacteria is evidenced by an elevation of cAMP levels in macrophages ingesting *Mycobacterium* (Lowrie and Andrew 1988). A recent report demonstrated that ATP-mediated killing of intracellular mycobacteria was associated with phagosome-lysosome fusion (Fairbairn et al. 2001). Recently, it was reported that a resistant allele (*sst1*) is involved in controlling *M. tuberculosis* infection in mice.

However, *sst1* is not adequate to provide protection indicating that genes outside the locus are involved in protection (Kramnik et al. 2000). Other experimental studies in mice suggest that a complex lipid determines tissue specific replication of *M. tuberculosis* (Cox et al. 1999).

GRANULOMATOUS LESION DEVELOPMENT

Aerosol exposure to acid-fast bacilli generally leads to involvement of pulmonary lymph nodes and lungs, while animals exposed by ingestion of contaminated food and water usually develop primary foci in lymph nodes of the head and other lymph tissues associated with the gastrointestinal tract (Thoen 1988). The mucociliary clearance in the upper respiratory passages provides defense against infection by inhalation of mycobacteria. However, microorganisms on small particles such as dust and water droplets that do not impinge against the mucociliary layer can pass through terminal bronchioles, thus gaining access to alveolar spaces. The estimated size of terminal endings of bronchioles is about 20 μm as compared to 1–4 μm for an acid-fast bacillus. Mycobacteria multiply within macrophages, and after 10–14 days, CMI responses develop and host macrophages acquire an increased capacity to kill the intracellular bacilli. The CMI responses are mediated by lymphocytes, which release lymphokines (γ-IFN) that attract, immobilize, and activate additional blood-borne mononuclear cells at the site where virulent mycobacteria or their products exist. The cellular hypersensitivity that develops contributes to cell death and tissue destruction (caseous necrosis). In some instances, liquefaction and cavity formation occur due to enzymatic action on proteins and lipids. Rupture of these cavities into the bronchi allows aerosol spread of bacilli. Activated macrophages migrate to blind endings of lymphatic vessels and course to one or more of the thoracic lymph nodes, either bronchial or mediastinal. Ultrastructural studies show that engorged macrophages enlarge and develop marked increases in the number of lysosomes, Golgi complexes, and vesicles.

Lymph nodes are more commonly infected than other tissues because fluids in an animal eventually pass through the nodes where the meshwork of trabeculae entraps the organisms. The enlargement and presence of macrophages in impenetrable passageways between reticular cell fibers of the lymph node provide an environment for mycobacterial growth and development of the granulomatous lesion in the node. Occasionally, some phagocytized mycobacteria remain in the lung, and both lung and thoracic nodes are affected. Primary lesions often become localized in a node(s), which may become large and firm. Fibrous connective tissue development probably contributes to localization of the granulomatous lesions.

Granuloma formation is an attempt by the host to localize the infection and to allow inflammatory and immune mechanisms to destroy the bacilli. A few lesions may appear to be regressing and becoming encapsulated by well-organized connective tissue; such lesions may contain viable bacilli. Typically, the microscopic appearance of a granuloma (a tubercle) is focal and has some caseous necrosis in a central area encircled by a zone of epithelioid cells, lymphocytes, and some granulocytes. Mineralization may be present in necrotic centers; in more advanced lesions, several foci of mineralization may coalesce. The zone near the necrotic area often contains multinucleated giant cells that contain several nuclei, often in a horseshoe or ring shape near the cytoplasmic border. An outer boundary of fibrous connective tissue is usually present between the lesions and normal tissue. Occasionally, fibrous tissue is not apparent and the lesion assumes a more diffuse appearance.

Lesions of lymph nodes associated with the gastrointestinal tract, as with *M. avium* ss *avium* in cattle and swine, suggest infection by ingestion. The medial retropharyngeal lymph nodes are a frequent site of *M. bovis* infection and are the most commonly infected site in the head. These nodes receive afferent lymph vessels from the floor of the mouth and adjacent parts. Other lymph nodes of the head (mandibular, parotid, and lateral retropharyngeal) are occasionally involved. The liver is only infrequently involved; hepatic nodes have afferent lymph vessels from the liver, duodenum, and abomasal lymph nodes are commonly involved. The greater part of the blood supply to the liver is derived from the portal vein, which drains the blood and lymph from the intestine; therefore, mycobacteria can pass directly to a hepatic node from the intestine, or through the portal vein to the liver only, or subsequently into hepatic nodes. Occasionally, only mesenteric lymph nodes are infected. Localized tubercles have not been reported in the mucous membrane of the small intestine; mycobacteria are apparently able to diffuse into lymphatics of the lamina propria and be transported by phagocytes via the lymphatic vessels to mesenteric lymph nodes. In

some animals, lesions may develop in superficial cervical lymph nodes. Superficial iliac and popliteal lymph node lesions are seen only infrequently.

Infection with *M. avium* ss *paratuberculosis* occurs primarily in the young (<30 days of age) through the fecal-oral route; other routes that have been suggested include placental and transuterine. Typically, the newborn animal nurses on teats contaminated with feces and, after an incubation period of 6 months to 8 years, it develops a general unthriftiness, rough hair coat, chronic weight loss, and intermittent diarrhea. The clinical course usually lasts only a few months, terminating in severe diarrhea, emaciation, ventral edema, debilitation, and death. There are vaccines that will alter the clinical course of disease and limit the shedding of bacteria in feces (Harris and Barletta 2001). Progress has also been made in the genetic analysis and manipulation of *M. paratuberculosis* including the study of promoters and the development of a transformation system, expression of reporter genes, and transposon mutagenesis (Foley-Thomas et al. 1995; Bannatine et al. 1997; Williams et al. 1999; Harris et al. 1999, 2002). These studies will facilitate the development of new live–attenauted or subunit vaccines with improved efficacy in protecting against Johne's disease.

Paratuberculosis is characterized by a granulomatous inflammation of the terminal region of the small and large bowel and regional lymph nodes. Information on the occurrence of cellular and humoral immune responses in cattle infected with *M. avium* ss *paratuberculosis* (*M. paratuberculosis*) has been reviewed (Thoen and Haagsma 1996). Clinical Johne's disease usually involves impaired intestinal function associated with chronic inflammatory responses. Tissue changes are accompanied by increased leakage of plasma proteins across the intestinal wall and malabsorption of amino acids from the gut lumen. Lesions in cattle are primarily in the intestinal wall and characterized by diffuse granulomatous changes with little or no evidence of necrosis. Accumulations of lymphocytes and epithelioid cells are present in the lamina propria. In advanced clinical cases, inflammatory cells may be observed in the submucosa as a band of epithelioid cells along the muscularis mucosa. Granulomas containing numerous acid-fast bacilli are often present in lymph nodes associated with the intestinal tract in cattle. Caseous necrosis has been observed in such lesions in sheep and goats.

M. paratuberculosis penetrates the intestinal epithelial layers through the follicle-associated epithelium (FAE) or M cells (Momotani et al. 1988). The mechanisms by which *M. paratuberculosis* survives in macrophages of the host involve virulence factors and other proteins. Studies with a mouse macrophage cell line suggest that pathogenic wild type strains of *M. paratuberculosis* block phagosomal acidification, and this allows for the replication of bacteria (Cheville et al. 2001). The importance of iron uptake in *M. paratuberculosis* infections has been reviewed (Harris and Barletta 2001). NRAMP1, a membrane protein in lysosomes of macrophages, has been reported to control tuberculosis in mice by removing iron from the phagosome (Canonne-Hergaux et al. 1999). There is some evidence of dissemination of *M. paratuberculosis* from infected to noninfected macrophages allowing the organism to avoid cellular mechanisms of the host (Bassey and Collins 1997). Studies on bovine paratuberculosis indicate that T-helper cell activity is altered in animals with clinical disease (Chiodini and Davis 1993). It has also been reported that general cellular responses in infected cattle are depressed as compared to normal cattle (Kreeger and Snider 1992). Exposure of peripheral blood mononuclear cells (PBMCs) to *M. paratuberculosis* seems to down-regulate expression of several genes. However, a comparison of gene expression profiles of PBMCs of *M. paratuberculosis* infected cows with clinical disease and uninfected cows stimulated with concanavalin A revealed the profiles were similar, indicating that *M. paratuberculosis* repression of gene expression in PBMCs was not due to a failure of immune responsiveness in cows with clinical Johne's disease (Coussens et al. 2002). Moreover, other studies demonstrated a differential response of PBMCs to exposure with either *M. avium* ss *avium* or *M. avium* ss *paratuberculosis*, with the latter effecting a decrease in the killing efficiency of mononuclear cells (Weiss et al. 2002).

Although a great deal of new information and understanding has been acquired in recent years on the pathogenesis and immunogenesis of mycobacterial infections, definitive information on the mechanisms of virulence remains obscure. Further research on tuberculosis and other mycobacterial infections will be required to fully understand and appreciate the complex immunoregulatory mechanisms of the host-parasite interrelationship that determine protective immunity, nonresponsiveness, tissue damage, and disease.

REFERENCES

Abou-Zeid, C., T. Garbe, R. Lathigra, H. G. Wiker, M. Harboe, G. A. Rook, and D. B. Young. 1991.

Genetic and immunological analysis of *Mycobacterium tuberculosis* fibronectin-binding proteins. Infect. Immun. 59:2712–2718.

Abou-Zeid, C., T. L. Ratcliff, H.G. Wiker, M. Harboe, J. Bennedsen, and G. A. W. Rook. 1998. Characterization of fibronectin-binding antigen released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Infect. Immun. 56:3046–3051.

Andersen, P., D. Askgaard, L. Ljungquist, J. Bennedsen, and I. Heron. 1991. Proteins released from *Mycobacterium tuberculosis* during growth. Infect. Immun. 59:1905–1910.

Arruda, S., G. Bomfim, R. Knights, T. Huima-Byron, and L. W. Riley. 1993. Cloning of an *M. tuberculosis* DNA fragment associated with entry and survival inside cells. Science 261:1454–1457.

Barclay, R., and C. Ratledge. 1983. Iron-binding compounds of *M. avium*, *M. intracellulare*, *M. scrofulaceum*, and mycobactin-dependent *M. paratuberculosis* and *M. avium*. J. Bacteriol. 153:1138–1144

Barry, C. E., R. E. Lee, K. Mdluli, A. E. Sampson, B. G. Schroeder, R. A. Slayden, and Y. Yuan. 1998. Mycolic acids: structure, biosynthesis and physiological functions. Prog. Lipid Res. 37:143–179.

Bassey, E. O., and M. T. Collins. 1997. Study of T lymphocyte subsets of healthy and *Mycobacterium avium* subsp. *paratuberculosis* infected cattle. Infect. Immun. 65:4869–4872.

Belisle, J. T., V. D. Vissa, T. Sievert, K. Takayama, P. J. Brennan, and G. S. Besra. 1997. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science 276:1420–1422.

Berthet, F. X., J. Rauzier, E. M. Lim, W. Philipp, B. Gicquel, and D. Portnoi. 1995. Characterization of the *Mycobacterium tuberculosis erp* gene encoding a potential cell surface protein with repetitive structures. Microbiology 141:2123–2130.

Brennan, M. J., G. Delogu, Y. Chen, S. Bardarov, J. Kriakov, M. Alavi, and W. R. Jacobs, Jr. 2001. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infect. Immun. 69:7326–7333.

Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285:732–736.

Canonne-Hergaux, F., S. Gruenheid, G. Govoni, and P. Gros. 1999. The Nramp1 protein and its role in resistance to infection and macrophage function. Proc. Assoc. Am. Physicians 111:283–289.

Chan, J., Y. Xing, R. S. Maghiozzo, and B. R. Bloom. 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. 175:1111–1122.

Cheville, N. F., J. Hostetter, B. V. Thomsen, F. Simutis, Y. VanLoubbeeck, and E. Steadham. 2001. Intracellular trafficking of *Mycobacterium avium* ss. *paratuberculosis* in macrophages. Deutsch Tierarztliche Wochenschrift 108:233–238.

Chiodini, R. J., and W. C. Davis. 1993. The cellular immunology of bovine paratuberculosis: immunity may be regulated by CD4+ helper and CD8+ immunoregulatroy T lymphocytes which down-regulate gamma/delta+ T-cell cytotoxicity. Microb. Pathog. 14:355–367.

Cole, S. T., K. Eiglmeier, et al. 2001. Massive gene decay in the leprosy bacillus. Nature 409:1007–1011.

Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S., Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M.A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–544.

Coussens, P. M., C. J. Colvin, K. Wiersma, A. Abouzied, and S. Sipkovsky. 2002. Gene expression profiling of peripheral blood mononuclear cells from cattle infected with *Mycobacterium paratuberculosis*. Infect. Immun. 70:5494–5502.

Cox, J. S., B. Chen, M. McNeil, and W. R. Jacobs, Jr. 1999. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. Nature 402:79–83.

Denis, M. 1991. Killing of *Mycobacterium tuberculosis* within human monocytes: activation of cytokines and calcitrol. Clin. Expt. Immunol. 84:200–206.

D'Souza, C. D., A. M. Cooper, A. A. Frank, R. J. Mazzaccaro, B. R. Bloom, and I. M. Orme. 1997. An anti-inflammatory role for γδ T lymphocytes in acquired immunity to *Mycobacterium tuberculosis*. J. Immunol. 158:1217–1221.

Fairbairn, I. P., C. B. Stober, D. S. Kumararatne, and D. A. Lammas. 2001. ATP-Mediated killing of intracellular Mycobacteria by macrophages is a P2X7-dependent process inducing bacterial death

by phagosome-lysosome fusion. J. Immunol. 167:3300–3307.

Fleischmann, R. D., D. Alland, J. A. Eisen, L. Carpenter, O. White, J. Peterson, R. DeBoy, R. Dodson, M. Gwinn, D. Haft, E. Hickey, J. F. Kolonay, W. C. Nelson, L. A. Umayam, M. Ermolaeva, S. L. Salzberg, A. Delcher, T. Utterback, J. Weidman, H. Khouri, J. Gill, A. Mikula, W. Bishai, W. R. Jacobs Jr, J. C. Venter, and C. M. Fraser. 2002. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. J. Bacteriol. 184:5479–5490.

Flesch, I. E. A., and S. H. E. Kaufmann. 1990. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin 4, and tumor necrosis factor. Infect. Immun. 58:2675–2677.

Foley-Thomas, E. M., D. L. Whipple, L. E. Bermudez, and R. G. Barletta. 1995. Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. Microbiology 141:1173–1181.

Harris, N. B., and R. G. Barletta. 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. 14:492–512.

Harris, N. B., D. K. Zinniel, M. K. Hsieh, J. D. Cirillo, and R. G. Barletta. 2002. Cell sorting of formalin-treated pathogenic *Mycobacterium paratuberculosis* expressing GFP. Biotechniques 32:522–527.

Harris, N. B., Z. Feng, X. Liu, S. L. Cirillo, J. D. Cirillo, and R. G. Barletta. 1999. Development of a transposon mutagenesis system for *Mycobacterium avium* subsp. *paratuberculosis*. FEMS Microbiol. LettS. 175:21–26.

Jeevan, A., and G. L. Asherson. 1988. Recombinant interleukin-2 limits the replication of *Mycobacterium lepraemurium* and *Mycobacterium bovis* BCG in mice. Infect. Immun. 56:660–664.

Kaneene, J. B., and C. O. Thoen. 2004. Tuberculosis. In Zoonosis update. J. Am. Vet. Med. Assoc. 224(5):685–691.

Kramnik, I., W. F. Dietrich, P. Demant, and B. R. Bloom. 2000. Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*. Proc. Nat. Acad. Sci. U S A 97:8560–8565.

Kreeger, J. M., and T. G. Snider. 1992. Measurement of lymphoblast proliferative capacity of stimulated blood mononuclear cells from cattle with chronic paratuberculosis. Am. J. Vet. Res. 53:392–395.

Labidi, A., and C. O. Thoen. 1989. Genetic relatedness of *Mycobacterium tuberculosis* and *M. bovis*. Acta Leprol. 7:217–221.

Lowrie, D. B., and P. W. Andrew. 1988. Macrophage antimycobacterial mechanisms. Brit. Med. Bull. 44:624–629.

McKinney, J. D., Z. U. Honer, K. Bentrup, E. J. Munoz-Elias, A. Miczak, B. Chen, W. T. Chan, D. Swenson, J. C. Sacchettini, W. R. Jacobs, Jr., and D. G. Russell. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406:735–738.

McNeil, M. R., and P. J. Brennan. 1991. Structure, function, and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis, and drug resistance; some thoughts and possibilities arising from recent structural information. Res. Microbiol. 142:451–463.

Momotani, E., D. L. Whipple, A. B. Thiermann, and N. F. Cheville 1988. Role of M cells and macrophages in the entrance of *Mycobacterium tuberculosis* into domes of ileal Peyer's patches in calves. Vet. Pathol. 25:131–137.

Noordhoek, G. T., J. D. A. vanEmbden, and A. H. J. Kolk. 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among laboratories. J. Clin. Microbiol. 34:2522–2525.

Orme, I. M. 1993. The role of CD8 T cells in immunity to tuberculosis infections. Trends Microbiol. 1:77.

Orme, I. M., and A. M. Cooper. 1999. Cytokine/chemokine cascades in immunity to tuberculosis. Immunol. Today. 20:307–312.

Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams. 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. 151:518.

Ratliff, T., L. R. McCarthy, W. B. Telle, and E. J. Brown. 1993. Purification of a mycobacterial adhesion for fibronectin. Infect. Immun. 61:1889–1894.

Romagnani, S. 1992. Induction of T_H1 and T_H2 responses: A key role for the "natural" immune response? Immunol. Today 13:379–381.

Rook, G. A. W. 1988. Role of activated macrophages in the immunopathology of tuberculosis. Br. Med Bull. 44:611–623.

Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing cytokine profiles of functional subsets of human CD4 and CD8 cell clones. Science 254:279–282.

Schlesinger, L. S., C. G. Bellinger-Kawahara, N. R. Payne, and M. A. Horwitz. 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. J. Immunol. 144:2771–2780.

St. John, G., N. Brot, J. Ruan, H. Erdjument-Bromage, P. Tempst, H. Weissbach, and C. Nathan. 2001. Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. Proc. Natl. Acad. Sci. U S A 98:9901–9906.

Stenger, S., J. Rosat, B. R. Bloom, A. M. Krensky, and R. L. Modlin. 1999. Granulysin: a lethal weapon of cytolytic T cells. Immunol. Today 20:390–394.

Teitelbaum, R., M. Cammer, M. L. Maitland, N. E. Freitag, J. Condeelis, and B. R. Bloom. 1999. Mycobacterial infection of macrophages results in membrane-permeable phagosomes. Proc. Nat. Acad. Sci. 96:15190–15195.

Thoen, C. O. 1988. Tuberculosis. J. Am. Vet. Med. Assoc. 193:1045–1047.

———. 1997. Tuberculosis. In Diseases of poultry, 10th ed. B. W. Calnek (ed.). Ames: Iowa State University Press, pp. 167–178.

———. 1994. Tuberculosis in wild and domestic mammals. In Tuberculosis: pathogenesis, protection, and control. B. R. Bloom (ed.). Washington, DC: ASM Press, pp. 157–162.

Thoen, C. O., and A. G. Karlson. 1984. Experimental tuberculosis in rabbits. In The mycobacteria: a sourcebook. G. P. Kubica and L. G. Wayne (eds.). New York: Marcel Dekker, pp. 978–89.

Thoen, C. O., and D. E. Williams. 1994. Handbook on zoonoses, 2nd ed. G. W. Beran (ed.). Boca Raton: CRC Press, pp. 41–60.

Thoen, C. O., and J. A. Haagsma. 1996. Molecular techniques in the diagnosis and control of paratuberculosis in cattle. J. Am. Vet. Med. Assoc. 209:734–737.

Thoen, C. O., and R. Chiodini. 1993. Mycobacterium. In Pathogenesis of bacterial infections in animals. 2nd ed. C. L. Gyles and C. O. Thoen (eds.). Ames: Iowa State University Press, pp. 44–56.

Thoen, C. O., and T. Schliesser. 1984. Mycobacterial infections in cold-blooded animals. In the mycobacteria: a sourcebook. G. P. Kublica and L. G. Wayne (eds.). New York: Marcel Dekker, pp. 1279–1311.

Thoen, C. O., K. J. Throlsen, L. D. Miller, E. M. Himes, and R. L. Morgan. 1988. Pathogenesis of *Mycobacterium bovis* infection in American bison. Am. J. Vet. Res. 49:1861–1865.

Thoen, C. O., W. J. Quinn, L. D. Miller, L. L. Stackhouse, B. F. Newcomb, and J. M. Ferrell. 1992. *Mycobacterium bovis* infection in North American elk (*Cervus elaphus)*. J. Vet. Diagn. Invest. 4:423–427.

Verghese, M. W., and R. Snyderman. 1983. Hormonal activation of adenylate cyclase in macrophage membranes is regulated by guanine nucleotides. J. Immunol. 130:869–873.

Wei, J., J. L. Dahl, J. W. Moulder, E. A. Roberts, P. O'Gaora, D. B. Young, and R. L. Friedman. 2000. Identification of *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. J. Bacteriol. 182:377–384.

Weiss, D. J., O. A. Evanson, A. Moritz, M. Q. Deng, and M. S. Abrahamsen. 2002. Differential responses of bovine macrophages to *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. Infect. Immun. 70:5556–5561.

Wiker, H. G., and M. Harboe. 1992. The antigen 85 complex: A major secretion product of *Mycobacterium tuberculosis*. Microbiol. Rev. 56:648–661.

Williams, S. L., N. B. Harris, and R. G. Barletta. 1999. Development of a firefly luciferase-based assay for determining antimicrobial susceptibility of *Mycobacterium avium* subsp. *paratuberculosis*. J. Clin. Microbiol. 37:304–309.

Zhang, L., M. B. Goren, T. J. Holzer, and B. R. Anderson. 1988. Effect of *Mycobacterium tuberculosis*-derived sulfolipid I on human phagocytic cells. Infect. Immun. 56:2876–2883.

Zhao, W., J. S. Schorey, R. Groger, P. M. Allen, E. J. Brown, and T. L. Ratliff. 1999. Characterization of the fibronectin binding motif for a unique mycobacterial fibronectin attachment protein, FAP. J. Biol. Chem. 274:4521–4526.

7
Corynebacterium and *Arcanobacterium*

B. H. Jost and S. J. Billington

CORYNEBACTERIUM

The group of organisms informally known as the corynebacteria contain many species that infect people and animals. During the past 20 years, the taxonomy of these bacteria has undergone dramatic changes. Several *Corynebacterium* spp., including *Arcanobacterium pyogenes*, have been assigned to new genera or further differentiated into new species, and several novel species have been described. The species of *Corynebacterium* that primarily infect animals are listed in table 7.1. Members of this genus are short, club-shaped, gram-positive rods, with cell walls that characteristically contain meso-diaminopimelic and short-chain mycolic acids.

CORYNEBACTERIUM BOVIS

Corynebacterium bovis is considered a commensal of the bovine udder. The organism inhabits the teat canal, colonizing around Furstenberg's rosette, and is routinely isolated from clinically normal quarters, especially during lactation. The organism is present in up to 40% of quarters, but its incidence decreases with routine teat disinfection and dry cow therapy (Honkanen-Buzalski et al. 1984). Mammary secretions support the growth of *C. bovis* during lactation, but are inhibitory during the nonlactating period. *C. bovis* colonization invokes a mild inflammatory response in the teat cistern, Furstenberg's rosette, and mammary parenchyma, with a concomitant infiltration of neutrophils (Ngatia et al. 1991), but its presence causes only a slight increase in somatic cell counts and does not adversely affect milk production (Honkanen-Buzalski and Bramely 1984). Furthermore, results from several trials have shown that quarters infected naturally or experimentally with *C. bovis* were more resistant to infection with major mastitis pathogens, such as *Staphylococcus aureus* and *Streptococcus dysgalactiae*. Conversely, colonization provided no protection from infection with other streptococci (Pankey et al. 1985). The basis of this resistance is unknown, but several explanations have been proffered, including competitive growth inhibition, neutrophil-mediated effects, and increased humoral immunity. One study found that plasma cells, especially those producing IgA, were found in higher concentrations in Furstenberg's rosettes and parenchymal tissue in *C. bovis*-colonized quarters (Sordillo et al. 1988). While *C. bovis* is generally considered a harmless, if not beneficial, commensal, it is infrequently the cause of clinical mastitis.

C. bovis can also cause hyperkeratotic dermatitis of nude and SCID mice, characterized by hyperkeratosis resulting in scaly skin. Hirsute, immunocompetent mice were susceptible to infection with *C. bovis* but did not develop the lesions typical of this disease. Rarely, *C. bovis* has been isolated from cases of human disease and also from a testicular abscess in a rabbit.

CORYNEBACTERIUM KUTSCHERI

Corynebacterium kutscheri is a commensal of the oral and nasal cavities and colorectum of laboratory rodents, including mice, rats, hamsters, and guinea pigs (Amao et al. 1995). Frequently, up to 100% of rodents in a facility will be colonized, and the organism is transmitted by the fecal/oral route. There appears to be strain and sex variations in colonization susceptibilities, with male mice more likely to carry *C. kutscheri*. Infection is usually subclinical in conventionally raised mice and rats, but

Table 7.1. Hosts and Disease Production of *Corynebacterium* spp. Pathogenic for Animals

Organism	Major hosts	Additional hosts	Disease
C. amycolatum	Humans	Cattle[a]	Bacteremia, endocarditis, septic arthritis (humans), mastitis (cattle)
C. auriscanis	Dogs[a]		Otitis externa
C. bovis	Cattle	Hairless mice, humans[a], rabbit[a]	Commensal, but can cause clinical mastitis (cattle), hyperkeratotic dermatitis (mice), abscesses (humans, rabbit)
C. camporealensis	Sheep[a]		Subclinical mastitis
C. cystitidis	Cattle[a]		Cystitis, pyelonephritis
C. felinum	Scottish wild cat[a]		Unknown
C. glucuronolyticum	Humans[a], swine[a]		Genitourinary tract infections (humans)
C. kutscheri	Laboratory rodents (guinea pigs, hamsters, mice, rats)	Humans[a], voles[a]	Colonization of the upper respiratory and gastrointestinal tracts, subclinical infections, abscesses, pneumonia, pseudotuberculosis (rodents), septic arthritis (humans)
C. mastiditis	Sheep[a]		Mild mastitis
C. minutissimum	Humans	Cattle[a]	Skin infections (humans), mastitis (cattle)
C. phocae	Seals[a]		Isolated from the nasal cavity
C. pilosum	Cattle	Horses[a], humans[a]	Cystitis, pyelonephritis, endocarditis (humans)
C. pseudotuberculosis	Goats, horses, sheep	Camels, cattle, deer[a], humans[a], swine[a]	Caseous lymphadenitis (goats, sheep, camels), abscesses (deer, horses, swine), mastitis (cattle), lymphadenitis (humans)
C. renale	Cattle	Deer[a], goats[a], sheep[a], laboratory rodents	Cystitis, pyelonephritis, osteomyelitis (goats)
C. testudinoris	Tortoise[a]		Isolated in mixed infection from a necrotic lesion
C. ulcerans	Humans	Camels[a], cat[a], cattle, primates[a], otters[a], squirrels[a]	Pharyngitis, sinusitis, skin infections (humans), caseous lymphadenitis (camels), mastitis (cattle), respiratory infections (primates)
C. urealyticum	Humans	Dogs[a]	Cystitis, pyelonephritis

[a]Rarely reported.

disease is seen following severe immune suppression, for example as a result of experimental exposure to gamma irradiation, corticosteroids, other infectious diseases, or dietary deficiencies. Disease manifests as pseudotuberculosis in mice and rats, with high morbidity and mortality (Weisbroth and Scher 1968; Brownstein et al. 1985). *C. kutscheri* was also the cause of septic arthritis in an elderly woman.

CORYNEBACTERIUM PSEUDOTUBERCULOSIS

Corynebacterium pseudotuberculosis, a facultative intracellular pathogen, is the etiologic agent of caseous lymphadenitis (CLA) in sheep and goats, as well as a cause of ulcerative lymphangitis and ventral abscessation in horses. CLA is a chronic, contagious disease, characterized by the formation of

abscesses in the skin, internal and external lymph nodes, and internal organs. The prevalence of CLA in sheep can be as high as 50–80%, which results in economic losses because of decreased meat yield from carcass trimming or condemnation, decreased wool and leather yield, decreased reproductive efficiency, and mortality in severe cases. *C. pseudotuberculosis* can also be isolated from abscesses in cattle, swine, camels, and deer and, infrequently, from cases of bovine mastitis. The occupational exposure of veterinarians and sheep handlers to *C. pseudotuberculosis* can result in lymphadenitis, although cases are uncommon. The ability to reduce nitrate *in vitro* distinguishes two biotypes of *C. pseudotuberculosis* that affect sheep and goats (*ovis*; nitrate negative), or horses (*equi*; nitrate positive). Both biotypes can be isolated from cases of bovine disease. *C. pseudotuberculosis* has also been isolated from the cervical canal of clinically normal sows.

Clinical Signs and Transmission

CLA presents as both an external form, characterized by infection of subcutaneous tissue and peripheral lymph nodes, and an internal form, characterized by abscess formation in the lungs, liver, kidneys, spleen, and the internal lymph nodes. The latter form is also known as "thin-ewe" syndrome in the United States. Both forms can occur simultaneously. External abscesses are generally found near peripheral lymph nodes, where they subsequently enlarge and rupture, draining thick, greenish-white, infective, purulent material into the surrounding environment. The abscess may heal, but it often recurs months later, as a result of the inability of the animal to totally eliminate the bacteria. Low temperatures and damp conditions prolong survival of *C. pseudotuberculosis* in the environment, including on feeders, fences, and shearing implements, and in sheep dips and soil. Transmission occurs through the contamination of wounds inflicted during shearing, castration, tail docking, or other trauma. Less commonly, infection can also be spread by pulmonary discharge from animals with abscesses in the lungs or by ingestion of contaminated material (Williamson 2001). In horses, *C. pseudotuberculosis* causes ulcerative lymphangitis, which is characterized by inflammation of the subcutaneous lymphatic vessels with formation of abscesses and ulcers on the ventral abdomen and chest. Infection can spread via the lymphatic system resulting in abscessation of the internal organs.

Virulence Factors

C. pseudotuberculosis possesses a surface lipid coat, which exists external to the cell wall. These lipids are toxic and are thought to protect the organism from the action of hydrolytic enzymes in host-cell lysosomes, and allow it to persist inside host cells (Williamson 2001). *C. pseudotuberculosis* also expresses a potent exotoxin, phospholipase D (PLD), which can hydrolyze sphingomyelin in host-cell membranes, causing membrane leakage and cytolysis, leading to enhanced vascular permeability. PLD can also activate complement by the alternate pathway, and while not directly hemolytic, exhibits synergistic hemolysis with the cholesterol oxidase of *Rhodococcus equi*. The PLD may also be involved in carbon acquisition from cell membranes once the organism is inside the macrophage. Molecular characterization of the *pld* gene led to the identification of Histidine_{20} as an important active site residue of this enzyme (Haynes et al. 1992). The PLD proteins expressed by the two biotypes of *C. pseudotuberculosis* only vary by two nonconserved amino acid substitutions in the mature protein (McNamara et al. 1995). The only other known *C. pseudotuberculosis* virulence factor is the *fagBCD* operon, which encodes a putative iron uptake system. These genes are immediately adjacent to the *pld* gene, although they are divergently transcribed. A *fagB* knockout mutant had reduced virulence in a goat model of CLA (Billington et al. 2002).

Pathogenesis

Disease begins when the organism enters the host through a break in the skin or, less frequently, via inhalation or ingestion, where the bacteria multiply locally forming micro-abscesses. From this site, *C. pseudotuberculosis* is phagocytosed by neutrophils and macrophages, but the organisms remain viable, probably due to their lipid-rich cell surface. Surprisingly, details of the interaction of the organism with macrophages are virtually nonexistent and represent fertile ground for future study. The phagocytes disseminate the organism, via the blood or lymph, to secondary sites. The toxicity of cell-surface lipids and PLD or other factors eventually result in phagocyte lysis, with concomitant deposition of bacteria, resulting in abscessation at these secondary sites, which include the peripheral and internal lymph nodes as well as the visceral organs. Enhanced vascular permeability as a result of the action of PLD may also promote the spread of infection, both locally and via the lymphatics. The finding

that PLD can activate chemotactic factors in sheep serum to promote neutrophil migration (Yozwiak et al. 1993) could enhance phagocyte-mediated dissemination of the bacteria by promoting bacteria: phagocyte proximity. PLD facilitates the persistence and spread of *C. pseudotuberculosis* within the host, as a PLD knockout mutant was unable to establish a primary infection or cause chronic abscessation of the peripheral lymph nodes (McNamara et al. 1994).

Immunity and Vaccines

Vaccination against CLA is efficacious, since anti-PLD antibodies limit PLD-mediated tissue damage and bacterial dissemination. Cell-mediated immunity, including TNF-α and IFN-γ secretion by $CD4^+$ T cells, is also important for prevention of bacterial proliferation and total elimination of the disease. The vaccine currently available in the United States contains inactivated *C. pseudotuberculosis* whole cell wall and culture supernatant (Case-Bac, Colorado Serum Company), and its use is licensed in sheep but not goats. Vaccination with Case-Bac provides protection against *C. pseudotuberculosis* challenge in sheep (Piontkowski and Shivvers 1998), but its efficacy in goats is questionable. In Canada, Australia, and New Zealand, two vaccines are available—Glanvac 3 and Glanvac 6 (Commonwealth Serum Laboratories, Melbourne, Australia)—which consist of formalin-inactivated *C. pseudotuberculosis* culture supernatant in combination with clostridial antigens. This vaccine is licensed for use in both sheep and goats. Vaccination with Glanvac commonly results in lameness and injection-site cutaneous granuloma formation, probably due to reactive cell-wall lipids. However, it is also efficacious, with a 74% reduction in CLA in vaccinated sheep (Paton et al. 1995).

Several experimental vaccines have been developed and their efficacy tested. Parenteral vaccination with a *pld* knockout strain, Toxminus, elicited strong humoral and cell-mediated immune responses and protected sheep against challenge with wild-type *C. pseudotuberculosis* (Hodgson et al. 1992). Protection against wild-type challenge was also achieved in sheep vaccinated by a single, oral dose of Toxminus expressing a genetically toxoided PLD (His_{20} to Tyr), but not in sheep vaccinated with only Toxminus (Hodgson et al. 1994). Recently, intramuscular vaccination with plasmid DNA encoding genetically toxoided PLD linked to CTLA4-Ig resulted in protection of 45% of vaccinated sheep following challenge (De Rose et al. 2002). However, the route of immunization was critical to the development of an immune response.

Corynebacterium renale, *Corynebacterium pilosum*, and *Corynebacterium cystitidis*

Corynebacterium renale, a cause of bovine cystitis and pyelonephritis, was originally classified into three serologic types. Subsequent reclassification resulted in three distinct species: *C. renale*, *C. pilosum,* and *C. cystitidis*. These organisms can exist as normal flora in the reproductive tract of cattle, although *C. cystitidis* is found only rarely in bulls (Yanagawa 1986). Whereas each species infects cattle (Rebhun et al. 1989), *C. renale* is by far the most prevalent. Infection with *C. renale* results in hemorrhagic cystitis leading to ureteritis and pyelonephritis. *C. cystitidis* causes severe hemorrhagic cystitis with ulceration of the bladder, also progressing to ureteritis and pyelonephritis. In contrast, *C. pilosum* is less pathogenic, and infection results in mild, uncomplicated cystitis, although ascending pyelonephritis can occur rarely (Hiramune et al. 1971). Infection begins with adhesion of the bacteria to the epithelium of the lower urinary tract. From there, the bacteria ascend via vesiculourethral reflux to the kidneys, resulting in chronic pyelonephritis. *C. renale* is also an uncommon cause of ovine cystitis and pyelonephritis, and caprine osteomyelitis.

Virulence Factors

All three species possess pili, which mediate adhesion to host epithelial cells of the urinary bladder, renal pelvis, and reproductive tract (Hayashi et al. 1985). The pili of *C. renale* and *C. pilosum* are distinct, as only minor antigenic cross-reactions between pili were observed (Kudo et al. 1987). Organisms with reduced piliation adhere less well, and adhesion can be blocked by monoclonal antibodies directed against the pili. Furthermore, piliated cells were less susceptible to nonopsonic phagocytosis by neutrophils or macrophages. However, opsonization with specific antibody and complement resulted in equal phagocytosis of piliated and nonpiliated cells (Kubota and Yanagawa 1988). All three species hydrolyze urea in order to obtain nitrogen while inhabiting the urinary tract. However, oral administration of acetohydroxamic acid, a urease inhibitor, prevented pyelonephritis in a rat model, suggesting that urease expression by these organisms may be a primary virulence determinant. Most isolates of *C. renale*, but not *C. pilosum* or *C. cystitidis,* express renalin, an extracellular

CAMP-like protein. In a nonenzymic fashion, renalin reacted with ceramide on red blood cells, exposed by the action of *S. aureus* sphingomyelinase C (β toxin), resulting in synergistic hemolysis. Therefore, this protein may play a role in lysis of host cells (Bernheimer and Avigad 1982).

Immunity

The role of humoral and cell-mediated immunity in protection against infection is not well understood. Serum antibodies are only detected when infection progresses from cystitis to pyelonephritis. However, in mice immunized with killed *C. renale* cells, humoral antibodies did not protect against developing pyelonephritis. Furthermore, in mice at least, selection for nonpiliated cells occurs in the host, although this process is independent of the antibody status of the animal.

CORYNEBACTERIUM ULCERANS

Corynebacterium ulcerans is an uncommon cause of bovine mastitis (Watts 1988). Clinical signs are generally mild but can be severe, with loss of the affected quarter (Hedlund and Pohjanvirta 1989). All isolates of *C. ulcerans* produce a toxic PLD, which is genetically similar to that produced by *C. pseudotuberculosis* and *Arcanobacterium haemolyticum* (McNamara et al. 1995). Presumably, expression of this toxin is important in the pathogenesis of infections by this organism, although no definitive studies have been performed. *C. ulcerans* causes uncomplicated pharyngitis in humans, although this organism can carry a bacteriophage encoding the diphtheria toxin gene. As a consequence, infection with toxigenic *C. ulcerans* may result in clinical diphtheria or skin lesions that mimic typical, cutaneous diphtheria. Infected cows may shed *C. ulcerans* for months to years, and cases of human diphtheria have arisen after consuming raw milk and milk by-products (Barrett 1986).

ARCANOBACTERIUM

The genus *Arcanobacterium* was created to accommodate the previously designated *Corynebacterium haemolyticum*. Initially, this genus consisted of the single species, but in the past five years, it has grown to include five additional species, four of which are pathogenic for animals: *A. hippocoleae*, *A. phocae*, *A. pluranimalium*, and *A. pyogenes*. Members of this genus are gram-positive, nonmotile, short, rod-shaped bacteria, and all species identified to date have been isolated from mammalian sources. With the exception of *A. pyogenes*, little is known about the *Arcanobacterium* spp. that infect animals. Table 7.2 lists the hosts and diseases caused by these species.

ARCANOBACTERIUM PYOGENES

Arcanobacterium pyogenes, recently reclassified from the genus *Actinomyces*, is the predominant animal pathogen within the *Arcanobacterium* genus. *A. pyogenes* is a widely distributed inhabitant of the mucous membranes of domestic animals and is found associated with the upper respiratory, gastrointestinal, and genital tracts (Timoney et al. 1988; Narayanan et al. 1998; Jost et al. 2002b). Infection

Table 7.2. Hosts and Disease Production of *Arcanobacterium* spp.

Organism	Host	Disease
A. bernardiae	Humans	Abscesses, septicemia
A. haemolyticum	Humans	Pharyngitis, wound infections
A. hippocoleae	Horses	Vaginitis
A. phocae	Seals	Unknown (isolated from intestine, lung, lymph nodes, oral and nasal cavities, peritoneal fluid, spleen)
A. pluranimalium	Deer	Lung abscess
	Porpoises	Unknown (isolated from the spleen)
A. pyogenes	Cattle	Liver abscess, mastitis, endometritis, abortion, endocarditis
	Deer	Pneumonia, intracranial abscesses
	Goats	Mastitis, miscellaneous abscesses
	Pigs	Pneumonia, septic arthritis, endocarditis
	Poultry	Osteomyelitis, nephritis
	Sheep	Pneumonia, abortion, endometritis

with *A. pyogenes* often occurs following a precipitating injury or infection, and this organism is one of the most common opportunistic pathogens in domestic ruminants and pigs.

Virulence Factors

A. pyogenes expresses several known and putative virulence factors, which are summarized in table 7.3. These factors are required for adherence, subsequent colonization, and to cause the host tissue damage associated with infection by *A. pyogenes*. The variety of factors expressed by this organism may explain why *A. pyogenes* is able to colonize many different host tissues and cause such a diverse range of diseases. Nevertheless, most aspects of the pathogenesis of infection caused by this important opportunist pathogen remain poorly characterized.

Beta-hemolysis is a characteristic of *A. pyogenes* grown on blood-containing media, a function of pyolysin (PLO), an exotoxin produced by all strains of *A. pyogenes*. PLO is lytic for red blood cells of a variety of animal species, as well as being dermonecrotic and lethal to laboratory animals via the intravenous (Lovell 1944) and intraperitoneal (Jost et al. 1999) routes. The PLO protein shows 35–41% amino acid identity with the cholesterol-binding (cholesterol-dependent) cytolysins (CDCs) (Billington et al. 1997). CDCs are produced by more than 20 species of gram-positive bacteria, and exert their cytolytic effects by forming large, oligomeric pores in eukaryotic cell membranes. Their activity is sensitive to free cholesterol, which appears to be the major host-cell receptor for these toxins (reviewed in Billington et al. 2000). PLO is important in the pathogenesis of *A. pyogenes* infections, since a *plo* mutant was significantly reduced for virulence in mice compared to the isogenic wild-type strain (Jost et al. 1999). However, the precise function of PLO in *A. pyogenes* pathogenesis is not fully understood. In addition to its hemolytic activity, PLO was also cytolytic for immune cells, murine macrophages, and bovine and ovine neutrophils (Ding and

Table 7.3. Virulence Factors of *Arcanobacterium pyogenes*

Factor	Activities in the host
Cholesterol dependent cytolysin, pyolysin (PLO)	Binds to cholesterol-containing cell membranes, forming pores resulting in cytolysis; the action of the toxin promotes hemolysis, cytolysis of immune cells, alteration of host cytokine expression
Extracellular matrix-binding proteins	
Collagen-binding protein, CbpA	Binds to collagen types I, II, and IV; probably promotes adhesion to collagen-rich tissues
Fibrinogen-binding protein	Binds to fibrinogen; may promote phagocytosis by neutrophils
Fibronectin-binding protein	Binds to fibronectin; may promote adhesion
Exoenzymes	
DNase	Degrades host nucleic acids, making nucleotides available as nutrients
Neuraminidases, NanH, NanP	Cleaves sialic acids from host molecules; may expose receptors for increased adhesion; reduces mucus viscosity; makes IgA more susceptible to proteolytic attack
Proteases	Degrades host proteins, releasing amino acids as nutrients; may degrade proteins such as IgA, which are involved in host defense
Other	
Ability to invade epithelial cells	Hides in a protected niche; escapes host defenses and certain antimicrobial drugs
Ability to survive within macrophages	Evades macrophage killing

Lämmler 1996; Jost et al. 1999). This cytolytic activity may provide protection for the invading organism against the immune response. However, the function of PLO in virulence may also involve modulation of the immune response through the alteration of expression of various cytokines and other inflammatory substances, as is seen with other CDCs (reviewed in Billington et al. 2000).

Adhesion to epithelial cells is a primary requisite for the ability of bacteria to colonize host mucosal surfaces. *A. pyogenes* expresses the ability to bind collagen (Esmay et al. 2002), fibrinogen (Lämmler and Ding 1994), and fibronectin (Jost and Billington 2002). Collagen binding has been ascribed to CbpA, an MSCRAMM-like (microbial surface components recognizing adhesive matrix molecules) protein expressed on the cell surface of ~50% of *A. pyogenes* isolates (Esmay et al. 2002). A *cbpA* knockout mutant had significantly reduced binding to epithelial and fibroblast cell lines. Therefore, it is likely that the ability to bind to collagen promotes adherence and subsequent colonization in collagen-rich tissue. While not as well characterized, it would appear likely that the ability to bind fibronectin and fibrinogen would also promote adhesion.

All *A. pyogenes* strains express neuraminidase activity encoded by the *nanH* gene (Jost et al. 2001), and approximately two-thirds of isolates also contain a second neuraminidase gene, *nanP* (Jost et al. 2002a). Insertional inactivation of both genes resulted in a strain with a 53% reduction in its ability to bind to the HeLa epithelial cell line compared with the wild type (Jost et al. 2002a), indicating that the neuraminidases of *A. pyogenes* play a role in the adherence of this organism to host epithelial cells. It is possible that removal of sialic acid moieties from host molecules by *A. pyogenes* neuraminidases exposes cryptic receptors for bacterial attachment.

A. pyogenes produces four distinct Ca^{2+}-dependent serine proteases with caseinase and/or gelatinase activity (Schaufuss et al. 1989; Takeuchi et al. 1995). While, there is currently no compelling evidence to support a role for *A. pyogenes* proteases in pathogenesis, proteases are expressed during infection. Of randomly selected pigs, 35% had antibodies to proteases, while 93% of pigs with *A. pyogenes* abscesses were antibody positive (Takeuchi et al. 1979), suggesting that protease expression by *A. pyogenes* may play a role in pathogenesis. In addition, all *A. pyogenes* isolates secrete a DNase, which may aid in the depolymerization of highly viscous DNA released from disintegrating neutrophils in inflammatory lesions, making nucleotides available as nutrients.

Pathogenesis

Following a physical or microbial insult to the host, commensal *A. pyogenes* can disseminate to cause abortion as well as a variety of suppurative infections involving the joints, skin, and visceral organs. *A. pyogenes* can act as a primary pathogen, but it is more commonly isolated in mixed infections. The more common and economically important diseases are summarized in table 7.2.

Liver abscesses can occur in all types of cattle, but the most economically significant disease occurs in feedlot cattle. The major effects of liver abscessation are seen in reduced feed intake and weight gain, resulting in decreased feed efficiency. *Fusobacterium necrophorum* (see chapter 27) is generally considered to be the causative agent, although *A. pyogenes* is the second most commonly isolated bacterium, present in up to 90% of abscesses (Narayanan et al. 1998) and can be isolated in pure culture. The prevalence of liver abscesses in cattle fed an all-grain diet can be 60–80%. Intensive grain feeding results in ruminal acidosis, with the resulting damage to the rumen wall probably facilitating the entry of *A. pyogenes* into the blood. Bacteria travel via the circulation to the liver, and deposition of bacteria in the tissue results in abscess formation (Narayanan et al. 1998).

A. pyogenes can cause severe, clinical mastitis characterized by thick, purulent secretions, frequently in dry cows but also in lactating animals following teat injury. The spread of mastitis is probably due to contact of teats with a contaminated environment, such as milking apparatus, or *A. pyogenes* from udder infections, abscesses, and/or genital tracts. *A. pyogenes* may also be transmitted by biting flies. Once infected, the prognosis is poor, as antibiotic therapy is often ineffective, and cows can lose the function of the affected quarter. While not a major cause of mastitis, the incidence of *A. pyogenes* mastitis within a herd can be as high as 18% (Jones and Ward 1989). Furthermore, *A. pyogenes*, either alone or with other bacteria, is often associated with bovine uterine infections, resulting in severe endometritis and infertility (Lewis 1997).

Vaccines

Previous vaccination experiments using whole cells or culture supernatant were largely unsuccessful in protecting domestic animals from *A. pyogenes* infections. However, PLO shows promise

as a subunit vaccine. The serum from mice immunized with formalin-inactivated, or genetically toxoided, recombinant PLO exhibited high neutralizing titers to PLO hemolytic activity and were protected from challenge with *A. pyogenes* (Jost et al. 1999, 2003). It is uncertain how these results will translate to the domestic species in which *A. pyogenes* is a pathogen. However, in dairy and feedlot cattle vaccinated with recombinant PLO, high neutralizing antibody titers have been obtained (Jost and Billington 2002).

CONCLUSIONS

Despite the importance of this group of bacteria, especially in production animal health, only sparse information is available with respect to the molecular mechanisms of virulence. In the case of *C. pseudotuberculosis*, details of the interaction of this organism with host phagocytes, including mechanisms of intracellular survival and neutrophil chemotaxis, are virtually nonexistent and represent fertile ground for future study. Similarly, we are only now starting to dissect the virulence mechanisms of *A. pyogenes*. Of particular interest are how this organism can cause disease in a wide diversity of hosts, and how such a potent pathogen regulates expression of its virulence determinants to reside as a commensal. Molecular techniques such as genome sequencing and DNA micro-array analysis are becoming more readily accessible, and their application will provide insights into the unique virulence attributes of these bacteria.

REFERENCES

Amao, H., Y. Komukai, M. Sugiyama, K. W. Takahashi, T. Sawada, and M. Saito. 1995. Natural habitats of *Corynebacterium kutscheri* in subclinically infected ICGN and DBA/2 strains of mice. Lab. Animal Sci. 45:6–10.

Barrett, N. J. 1986. Communicable disease associated with milk and dairy products in England and Wales: 1983–1984. J. Infect. 12:265–272.

Bernheimer, A. W., and L. S. Avigad. 1982. Mechanism of hemolysis by renalin, a CAMP-like protein from *Corynebacterium renale*. Infect. Immun. 36:1253–1256.

Billington, S. J., B. H. Jost, and J. G. Songer. 2000. Thiol-activated cytolysins: structure, function and role in pathogenesis. FEMS Microbiol. Lett. 182:197–205.

Billington, S. J., B. H. Jost, W. A. Cuevas, K. R. Bright, and J. G. Songer. 1997. The *Arcanobacterium (Actinomyces) pyogenes* hemolysin, pyolysin, is a novel member of the thiol-activated cytolysin family. J. Bacteriol. 179:6100–6106.

Billington, S. J., P. A. Esmay, J. G. Songer, and B. H. Jost. 2002. Identification and role in virulence of putative iron acquisition genes from *Corynebacterium pseudotuberculosis*. FEMS Microbiol. Lett. 208:41–45.

Brownstein, D. G., S. W. Barthold, R. L. Adams, G. A. Terwilliger, and J. G. Aftosmis. 1985. Experimental *Corynebacterium kutscheri* infection in rats: bacteriology and serology. Lab. Animal Sci. 35:135–138.

De Rose, R., J. Tennent, P. McWaters, P. J. Chaplin, P. R. Wood, W. Kimpton, R. Cahill, and J. P. Scheerlinck. 2002. Efficacy of DNA vaccination by different routes of immunisation in sheep. Vet. Microbiol. 90:55–63.

Ding, H., and C. Lämmler. 1996. Purification and further characterization of a haemolysin of *Actinomyces pyogenes*. J. Vet. Med. B 43:179–188.

Esmay, P. A., S. J. Billington, M. A. Link, J. G. Songer, and B. H. Jost. 2002. The *Arcanobacterium pyogenes* collagen binding protein, CbpA, promotes adhesion to host cells. Unpublished data. Infect. Immun. Submitted.

Hayashi, A., R. Yanagawa, and H. Kida. 1985. Adhesion of *Corynebacterium renale* and *Corynebacterium pilosum* to the epithelial cells of various parts of the bovine urinary tract from the renal pelvis to vulva. Vet. Microbiol. 10:287–292.

Haynes, J. A., J. Tkalcevic, and I. T. Nisbet. 1992. Production of an enzymatically inactive analog of phospholipase D from *Corynebacterium pseudotuberculosis*. Gene 119:119–121.

Hedlund, M., and T. Pohjanvirta. 1989. *Corynebacterium ulcerans* in dairy cattle—a potential risk for man. A review on *C. ulcerans* and a survey of its occurrence in mastitic milk in Eastern Finland. Suom. Eläinlääkärilehti 95:552–558.

Hiramune, T., S. Inui, N. Murase, and R. Yanagawa. 1971. Virulence of three types of *Corynebacterium renale* in cows. Am. J. Vet. Res. 32:237–242.

Hodgson, A. L., J. Krywult, L. A. Corner, J. S. Rothel, and A. J. Radford. 1992. Rational attenuation of *Corynebacterium pseudotuberculosis*: potential cheesy gland vaccine and live delivery vehicle. Infect. Immun. 60:2900–2905.

Hodgson, A. L., M. Tachedjian, L. A. Corner, and A. J. Radford. 1994. Protection of sheep against caseous lymphadenitis by use of a single oral dose of live recombinant *Corynebacterium pseudotuberculosis*. Infect. Immun. 62:5275–5280.

Honkanen-Buzalski, T., and A. J. Bramely. 1984. Observations on *Corynebacterium bovis* infection

of the bovine mammary gland. II. Experimental infections. J. Dairy Sci. 51:379–385.

Honkanen-Buzalski, T., T. K. Griffen, and F. H. Dodd. 1984. Observations on *Corynebacterium bovis* infection of the bovine mammary gland. I. Natural infection. J. Dairy Res. 51:371–378.

Jones, G. F., and G. E. Ward. 1989. Cause, occurrence, and clinical signs of mastitis and anorexia in cows in a Wisconsin study. J. Am. Vet. Med. Assoc. 195:1108–1113.

Jost, B. H., and S. J. Billington. 2002. Unpublished data.

Jost, B. H., H. T. Trinh, J. G. Songer, and S. J. Billington. 2003. Immunization with genetic toxoids of the *Arcanobacterium pyogenes* cholesterol dependent cytolysin, pyolysin, protects mice against infection. Infect. Immun. 71: in press.

Jost, B. H., J. G. Songer, and S. J. Billington. 1999. An *Arcanobacterium (Actinomyces) pyogenes* mutant deficient in production of the pore-forming cytolysin pyolysin has reduced virulence. Infect. Immun. 67:1723–1728.

———. 2001. Cloning, expression and characterization of a neuraminidase gene from *Arcanobacterium pyogenes*. Infect. Immun. 69:4430–4437.

———. 2002a. Identification of a second *Arcanobacterium pyogenes* neuraminidase, and involvement of neuraminidase activity in host cell adhesion. Infect. Immun. 70:1106–1112.

Jost, B. H., K. W. Post, J. G. Songer, and S. J. Billington. 2002b. Isolation of *Arcanobacterium pyogenes* from the porcine gastric mucosa. Vet. Res. Comm. 26:419–425.

Kubota, T., and R. Yanagawa. 1988. Phagocytosis of piliated and non-piliated *Corynebacterium renale* by murine polymorphonuclear leukocytes. Jpn. J. Vet. Sci. 50:199–207.

Kudo, Y., R. Yanagawa, and T. Hiramune. 1987. Isolation and characterization of monoclonal antibodies against pili of *Corynebacterium renale* and *Corynebacterium pilosum*. Vet. Microbiol. 13:75–85.

Lämmler, C., and H. Ding. 1994. Characterization of fibrinogen-binding properties of *Actinomyces pyogenes*. J. Vet. Med. B 41:588–596.

Lewis, G. S. 1997. Uterine health and disorders. J. Dairy Sci. 80:984–994.

Lovell, R. 1944. Further studies on the toxin of *Corynebacterium pyogenes*. J. Pathol. Bacteriol. 56:525–529.

McNamara, P. J., G. A. Bradley, and J. G. Songer. 1994. Targeted mutagenesis of the phospholipase D gene results in decreased virulence of *Corynebacterium pseudotuberculosis*. Mol. Microbiol. 12:921–930.

McNamara, P. J., W. A. Cuevas, and J. G. Songer. 1995. Toxic phospholipases D of *Corynebacterium pseudotuberculosis*, *C. ulcerans*, and *Arcanobacterium haemolyticum*: cloning and sequence homology. Gene 156:113–118.

Narayanan, S., T. G. Nagaraja, N. Wallace, J. Staats, M. M. Chengappa, and R. D. Oberst. 1998. Biochemical and ribotypic comparison of *Actinomyces pyogenes* and *A. pyogenes*-like organisms from liver abscesses, ruminal wall, and ruminal contents of cattle. Am. J. Vet. Res. 59:271–276.

Ngatia, T. A., N. E. Jensen, and B. B. Berg. 1991. Changes in the bovine udder quarters naturally infected by *Corynebacterium bovis*. Br. Vet. J. 147:463–468.

Pankey, J. W., S. C. Nickerson, R. L. Boddie, and J. S. Hogan. 1985. Effects of *Corynebacterium bovis* infection on susceptibility to major mastitis pathogens. J. Dairy Sci. 68:2684–2693.

Paton, M. W., S. S. Sutherland, I. R. Rose, R. A. Hart, A. R. Mercy, and T. M. Ellis. 1995. The spread of *Corynebacterium pseudotuberculosis* infection to unvaccinated and vaccinated sheep. Aust. Vet. J. 72:266–269.

Piontkowski, M. D., and D. W. Shivvers. 1998. Evaluation of a commercially available vaccine against *Corynebacterium pseudotuberculosis* for use in sheep. J. Am. Vet. Med. Assoc. 212:1765–1768.

Rebhun, W. C., S. G. Dill, J. A. Perdizet, and C. E. Hatfield. 1989. Pyelonephritis in cows: 15 cases (1982–1986). J. Am. Vet. Med. Assoc. 194:953–955.

Schaufuss, P., R. Sting, and C. Lämmler. 1989. Isolation and characterization of an extracellular protease of *Actinomyces pyogenes*. Zentralbl. Bakteriol. 271:452–459.

Sordillo, L. M., S. P. Oliver, A. J. Guidry, and J. T. Dermody. 1988. Humoral immune response of bovine mammary glands colonized with *Corynebacterium bovis*: enumeration of plasma cell populations in tissue and immunoglobulin concentrations in milk. J. Vet. Med. B 35:617–627.

Takeuchi, S., R. Azuma, Y. Nakajima, and T. Suto. 1979. Diagnosis of *Corynebacterium pyogenes* in pigs by immunodiffusion test with protease antigen. Natl. Inst. Anim. Health Q. 19:77–82.

Takeuchi, S., T. Kaidoh, and R. Azuma. 1995. Assay of proteases from *Actinomyces pyogenes* isolated from pigs and cows by zymography. J. Vet. Med. Sci. 57:977–979.

Timoney, J. F., J. H. Gillespie, F. W. Scott, and J. E. Barlough. 1988. Hagan and Bruner's microbiology and infectious diseases of domestic animals, 8th ed. Ithaca, NY: Cornell University Press.

Watts, J. 1988. Etiological agents of bovine mastitis. Vet. Microbiol. 16:41–66.

Weisbroth, S. H., and S. Scher. 1968. *Corynebacterium kutscheri* infection in the mouse. I. Report of an outbreak, bacteriology, and pathology of spontaneous infections. Lab. Animal Care 18:451–458.

Williamson, L. H. 2001. Caseous lymphadenitis in small ruminants. Veterinary Clinics of North America: Food Animal Practice 17:359–371.

Yanagawa, R. 1986. Causative agents of bovine pyelonephritis: *Corynebacterium renale*, *C. pilosum* and *C. cystitidis*. Prog. Vet. Microbiol. Immunol. 2:158–174.

Yozwiak, M. L., and J. G. Songer. 1993. Effect of *Corynebacterium pseudotuberculosis* phospholipase D on viability and chemotactic responses of ovine neutrophils. Am. J. Vet. Res. 54:392–397.

8
Rhodococcus

J. F. Prescott, J. Ren, and C. Dupont

The genus *Rhodococcus* currently contains 12 species, several of which are of interest because of their diverse metabolic activities, industrial applications or potential in bioremediation. Two species are significant as pathogens, *R. equi* in mammals and *R. fascians* in plants (Bell et al. 1998). This chapter focuses entirely on *R. equi*.

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 and, 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 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

R. equi is an actinomycete member of the 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 hours of culture at 37°C.

Rhodococcus equi is a soil organism, certain specific virulent types of which are associated particularly with horses or pigs.

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 factors (temperature, dust, soil pH) and management. 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 is a progressive increase of infection to sometimes untenable levels. Inhalation of contaminated dust particles leads to pneumonic infection in foals. Endemic farms are generally those used intensively to rear foals over many years, where summer temperatures are high, the 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

VIRULENCE PLASMIDS

Foal Virulent *Rhodococcus equi*

A major advance in understanding the virulence of *R. equi* was the discovery by Takai and others (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°C 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.

Two virulence plasmids from foal isolates have been sequenced (Takai et al. 2000). By sequence data, 69 open reading frames (ORFs) were identified on these virtually identical plasmids (fig. 8.1). An important finding was that a 27.5 kb region fulfilled the criteria for a typical pathogenicity island (PI). This region contains at least 25 ORFs including the surprising finding of seven members of the *vap* gene family (*vapA*, *C*, *D*, *E*, *F*, *G,* and *H*). By contrast to VapA, Vaps C, D, and E are secreted from the cell into the environment (Byrne et al. 2001). The function of the Vap proteins is unknown. Apart from *vap* family genes, 12 of 25 ORFs in the PI lack similarity to genes of known function in the databases and their function remains unknown (Takai et al. 2000). Two genes with similarity to regulatory genes are present on the PI, a LysR family transcriptional regulator and a two-component response regulator.

Pig Virulent *Rhodococcus 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

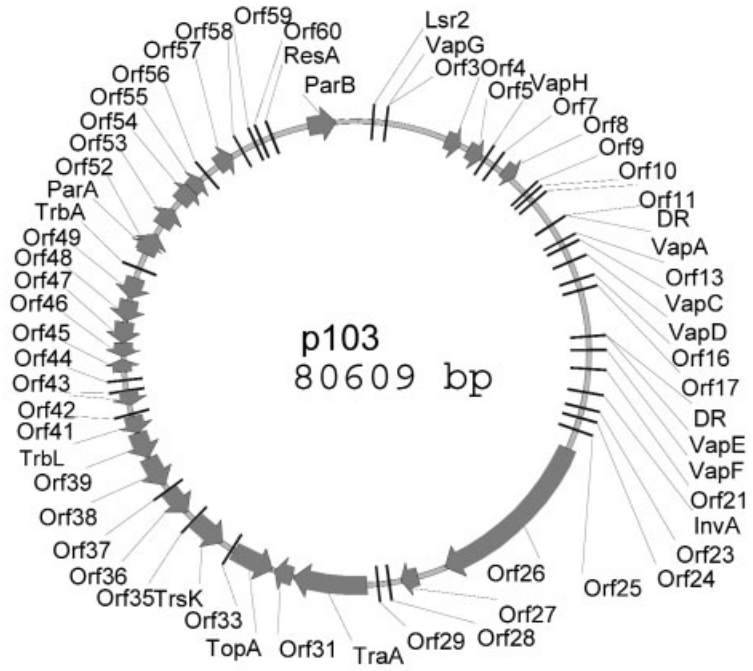


Figure 8.1. Map of the *Rhodococcus equi* virulence plasmid p103. The circle shows the orientation of the open reading frames, with genes having known or suspected function named. The 27.5 kb pathogenicity island runs from ResA to InvA. The conjugation region runs from about ORFs 23 to 40, and the replication region from about ORFs 41 to 60.

confers an "intermediate" virulence phenotype for mice (Takai et al. 1996b).

Other *Rhodococcus equi*

Opportunistic infections in immunocompromised human patients can be caused both by mouse high- 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, in which the virulence plasmid is always present (Takai 1997). In some cases, however, AIDS patients are infected with *R. equi,* which appear to have either a horse or pig source. Isolates from goats (Tkachuk-Saad et al. 1998) and cattle (Flynn et al. 2001) also lack a virulence plasmid.

Virulence of non-plasmid-containing human isolates of *R. equi* has also been associated with β-lactam antibiotic resistance. Compared to 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).

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 1997). 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 (1997) 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 *Mycobacterium 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 scavenging potentially harmful reactive oxygen intermediates (Chan et al. 1991). They may have a similar role in *R. equi*. 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 are more virulent than those with shorter chains (Gotoh et al. 1991). More recently, trehalose 6,6'-dimycolate, a compound identified in *R. equi* (Barton et al. 1989), was shown to be the main granulomatogenic mycolyl glycolipid isolated from a *Rhodococcus* sp. (Ueda et al. 2001).

A lipoglycan, lipoarabinomannam (LAM), may localize to both the plasma membrane and the outer lipid layer (Sutcliffe 1997) and play a role in virulence. The LAM of *R. equi* 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 may be particularly important in interaction of the organism with macrophages, as discussed below. Purified *R. equi* LAM 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). In *M. tuberculosis*, LAM can be released at the site of infection and modulate immune responses by blocking T-cell activation and proliferation (Strohmeier and Fenton 1999), although details are not well characterized.

Other unexplored candidates as virulence factors include capsular polysaccharides as well as cholesterol oxidase, choline phosphohydrolase, and phospholipase C exoenzymes. These exoenzymes may contribute to cellular membrane destruction and provide nutrient sources for the organism within the macrophage, as well as causing tissue damage.

Many aspects of virulence in *R. equi* are currently unknown and speculative, but details are becoming clarified. Part of the importance of the organism is that it is a pathogen of macrophages related to *M. tuberculosis* that, once details of its pathogenesis are fully understood, may enhance understanding of some of the key events that allow this organism to persist and replicate in macrophages.

PATHOGENESIS

Overview

The basis of the pathogenicity of *R. equi* is the ability to multiply in and eventually to destroy alveolar macrophages, but the mechanisms are not

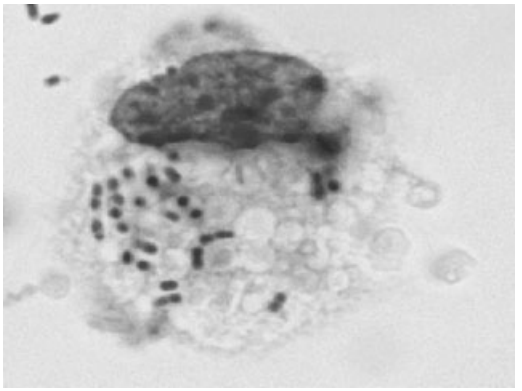


Figure 8.2. *Rhodococcus equi* growing in an equine macrophage *in vitro*. The ability of *R. equi* to persist in, and eventually destroy, alveolar macrophages is the basis of its pathogenicity.

understood. The infectivity of *R. equi in vitro* is largely or exclusively limited to cells of the monocyte-macrophage lineage (fig. 8.2). In contrast to macrophages, neutrophils from foals and adult horses are fully bactericidal, and such killing is markedly enhanced by specific opsonizing antibody (Hondalus 1997). The discussion below 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

It is apparent that 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), which bind one or more of C3b, C3bi, and C4b 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 proinflammatory 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 complement receptor type 3 (CR3) (Hondalus and Mosser 1994). The lipoarabinomannam of *R. equi* (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* lipoarabinomannam 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 mannose receptor may occur. Since opsonization of *R. equi* with specific antibody is followed by increased phagosome-lysosome fusion and by significantly enhanced *R. equi* killing by equine macrophages (Hietala and Ardans 1987; Zink et al. 1987), macrophage entry through non-Fc receptors may be important in determining the fate of the 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 (Tjelle et al. 2000). The evidence supports phagosome-endosome fusion as occurring not as complete fusion events but rather as brief exchanges ("kiss-and-run," or short-lasting fusion-fission events) of content and membranes, probably via microtubule-dependent transport (De Chastellier et al. 1995; Desjardins 1995). This maturation process is characterized by changes in the molecules associated with the phagosomal membrane from those typical of early endosomes (Rab5, annexin, NSF, α/β SNAP [soluble NSF-attachment proteins], transferrin receptor, mannose receptor) to those typical of late endosomes and lysosomes (Rab5, Rab7, mannose-6-phosphate receptor, cathepsin D, LAMP1 [lysosome-associated membrane-protein1], LAMP2). Proteins involved in budding from endosomal vesicles and their fusion with the identical protein on the phagosome (the GTPase Rab5 in early endosomes, Rab7 in late endosomes) may be important targets for pathogens such as *R. equi,* which persist in macrophages.

Fusion may also be dependent on a proton gradient between the interacting vacuoles. Maturation of the phagosome is also characterized by progressive acidification with delivery of the vacuolar proton pump from 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) (Clemens 1996). For bacteria that do not interfere with the process, phagocytosis to phagolysosome formation takes about 5 minutes (Mayorga et al. 1991).

Phagocytosis: Degradation of Bacteria in the Phagolysosome

Phagocytosed bacteria are degraded in the late phagolysosome by the same mechanisms employed by lysosomes, involving a wide range of acid-resistant hydrolases within the acid environment of the phagolysosome. Materials may also be transported for further degradation from the phagolysosome to late endosomes or lysosomes. Peptides are acquired by class II MHC molecules within the phagosome or in endocytic organelles before transport to the plasma membrane for antigen presentation. Peptides may also be acquired by class I MHC molecules from phagocytosed bacteria, suggesting that degraded materials reach the cytosol in some way.

Behavior of *Rhodococcus equi* in Macrophages

How virulent *R. equi* survive and replicate in macrophages offers fertile ground for future study. Intramacrophage *R. equi* appear to be located exclusively within membrane-enclosed vacuoles and persistence correlates with the absence of phagosome-lysosome fusion (Hietala and Ardans 1987; Zink et al. 1987). It has been shown that plasmid-encoded products contribute to the ability of *R. equi* to survive and replicate in macrophages. Preliminary observations by Haas (2002) suggest that maturation of the phagosome is more efficiently diverted in strains possessing the virulence plasmid compared to plasmid-negative isogenic strains that do not, and that cytotoxicity of *R. equi* for J774E murine macrophages is strongly up-regulated by the possession of the virulence plasmid. The presence of transferrin in early endosomes would explain the importance of iron restriction in regulating transcription of some virulence plasmid PI genes, discussed below. NRAMP1 (natural-resistance-associated macrophage protein 1) 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 (Zwilling et al. 1999), 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 (Pierre and Fontecave 1999). 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, dramatic down-regulation of NRAMP1 transcription was observed in equine macrophages following infection with *R. equi* (Watson et al. 2002), but whether this is different between plasmid positive and negative strains was not explored.

Although details are sparse, it can be speculated that *R. equi* may therefore behave like *M. tuberculosis* and related mycobacteria that interfere with phagosome maturation, residing in a vacuole that is only mildly acid (pH 6.5) and that resists lysosomal fusion (Armstrong and Hart 1975). The characteristics of the *M. tuberculosis* phagosome are those of

immaturity and of a persistent interaction between the phagosome and early endosomes (Clemens 1996; Clemens and Horwitz 1996). The *M. tuberculosis* phagosome shows persistence of functional Rab5, consistent with maintenance of its interaction with early endosomes and preservation of its early endosomal characteristics (Clemens et al. 2000). *Mycobacterium tuberculosis* interferes with the acquisition of the fusion facilitating molecules, either by avoiding loss of Rab5 and/or early endosome SNARES (soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor), or by blocking acquisition of other facilitating molecules (such as Rab7) (Russell 1998). The specific lack of the vacuolar proton pump (Russell 1998) appears to be responsible for the mildly acid conditions. Inhibition of acidification blocks vesicular delivery to lysosomes (Clague et al. 1994), and the pH of the phagosome is probably important in controlling fusion events. The 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,* inhibits phagolysosome formation (Gordon et al. 1980). 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 nitric oxide synthase (iNOS). Macrophage production of toxic superoxides from the relatively inert oxygen molecule involves production by a membrane-bound NADPH oxidase that is activated in the "respiratory burst" that occurs when opsonized bacteria initiate the phagocytosis process. Nitric oxide synthetases, especially iNOS, catalyze the oxidation of a guanidino nitrogen of L-arginine to NO. The iNOS is usually transcribed in response to cytokines (IFN-γ, IL-1, TNF-α) 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 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 that 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-α, results in nitric oxide production following iNOS transcription, and the second step, signaled 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 appears to have a considerable effect on expression of PI genes associated with replication of the organism. 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*. Recent studies by Ren and Prescott (2003) using a microarray of the virulence plasmid genes have shown that only genes on the PI are differentially transcribed inside equine macrophages in comparison to the isolate grown at 30°C *in vitro* under non-nutrient-limiting conditions. Six *vap* genes were transcribed, with two other genes, Orf9 and Orf10, being the most transcribed. Orf8, a two-component response-regulator homologue, was mildly up-regulated. The major influence of PI transcription was temperature (fig. 8.3), but temperature-up-regulated genes were found to fall into two groups. Group I, which contained 12 PI genes including the 6 *vap* genes, was further up-regulated by low iron and down-regulated by low magnesium, whereas group II genes, Orf3, 9, and 10, 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. An IdeR binding site was found at the -35 promoter region of this

gene, suggesting not only that the low iron environment of the macrophage is an important regulatory signal for production of VapA but also 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 *M. tuberculosis*, the IdeR also controls transcription of genes involved in macrophage survival, as well as in iron acquisition and iron storage (Gold et al. 2001). Inactivation experiments have demonstrated that *mgtC*, a magnesium regulated gene, is essential for bacterial survival in cultured macrophages and infection of mice in both *S. typhimurium* and *M. tuberculosis* (Moncrief and Maguire 1998), although the precise role of *mgtC* is unclear.

R. 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 four- and 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 formation, 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 *Mycobacterium tuberculosis* (McKinney et al. 2000) and *Candida albicans* (Lorenz and Fink 2001). It is interesting that the isocitrate lyase (*aceA*) gene of *R. equi* is cotranscribed with the 3-hydroxyacyl-CoA dehydrogenase (*fadB*) gene involved in beta-oxidation of fatty acids and, 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* protect it from exonuclease attack. A stable *aceA* transcript may provide the high isocitrate lyase activity required for successful

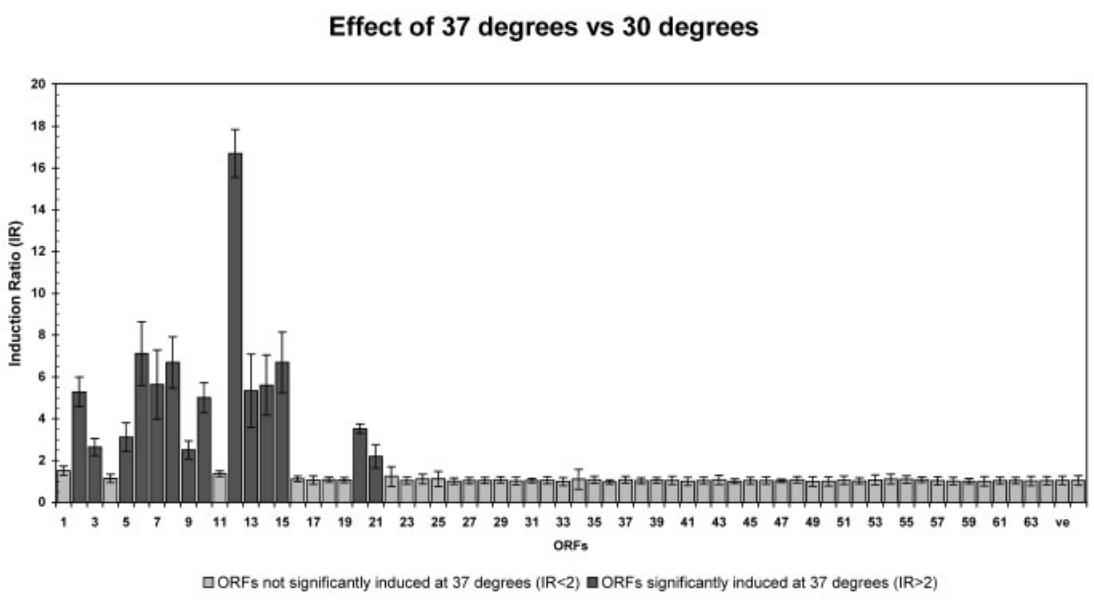


Figure 8.3. The effect of growth at 37°C compared to growth at 30°C on transcription of pathogenicity island genes of the virulence plasmid from a foal isolate of *R. equi*. ORFs 2, 6, 12, 14, 15, and 20 are *vap* genes. *VapA* (ORF 12) is the gene most responsive to temperature under these conditions.

competition for isocitrate between this glyoxylate shunt enzyme and isocitrate dehydrogenase (Kelly et al. 2002). The cholesterol oxidase gene, *choE*, of *R. equi* has been identified and mutated, but its role in survival of the organism in macrophages has not been assessed (Navas et al. 2001). How *R. equi* regulates its metabolic activities to survive in the nutrient-limited environment of the macrophage requires extensive investigation.

Interestingly, the plant pathogen *R. fascians* not only possesses a linear plasmid necessary for virulence but also has an 18 kb chromosomal region essential for persistence that is syntenic with chromosomal regions of *Mycobacterium* species that encompass persistence loci (Vereecke et al. 2002). This locus includes a malate synthase gene involved in the glyoxylate shunt as well as genes involved in glycine cleavage, thought to be involved in maintence of the NAD pool under oxygen-limiting conditions in *M. tuberculosis*.

In summary, considerable work is required to understand the regulation of virulence and metabolic genes within the macrophage, but initial evidence supports expression of virulence genes being a function of temperature and of iron, magnesium, and hydrogen peroxide concentrations.

Lesions

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

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 of 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 and others (2001) showed that purified VapA- and VapC-antibody provided protection of foals against experimental pneumonic infection equivalent to that obtained with hyperimmune plasma. However, foals born to vaccinated mares are not protected against infection despite passive transfer of anti-*R. equi* antibody. This failure may relate to the isotype of antibody produced by the vaccines used experimentally, but this has not been investigated in detail.

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 her coworkers (1993, 1995, 1996) 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. (1993) 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. There is some evidence that CD8+ T lymphocytes may play a minor role in clearance (reviewed by Hines et al. 1997).

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 to $CD4^+$ 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.

Immunization of foals under natural settings with a VapA extract in aluminium hydroxide adjuvant resulted in development of pneumonia in immunized but not in nonimmunized foals, and an IgGb- and IgGT-dominated isotype response compared to 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 to 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.

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 are important correlates of protective immunity. It is interesting that IgGa and IgGb fix complement whereas IgGT does not, and indeed IgGT may inhibit complement fixation by IgGa and IgGb (Lopez et al. 2002).

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 plays a crucial role. The number of bacteria inhaled by foals is almost certainly also important. For example, the dose of 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 that predispose 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.

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

It is anticipated that molecular details of how *R. equi* subverts normal macrophage killing mechanisms and the foal's protective immune response will be investigated in the next years and will lead to a greater understanding of this fascinating pathogen. The availability of a partial genome sequence of the organism (Rahman et al. 2003) will allow researchers to identify potential virulence genes of interest for future study. The ability to introduce both random (Mangan and Meijer 2001; Ashour and Hondalus 2003) and particularly targeted mutations (Navas et al. 2001) will be crucial in these endeavors

REFERENCES

Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. Ann. Rev. Immunol. 17:593–623.

Armstrong, J. A., and P. D. Hart. 1975. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. J. Exp. Med. 142:1–16.

Ashour, J., and M. K. Hondalus. 2003. Phenotypic mutants of the intracellular actinomycete *Rhodococcus equi* created by in vivo Himar1 transposon mutagenesis. J. Bacteriol. 185:2644–2653.

Barton, M. D., M. Goodfellow, and D. E. Minnikin. 1989. Lipid composition in the classification of *Rhodococcus equi*. Zblatt. Bakteriol. 272:154–170.

Bell, K. S., J. C. Phil, D. W. J. Aw, and N. Christofi. 1998. The genus Rhodococcus. J. Appl. Microbiol. 85:195–210.

Benoit, S., A. Benachour, S. Taouji, Y. Auffray, and A. Hartke. 2001. Induction of *vap* genes encoded by the virulence plasmid of *Rhodococcus equi* during acid tolerance response. Res. Microbiol. 152:439–449.

———. 2002. H_2O_2, which causes macrophage-related stress, triggers induction of expression of virulence-associated plasmid determinants in *Rhodococcus equi*. Infect. Immun. 70:3768–3776.

Boland, C. A., and W. G. Meijer. 2000. The iron dependent regulatory protein IdeR (DtxR) of *Rhodococcus equi*. FEMS Microbiol. Lett. 191:1–5.

Byrne, B. A., J. F. Prescott, G. H. Palmer, S. Takai, V. M. Nicholson, D. C. Alperin, and S. A. Hines. 2001. Virulence plasmid of *Rhodococcus equi* contains inducible gene family encoding secreted proteins. Infect. Immun. 69:650–656.

Chan, J., X. D. Fan, S. W. Hunter, P. J. Brennan, and B. R. Bloom. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect Immun. 59:1755–1761.

Clague, M. J., S. Urbe, F. Aniento, and J. Gruenberg. 1994. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. J. Biol. Chem. 269:21–24.

Clemens, D. L. 1996. Characterization of the *Mycobacterium tuberculosis* phagosome. Trends Microbiol. 4:113–118.

Clemens, D. L., and M. A. Horwitz. 1996. The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously added transferrin. J. Exp. Med. 184:1–7.

Clemens, D. L., B-Y. Lee, and M. A. Horwitz. 2000. Deviant expression of Rab5 on phagosomes containing the intracellular pathogens *Mycobacterium tuberculosis* and *Legionella pneumophila* is associated with altered phagosomal fate. Infect. Immun. 68:2671–2684.

Darrah, P. A., M. K. Hondalus, Q. Chen, H. Ischiropoulos, and D. M. Mosser. 2000. Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. Infect. Immun. 68:3587–3593.

De Chastellier, C., L. Lang, and L. Thilo. 1995. Phagocytic processing of the macrophage endoparasite, *Mycobacterium avium*, in comparison to phagosomes which contain *Bacillus subtilis* or latex beads. Eur. J. Cell Biol. 68:167–182.

Desjardins, M. 1995. Biogenesis of phagolysosomes; the "kiss and run" hypothesis. Trends Cell Biol. 5:183–186.

Flynn, O., F. Quigley, E. Costello, D. O'Grady, A. Gogarty, J. McGuirk, and S. Takai. 2001. Virulence-associated protein characterisation of *Rhodococcus equi* isolated from bovine lymph nodes. Vet. Microbiol. 78:221–228.

Garton , N. J., M. Gilleron, T. Brando, H-H. Dan, S. Giguère, G. Puzo, J. F. Prescott, and I. C. Sutcliffe. 2002. A novel lipoarabinomannam from the equine pathogen *Rhodococcus equi*: structure and effect on macrophage cytokine production. J. Biol. Chem. 277:31722–31733.

Giguère S., B. N. Wilkie, and J. F. Prescott. 1999. Modulation of cytokine response of pneumonic foals by virulent *Rhodococcus equi*. Infect. Immun. 67:5041–5047.

Gold, B., G. M. Rodriguez, S. A. Marras, M. Pentecost, and I. Smith. 2001. The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. Mol. Microbiol. 42: 851–865.

Gordon, A. H., P. D. Hart, and M. R. Young. 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature 286:79–80.

Gotoh, K., M. Mitsuyama, S. Imaizumi, I. Kawamura, I., and I. Yano. 1991. Mycolic acid-containing glycolipid as a possible virulence factor of *Rhodococcus equi* for mice. Microbiol. Immunol. 35:175–185.

Haas, A., A. Lührmann, N. Mauder, E. Fernandez-Mora, J. Schulze-Lührmann. 2002. Cell biology of *R. equi*-infected macrophages: phagosome biogenesis and host cell toxicity. Dorothy Russell Havemeyer Foundation Workshop on *Rhodococcus equi* and equine rhodococcal pneumonia. Pullman, WA.

Hietala, S. K., and A. A. Ardans. 1987. Interaction of *Rhodococcus equi* with phagocytic cells from *R.*

equi-exposed and non-exposed foals. Vet. Microbiol. 14:307–320.

Hines, M. T., K. M. Paasch, D. C. Alperin, G. H. Palmer, N. C. Westhoff, and S. A. Hines. 2001. Immunity to *Rhodococcus equi*: antigen-specific recall responses in the lungs of adult horses. Vet. Immunol. Immunopathol. 79: 101–113.

Hines, S. A., D. M. Stone, M. T. Hines, D. C. Alperin, D. P. Knowles, L. K. Norton, M. J. Hamilton, W. C. Davis, and T. C. McGuire. 2003. Clearance of virulent but not avirulent *Rhodococcus equi* from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4(+) and CD*(+) T lymphocytes. Clin. Diagn. Lab. Immunol. 10:208–215.

Hines, S. A., S. T. Kanaly, B. A. Byrne, and G. H. Palmer. 1997. Immunity to Rhodococcus equi. Vet. Microbiol. 56:177–185.

Hondalus, M. K. 1997. Pathogenesis and virulence of *Rhodococcus equi*. Vet. Microbiol. 56:257–268.

Hondalus, M. K., and D. M. Mosser. 1994. Survival and replication of *Rhodococcus equi* in macrophages. Infect. Immun. 62:4167–4175.

Hooper-McGrevy, K., B. N. Wilkie, and J. F. Prescott. 2003. Immunoglobulin G sub-isotype response of pneumonic and healthy, exposed foals and adult horses to *Rhodococcus equi* virulence-associated proteins. Clin. Diagn. Lab. Immunol. (In press)

Hooper-McGrevy, K. E., S. Gugère, B. N. Wilkie, and J. F. Prescott. 2001. Evaluation of equine immunoglobulin specific for *Rhodococcus equi* virulence-associated proteins A and C for use in protecting foals against *Rhodococcus equi*-induced pneumonia. Am. J. Vet. Res. 62:1307–1313.

Kanaly S. T., S. A. Hines, and G. H. Palmer. 1993. Failure of pulmonary clearance of *Rhodococcus equi* infection in $CD4^+$ T-lymphocyte-deficient transgenic mice. Infect. Immun. 61:4929–4932.

———. 1995. Cytokine modulation alters pulmonary clearance of *Rhodococcus equi* and development of granulomatous pneumonia. Infect. Immun. 63:3037–3041.

———. 1996. Transfer of $CD4^+$ Th1 cell line to nude mice effects clearance of *Rhodococcus equi* from the lung. Infect. Immun. 64:1126–1132.

Kasuga-Aoki, H., S. Takai, Y. Sasaki, S. Tsubaki, H. Madarame, and A. Nakane. 1999. Tumour necrosis factor and interferon-gamma are required in host resistance against virulent *Rhodococcus equi* infection in mice: cytokine production depends on the virulence levels of *R. equi*. Immunology. 96: 122–127.

Kelly, B. G., D. M. Wall, C. A. Boland, and W. G. Meijer. 2002. Isocitrate lyase of the facultative intracellular pathogen *Rhodococcus equi*. Microbiol. 148:793–798.

Lopez, A. M., M. T. Hines, G. H. Palmer, D. C. Alperin, and S. A. Hines. 2002. Identification of pulmonary T-lymphocyte and serum antibody isotype responses associated with protection against *Rhodococcus equi*. Clin. Diagn. Lab. Immunol. 9:1270–1276.

Lorenz, M. C., and G. R. Fink. 2001. The gloxylate cycle is required for fungal virulence. Nature 412:83–86.

Mangan, M. W., and W. G. Meijer. 2001. Random insertion mutagenesis of the intracellular pathogen *Rhodococcus equi* using transposomes FEMS Microbiol. Lett. 205:243–246.

Mayorga, L. S., F. Bertini, and P. D. Stahl. 1991. Fusion of newly formed phagosomes with endosomes in intact cells and in a cell-free system. J. Biol. Chem. 266:6511–6517.

McKinney, J. D., K. Höner zu Bentrup, E. J. Munoz-Elias, A. Miczak, B. Chen, W. T. Chan D. Swenson, W. R. Jacobs, Jr., and D. G. Russell. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406:735–738.

Moncrief, M. B., and M. E. Maguire. 1998. Magnesium and the role of MgtC in growth of *Salmonella typhimurium*. Infect. Immun. 66:3802–3809.

Navas, J., B. González-Zorn, N. Ladron, P. Garrido, and J. A. Vázquez-Boland. 2001. Identification and mutagenesis by allelic exchange of *choE*, encoding a cholesterol oxidase from the intracellular pathogen *Rhodococcus equi*. J. Bact. 183:4796–4805.

Nordengrahn, A, M. Rusvai, M. Merza, J. Ekstrom, B. Morein, and S. Belak. 1996. Equine herpesvirus type 2 (EHV-2) as a predisposing factor for *Rhodococcus equi* pneumonia in foals: prevention of the bifactorial disease with EHV-2 immunostimulating complexes. Vet. Microbiol. 51:55–68.

Nordmann, P., M. Keller, F. Espinasse, E. J. Ronco. 1994. Correlation between antibiotic resistance, phage-like particle presence, and virulence in *Rhodococcus equi* human isolates. J. Clin. Microbiol. 32:377–383.

Pierre, J. L., and M. Fontecave. 1999. Iron and activated oxygen species in biology: the basic chemistry. Biometals 12:195–199.

Power, C. A., G. Wei, and P. A. Bretscher. 1998. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. Infect. Immun. 66:5743–5750.

Prescott, J. F., V. M. Nicholson, M. C. Patterson, M. C. Zandona Meleiro, A. Caterino de Araujo. J. A. Yager, and M. A. Holmes. 1997. Use of

Rhodococcus equi virulence-associated protein for immunization of foals against *Rhodococcus equi* pneumonia. Am. J. Vet. Res. 58:356–359.

Rahman, M. T., L. L. Herron, V. Kapur, W. G. Meijer, B. A. Byrne, J. Ren, V. M. Nicholson, and J. F. Prescott. 2003. Partial genome sequencing of *Rhodococcus equi* ATCC 33701. Vet. Microbiol. (In press)

Ren, J., and J. F. Prescott. 2003. Analysis of virulence plasmid gene expression of intra-macrophage and in vitro grown *Rhodococcus equi* ATCC 33701. Vet. Microbiol. (In press)

Riess, F. G., M. Eflein, M. Benk, B. Schiffler, R. Benz, N. Garton, and I. Sutcliffe. 2003. The cell wall of the pathogenic bacterium *Rhodococcus equi* contains two channel-forming proteins with different properties. J. Bacteriol. 185:2952–2960.

Russell, D. G. 1998. What does "inhibition of phagosome-lysosome fusion" really mean? Trends Microbiol. 6:212–213.

———. 2001. *Mycobacterium tuberculosis*: Here today, and here tomorrow. Nature reviews Mol. Cell. Biol. 2:569–577.

Smith, R. L., M. T. Kaczmarek, L. M. Kucharski, and M. E. Maguire. 1998. Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* and *mgtCB* during invasion of epithelial and macrophage cells. Microbiol. 144:1835–1843.

Strohmeier, G. R., and M. J. Fenton. 1999. Roles of lipoarabinomannam in the pathogenesis of tuberculosis. Microbes Infect. 1:709–717.

Sutcliffe, I. C. 1997. Macroamphiphilic cell envelope components of *Rhodococcus equi* and closely related bacteria. Vet. Microbiol. 56:287–299.

Takai, S. 1997. Epidemiology of *Rhodococcus equi* infections: a review. Vet. Microbiol. 56:167–176.

Takai, S., K. Koike, S. Ohbushi, C. Izumi, and S. Tsubaki. 1991a. Identification of 15- to 17-kilodalton antigens associated with virulent *Rhodococcus equi*. J. Clin. Microbiol. 29:439–443.

Takai, S., M. Iie, Y. Watanabe, S. Tsubaki, and T. Sekizaki. 1992. Virulence-associated 15- to 17-kilodalton antigens in *Rhodococcus equi*: temperature-dependent expression and location of the antigens. Infect. Immun. 60:2995–2997.

Takai, S., N. Fukunaga, K. Kamisawa, Y. Imai, Y. Sasaki, and S. Tsubaki. 1996a. Expression of virulence-associated antigens of *Rhodococcus equi* is regulated by temperature and pH. Microbiol. Immunol. 40:591–594.

Takai, S., N. Fukunaga, S. Ochiai, Y. Imai, Y. Sasaki, S. Tsubaki, and T. Sekizaki. 1996b. Identification of intermediately virulent *Rhodococcus equi* from pigs. J. Clin. Microbiol. 34:1034–1037.

Takai, S., S. A. Hines, T. Sekizaki, V. M. Nicholson, D. Alperin, M. Osaki, D. Takamatsu, M. Nakamura, K. Suzuki, N. Ogino, T. Kakuda, H. Dan, and J. F. Prescott. 2000. DNA sequence and comparison of virulence plasmids from *Rhodococcus equi* ATCC 33701 and 103. Infect. Immun. 68:6840–6847.

Takai, S., T. Sekizaki, T. Ohbushi, C. Izumi, T. Sungawara, Y. Watanabe, and S. Tsubaki. 1991b. Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. Infect. Immun. 59:4056–4060.

Tan, C., J. F. Prescott, M. C. Patterson, and V. M. Nicholson. 1995. Molecular characterization of a lipid-modified virulence-associated protein of *Rhodococcus equi* and its potential in protective immunity. Can. J. Vet. Res. 59:51–59.

Tjelle, T. E., T. Lovdal, and T. Berg. 2000. Phagosome dynamics and function. Bioessays 22:255–263.

Tkachuk-Saad, O., and J. Prescott. 1991. *Rhodococcus equi* plasmid: isolation and partial characterization. J. Clin. Microbiol. 29:2696–2700.

Tkachuk-Saad, O., P. Lusis, R. D. Welch, and J. F. Prescott. 1998. *Rhodococcus equi* infection in goats. Vet. Rec. 143:311–312.

Ueda, S., N. Fujuwara, T. Naka, I. Sakaguchi, Y. Ozeki, I. Yano, T. Kasama, and K. Kobayashi, 2001. Structure-activity relationship of mycolyl glycolipids derived from *Rhodococcus* sp. 4306. Microbial Pathogen. 30:91–99.

Vereecke, D., C. Cornelis, W. Temmerman, M. Holsters, and K. Goethals. 2002. Versatile persistence pathways for pathogens of animals and plants. Trends Microbiol. 10:485–488.

Watson, J. L., C. M. Bosio, S. K. Hietala, J. Bea, and W. D. Wilson. 2002. Preliminary investigations of innate immunity to *Rhodococcus equi* infection. Dorothy Russell Havemeyer Foundation Workshop on *Rhodococcus equi* and equine rhodococcal pneumonia. Pullman, WA.

Zink, M. C., J. A. Yager, J. F. Prescott, and M. A. Fernando. 1987. Electron microscopic investigation of intracellular events after ingestion of *Rhodococcus equi* by foal alveolar macrophages. Vet. Microbiol. 14:295–305.

Zwilling, B. S., D. E. Kuhn, L. Wikoff, D. Brown, and W. Lafuse. 1999. Role of iron in Nramp1-mediated inhibition of mycobacterial growth. Infect. Immun. 67: 1386–1392.

9
Listeria

C. Czuprynski

The genus *Listeria* contains five species: *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri,* and *L. welshimeri.* Of these, *L. monocytogenes* is the only significant pathogen, causing septicemia, abortion, and central nervous system (CNS) infection in a wide range of animal species including humans. The latter three *Listeria* species are relatively avirulent and will not be considered further in this review. *L. ivanovii* shares certain characteristics with *L. monocytogenes* (i.e., hemolysis) and is occasionally associated with abortion in ruminants, but it does not cause CNS infection and is not of pathogenic significance in other animal species or in humans. Genomic analysis indicates multiple gene acquisitions and deletion events between *L. monocytogenes* strain EGD (serotype 1/2a) and *L. innocua* (Glasser et al. 2001). The encoded proteins of *L. monocytogenes* have considerable similarity with those of *Bacillus subtilis* (Cabanes et al. 2002). A serotype 4b strain of *L. monocytogenes* (ScottA) is also being sequenced. It will be interesting to compare its genome with that of strain EGD, since there are differences in the distribution of the two serotypes in sporadic cases versus large outbreaks of listeriosis in humans (Notermans et al. 1998; Hurd et al. 2000; Aureli et al. 2000).

From a practical standpoint, *L. monocytogenes* is primarily of veterinary medical concern 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). Although the number of reported human cases is low compared to other foodborne bacterial pathogens, such as *Campylobacter* and *Salmonella*, the mortality rate for human listeriosis cases is the highest among human foodborne bacterial pathogens (an estimated 2,500 cases and 500 deaths per year in the United States) (Notermans et al. 1998; Hurd et al. 2000). The veterinarian must be aware of both the direct effects of *L. monocytogenes* on infected ruminants, and the public health concerns surrounding the entry of *Listeria*-contaminated meat and dairy products into the human food chain (fig. 9.1).

CHARACTERISTICS OF THE BACTERIUM

L. monocytogenes has several characteristics that make it a unique and problematic pathogen. As might be expected for a bacterium capable of free-living growth, *L. monocytogenes* can multiply in diverse environmental conditions. It is relatively resistant to high salt concentrations and can grow over a pH range of 5 to 9. This latter property is related to some outbreaks of listeriosis in ruminants (i.e., poorly prepared silage that does not achieve appropriate acidity) (Low and Renton 1985; Fenlon 1986). Of particular note is its ability to grow at temperatures from 4°C to 45°C. The ability to grow at low temperatures (albeit slowly) has been exploited by both the organism and the laboratory diagnostician. *L. monocytogenes* can multiply to high numbers in silage (Fenlon 1986) or in food products maintained at room or refrigeration temperature (Farber and Peterkin 1991; Aureli et al. 2000). This poses a threat to the animal or person that ingests the contaminated material, and presents a mechanism by which the laboratory worker can select and enrich for listeriae in clinical or environmental specimens (i.e., cold enrichment).

In the laboratory, *L. monocytogenes* forms small translucent colonies in 24 to 48 hours when incubated

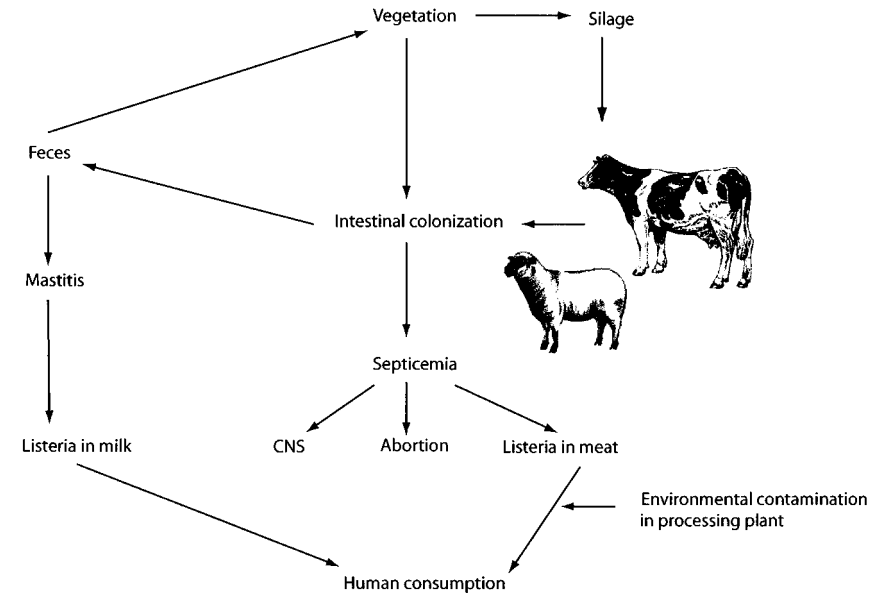


Figure 9.1. Overview of listeriosis in ruminants and possible route of introduction into food products for human consumption.

aerobically or anaerobically at 37°C on common bacteriological media such as trypticase soy agar or brain heart infusion agar. *L. monocytogenes* is hemolytic when blood is included in the agar, although the zone of hemolysis can be narrow. *L. monocytogenes* produces a CAMP reaction with the hemolysin of *Staphylococcus aureus*. In contrast, *L. ivanovii* typically produces double-zone hemolysis and is negative in the CAMP reaction with *S. aureus* (Schuchat et al. 1991). Gram stain reveals thin, nonspore-forming, gram-positive rods that can vary in length. Other diagnostic characteristics include being oxidase negative, catalase positive, H_2S negative, and motile at room temperature but not at 37°C. This latter property reflects the production of peritrichous flagella at 25°C or lower, whereas flagella are rare when the organisms are incubated at body temperature (35° to 39°C). The emergence of *L. monocytogenes* as a foodborne pathogen has led diagnosticians to devise improved selective media (modified Oxford agar) as well as novel methods such as antibody-based tests, or use of DNA probes and PCR amplification.

There are 11 recognized serotypes for the somatic and flagellar antigens of *L. monocytogenes,* with nearly all cases of animal and human infection 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 serotypes (Notermans et al. 1998; Aureli et al. 2000; Hurd et al. 2000). The reason for this association is unclear, but it might reflect a property of the serotype 4b strains that allows them to better survive within the gastrointestinal tract and translocate across the intestinal mucosa to establish systemic infection (Barbour et al. 2001; Czuprynski et al. 2002). Using ribotyping and other techniques, Wiedmann and coworkers (1997b) identified three lineages of *L. monocytogenes* strains, which appear to segregate into those that are associated with animal cases, sporadic human cases, and epidemic human episodes.

SOURCES OF THE BACTERIUM

L. monocytogenes is widely distributed in nature, having been isolated from soil, vegetation, water, feces, and tissues from a wide variety of vertebrate and invertebrate species (Gray and Killinger 1966). The organism is hardy and persistent in the environment. In particular, *Listeria* species can be isolated from drains and other surfaces in food processing

facilities even when good hygienic practices are in place (Farber et al. 1996; Notermans et al. 1998; Aureli et al. 2000). The same may be true on the farm. As a result, the exposure of animals to *L. monocytogenes* is likely unavoidable.

BACTERIAL VIRULENCE FACTORS

Listeria monocytogenes has served as an exemplary model for intracellular parasitism. Work done by several laboratories has elucidated a complex interplay among virulence factors that are involved in the attachment and internalization of *L. monocytogenes*, and its ability to survive and multiply within the cytoplasm of infected cells (Decatur and Portnoy 2000; Cabanes et al. 2002; Lecuit et al. 2002). Although this work has been done principally using tissue culture cells and mouse infection models, there is reason to believe that the general principles they have delineated are operative in *L. monocytogenes* infections of ruminants and humans as well.

As illustrated in the simplified overview in figure 9.2, *L. monocytogenes* invades cells (both professional phagocytes and nonphagocytic tissue cells are susceptible) via one or more members of a family of covalently linked surface proteins named internalin (A) (Gregory et al. 1997; Cabanes et al. 2002; Lecuit et al. 2002). The internalized listeriae then escape from the phagosome via the action of their hemolysin (listeriolysin or LLO) and a phosphatidylinositol-specific phospholipase C (B) (Schlüter et al. 1998; Decatur and Portnoy 2000; Glomski et al. 2002; Kohda et al. 2002). Within the cytoplasm, the listeriae multiply with an estimated doubling time of approximately 1 hour (C). The bacterial protein ActA directs the nucleation of host-cell-derived actin filaments onto the terminal end of the cytoplasmic listeriae (Beckerle 1998; Darji et al. 1998; Moors et al. 1999). The continued addition of F-actin monomers serves to propel the bacilli about the cytoplasm and toward the cell's periphery (D). Projections of the infected cell's cytoplasmic membrane then invaginate into adjacent cells, allowing the listeriae to enter that cell (E). The listeriae then escape from the double-membrane vacuole that encloses them via the action of the hemolysin and a phosphatidylcholine-dependent phospholipase C (lecithinase) (F) (Portnoy et al. 1989; Schlüter et al. 1998), and the process of intracellular multiplication is repeated (G). As a result of this series of events, *L. monocytogenes* is able to multiply and spread without direct exposure to the cells and soluble factors of the extracellular milieu. This centripetal spread of organisms within foci of infection can sometimes be dramatically visualized via Gram staining of lesions (Havell 1989; Gregory and Wing 2002).

Gene products 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) (Glasser et al. 2001; Cabanes et al. 2002). Invasion involves at least two members of the internalin (Inl) gene cluster. 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,

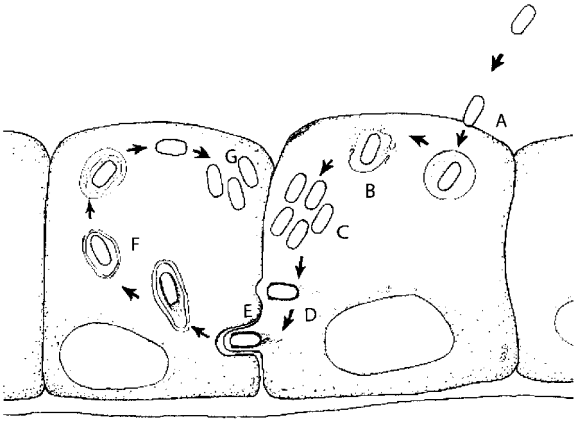


Figure 9.2. Simplified illustration of the events involved in the invasion, intracellular growth, and cell-to-cell spread of *L. monocytogenes*.

which can be found on the basolateral surface of intestinal epithelial cells. Following an elegant series of experiments, Lecuit et al. (2002) proposed that this interaction is paramount in the ability of *L. monocytogenes* to invade intestinal epithelial cells and cause systemic infection via the gastrointestinal tract. However, the possible role of other *L. monocytogenes* surface proteins in this process cannot be excluded at this time. InlB is a second member of the internalin family that is important for cell invasion and virulence (Gregory et al. 1997; Lecuit et al. 2002). It is interesting to note that the nonpathogenic *L. innocua* lacks more than 20% of the surface proteins (including InlB) encoded by the *L. monocytogenes* genome (Cabanes et al. 2002; Glasser et al. 2001).

Once inside the cell, L. *monocytogenes* relies on its cholesterol-binding, thiol-activated hemolysin (listeriolysin O, related to streptolysin O) and a phosphatidylinositol-dependent phospholipase C to lyse the phagosome membrane and escape into the cytoplasm (Decatur and Portnoy 2000). The LLO is more active at the acidic pH found within a phagosome (Glomski et al. 2002). In an ingenious strategy, the LLO contains a PEST amino acid sequence (proline, glutamine, serine, and threonine) that targets the LLO for intracellular degradation and thus reduces toxicity to the host cell (Decatur and Portnoy 2000). This mechanism undoubtedly contributes to the immense listerial burden that can be achieved in infected cells *in vivo* and *in vitro*. Evidence suggests that different domains of LLO are involved in lytic and stimulatory activities against mammalian cells (Kohda et al. 2002). Lysis of the double membrane is mediated by LLO and a lecithinase as listeriae spread from cell to cell (Schlüter et al. 1998).

ActA is a 90-kDa surface protein that directs the host-dependent arrangement of actin filaments that propel the listeriae about 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. The interaction of ActA with the actin cytoskeleton of mammalian cells has provided a powerful tool for understanding the regulation of the latter in cell shape and motility (Beckerle 1998). ActA is recognized by the immune system during infection, but it is not a target for protective immunity because of the intracellular location of the antigen (Darji et al. 1998).

Regulation of the above-described virulence factors is coordinated by a temperature-regulated positive regulatory factor (PrfA) (Renzoni et al. 1992). Environmental cues (e.g., temperature, pH, nutrients) influence *prfA* expression. The *prfA* expression is enhanced during infection of mammalian cells, beginning with the process of cell adhesion. Furthermore, exposure to mammalian cell lysates can enhance *prfA* expression. This suggests that *prfA* regulation may be a key event in triggering expression of virulence determinants by *L. monocytogenes*. Consistent with this hypothesis is knowledge that the nonpathogenic *L. innocua* does not contain the *prfA* gene (Glasser et al. 2001).

Mammalian cells are not simply passive targets in the invasion and intracellular multiplication of *L. monocytogenes*. The interaction of ActA with components of the actin cytoskeleton is well recognized, as described above. In addition, LLO has been reported to activate MEK-1 and ERK-2 of the MAP kinase pathway (Tang et al. 1998) and induce NF-κB signaling by activating the IκB kinase complex that phosphorylates 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 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

L. monocytogenes is an infrequent but serious pathogen of ruminants (both domestic and exotic) (Gray and Killinger 1966). In the United States, the greatest attention is usually placed on infections of dairy and beef cattle. In those areas of North America where sheep raising is an important industry, and in Europe, listeriosis in sheep and goats assumes greater importance. In both cattle and sheep, listeriosis can manifest itself in one of four ways: (1) as a CNS infection (meningoencephalitis in adults and meningitis in the young), (2) as abortion, (3) as a general-

ized septicemia with involvement of the liver and other organs, and (4) as mastitis.

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 or 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. When the animal moves, it tends to be in a single direction, 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. Unlike human meningitis, *L. monocytogenes* is seldom recovered from the cerebrospinal fluid in ruminant meningoencephalitis. Spontaneous recovery from severe clinical disease is rare and may be accompanied by permanent neurologic damage.

The pathogenesis of meningoencephalitis in ruminants is only partially understood. There is a definite seasonal association, with disease being more common in winter or early spring when the animals are housed indoors rather than on pasture. There is also a strong association between listeriosis and animals fed 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 greater than 5.0) is well documented (Fenlon 1986). Ribotyping has been used to document the identity of *L. monocytogenes* isolated from cattle and sheep and the silage they consumed (Wiedmann et al. 1997b). It is not clear how *L. monocytogenes* breaches the barrier of the gastrointestinal mucosal epithelium. One group has suggested it may penetrate through the dental pulp when sheep are cutting or losing teeth (Barlow and McGorum 1985). 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 has been presented for mice and rabbits inoculated with *L. monocytogenes* in the lip (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.

Grossly, the brains of ruminants suffering from encephalitis may appear normal. The typical histopathologic lesions observed are perivascular cuffing of mononuclear cells and occasional inflammatory foci (micro-abscesses) 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). Gram-stained tissue sections may reveal listeriae in large lesions, but they are infrequently observed in the smaller perivascular cuffs. Inducible nitric oxide synthase was observed in abscesses but not in perivascular cuffs of mononuclear cells in the brains of infected cattle; the reverse relationship was observed for expression of MHC class 2 antigens in the brains of infected cattle (Jungi et al. 1997; Shin et al. 2000). Studies with laboratory animals suggest that invasion of the CNS may be facilitated by extravasation of *Listeria*-infected blood monocytes (Drevets 1999).

L. monocytogenes appears to have a predilection for invading the fetoplacental unit in a variety of animal species (Gray and Killinger 1966). This may or may not follow obvious septicemia. Experimental infections in mice suggest that T cells and macrophages do not reach the infected placenta in sufficient numbers to effect protective immunity (Redline et al. 1988). Abortion typically occurs in the third trimester of pregnancy and may or may not 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 flocks of sheep (Vazquez-Boland et al. 1992). These outbreaks result in substantial economic losses to the farmer (Low and Renton 1985). The organism can be microscopically visualized or recovered by culture from the aborted fetus or placenta (Low and Renton 1985).

In addition to meningoencephalitis and abortion, *L. monocytogenes* has been associated with generalized septicemia and focal necrosis of the spleen and liver in various ruminant species (Gray and Killinger 1966; Low and Renton 1985). Considerable attention has been paid to the problem of *L.*

monocytogenes mastitis in dairy cattle (Gitter 1980). This can range in severity from subclinical to severe suppurative infection. *L. monocytogenes* can be commonly isolated from bovine feces (Weber et al. 1995) and in a dairy farm environment (Ueno 1996), thus providing the source of the organism for this environmental mastitis. Both serotype 1/2a and serotype 4b strains of *L. monocytogenes* can cause experimental mastitis in cattle (Bourry et al. 1995). A similar susceptibility has been reported for sheep (Bourry et al. 1995; Tzora et al. 1998). *L. monocytogenes* organisms can be found within neutrophils in mastitic bovine milk (Doyle et al. 1987). Evidence was presented that this intracellular site protected some of the listeriae against inactivation by short-term pasteurization, but others have not found this to be the case (Bunning et al. 1988). Long-term infection of the udder may ensue, with shedding of listeriae in milk exacerbated by periodic episodes of immunosuppression (Wesley et al. 1989). This has practical implications since successful treatment of *L. monocytogenes* mastitis is difficult to achieve (Gitter 1980).

Listeriosis in Nonruminant Animal Species

Clinical listeriosis is rare in horses, pigs, dogs, and cats. Healthy pigs may excrete *L. monocytogenes* in their feces, and *L. monocytogenes* can be recovered from pig carcasses at slaughter (Autio et al. 2000), but listeriosis in pigs is not of practical 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 for a Doberman (Schroeder and van Rensberg 1993). *L. monocytogenes* serotype 4 was recovered from a gray fox, and from a raccoon dog (*Nyctereutes procyonoides*), which had histopathological evidence of canine distemper (Black et al. 1996; Aoyagi et al. 2000). Cats can be experimentally infected with *L. monocytogenes* following subcutaneous injection and develop a strong delayed-type hypersensitivity response (Pedersen et al. 1998). Infection was severe in FIV-infected cats (Dean et al. 1998). An outbreak of encephalitis in commercial broiler chickens has also been reported; the source of the organism in that outbreak was not determined (Cooper 1989). Broiler chicks could be colonized with *L. monocytogenes* following oral challenge (Bailey et al. 1990). Leghorn chicks were protected against colonization by prior administration of a competitive exclusion culture of normal caecal bacteria (Hume et al. 1998). *L. monocytogenes* was isolated from a corneal lesion of a horse with keratitis (Sanchez et al. 2001). Listeriosis has been reported in animals at zoological parks. Perinatal septicemia caused by *L. monocytogenes* was reported for a stillborn Celebese ape (McClure and Strozier 1975), and acute death due to disseminated listeriosis was reported for bushy-tailed jirds (Tappe et al. 1984). *L. monocytogenes* can also infect fish; *L. monocytogenes* was recovered from the brain, spleen, kidneys, and fillets of farm-raised channel catfish (Wang et al. 1998). Laboratory investigations of listeriosis rely heavily on the use of rodents, rabbits, and guinea pigs, all of which are quite susceptible to listeriosis. The prevalence of spontaneous listeriosis in these species kept as pets is not known, but practitioners whose practice includes pocket pets might keep listeriosis in mind as a possible diagnosis for unexplained septicemia or abortion. Nonhuman primates appear to be relatively resistant to listeriosis. In a Japanese study, 20% of wild-caught monkeys had *L. monocytogenes* in their feces or intestinal contents (Yoshida et al. 2000), and cynomolgus monkeys were very resistant to experimental oral challenge (Farber et al. 1991).

Listeriosis in Humans

L. monocytogenes is considered a zoonotic agent. Infection may be transmitted directly from contaminated meat or milk, or indirectly by *L. monocytogenes*-containing manure that contaminates vegetables that are then consumed by people. In humans, 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). There have been several large outbreaks of human listeriosis. The largest of these in the United States occurred in Los Angeles County as a result of ingestion of *Listeria*-contaminated Mexican-style cheese made with unpasteurized milk. Of a total of 142 cases, 93 occurred in pregnant women and their fetuses. The case fatality rate was 63% for early neonatal or fetal infections and 37% for non-neonatal infections (Farber and Peterkin 1991; Schuchat et al. 1991). As in other outbreaks, most of the latter group of patients suffered from some underlying immunodeficiency. Other large prominent outbreaks include one associated with contaminated wieners in the United States in 1998 (Hurd et al. 2000) and a very large outbreak in Italy (292 persons hospitalized)

resulting from *Listeria*-contamination of a corn salad distributed to school cafeterias for student lunches (Aureli et al. 2000).

Earlier studies estimated the annual incidence of listeriosis in the United States was 7.4 cases per one million people, with a fatality rate of 23% (Anderson et al. 1992). More recently it has been estimated that there may be 2,500 cases and 500 deaths attributable to listeriosis in the United States each year (Notermans et al. 1998; Hurd et al. 2000). Foods incriminated with transmitting L. *monocytogenes* infection include soft cheeses, delicatessen items, undercooked chicken and hot dogs, and seafood. 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, because listeriosis has achieved considerable visibility as a food safety concern among regulatory agencies and the public, veterinary practitioners and diagnosticians will be expected to assist food-animal producers in reducing the likelihood of their raw product being contaminated with *L. monocytogenes.*

HOST-PATHOGEN INTERACTIONS IN LISTERIOSIS

Most of the available information on host defense against listeriosis is based on experimental studies of murine listeriosis, which is a popular and valuable model for assessing immunoregulation of antibacterial resistance to an intracellular pathogen. Despite the acknowledged role of *L. monocytogenes* as a pathogen of ruminants, there are few published reports about host defense against listeriosis in cattle or sheep. The information given below, therefore, is based principally on murine studies, with relevant reports from cattle and sheep stated where available.

Innate Immunity

Based on the ubiquitous distribution of *L. monocytogenes* and the generally infrequent occurrence of listeriosis, it is reasonable to assume that innate immune mechanisms are effective in controlling occasional exposure to low numbers of organisms. The first barrier may be posed by the low pH and competing microbes of the gastrointestinal tract. *L. monocytogenes* generally does not compete well nor multiply to high numbers in the gastrointestinal tracts of experimentally infected animals (Czuprynski and Haak-Frendscho 1997). Host secretions may also contain components that inhibit *L. monocytogenes*. Bovine lactoferrin has been reported to reduce the invasion of an intestinal epithelial cell line, and inactivate LLO, *in vitro* (Moriishi et al. 1999; Valenti et al. 1999). Lipids in bovine milk were inhibitory for *L. monocytogenes in vitro* (Sprong et al. 2001) and inhibited intestinal colonization by *L. monocytogenes* in rats (Sprong et al. 1999).

Being an intracellular pathogen, resistance to *L. monocytogenes* requires a cellular immune response. Antibodies are generated against *L. monocytogenes* extracellular antigens during infection; cell-associated antigens tend to elicit little or no antibody response (Bhunia 1997; Darji et al. 1998). Antibodies produced during naturally occurring or experimental listeriosis by themselves are not protective, although they may enhance the ability of phagocytic cells to ingest *L. monocytogenes* (Czuprynski et al. 1984). More recently, it has been shown that a monoclonal antibody produced against the LLO of *L. monocytogenes* can protect mice against experimental infection (Edelson and Unanue 2000), presumably by decreasing the effectiveness of LLO in infected cells (Kohda et al. 2002).

The first cells to enter foci of infection are neutrophils. Neutrophils from several mammalian species (including bovine) can kill *L. monocytogenes in vitro* (Czuprynski et al. 1984, 1989). Mononuclear phagocytes also ingest *L. monocytogenes* and, depending on the activation state of the cell, can inactivate a proportion of the ingested listeriae (Czuprynski et al. 1984, 1989; Portnoy et al. 1989). Mice treated with a monoclonal antibody that prevents influx of neutrophils, or depletes mice of circulating neutrophils, are exquisitely sensitive to listeriosis (Conlan and North 1991; Czuprynski et al. 1994). Although it has been proposed that neutrophils protect the host by both destroying infected cells that harbor the intracellular listeriae (Gregory and Wing 2002) and ingesting and killing the listeriae that are released from the lysed cells (Czuprynski et al. 1984), it is also possible that neutrophils release mediators (i.e., cytokines) that activate the anti-*Listeria* activity of hepatocytes and macrophages (Edelson and Unanue 2000; Gregory and Wing 2002).

Adaptive Immunity

The pioneering studies of Mackaness and coworkers in the 1960s demonstrated the preeminent role of

cellular immunity in host defense against listeriosis (Miki and Mackaness 1964). For many years, investigators adhered to a simple paradigm in which T-helper cells released IFN-γ that activated macrophages to kill intracellular listeriae. While macrophage activation certainly occurs in listeriosis, recent evidence indicates that the protective host response to listeriosis is considerably more complex. It has been demonstrated that considerable multiplication of *L. monocytogenes* occurs in hepatocytes and other nonprofessional phagocytic cells (Havell 1989**;** Conlan and North 1991; Gregory and Wing 2002). This finding indicates that additional defense mechanisms must be operative to achieve the sterilizing immunity observed in experimentally infected mice. $CD4^+$ and $CD8^+$ cytolytic T cells have been described that can lyse *L. monocytogenes*-infected macrophages (Kaufmann 1993)**.** Perhaps similar T cells attack other types of cells infected with *L. monocytogenes,* as occurs in viral infections. It is also likely that natural killer cells (NK cells) release IFN-γ early in infection, and that this is of considerable importance to host defense (Dunn and North 1991). Lest one get the impression that macrophages have been relegated to a minor role, IFN-γ-treated activated macrophages restrict the ability of *L. monocytogenes* to escape from the phagolysosome (Portnoy et al. 1989). Thus, macrophage activation will reduce the intracellular multiplication of listeriae and increase the ability of macrophages to inactivate ingested organisms.

Besides the cells described above, there is an abundant literature on the influence of various soluble mediators (cytokines) on antilisteria resistance. Administration of a number of recombinant cytokines (e.g., IFN-γ, IL-1, IL-2, TNF-α, GM-CSF) has been shown to enhance resistance to experimental listeriosis in mice (Kaufmann 1993; Czuprynski and Haak-Frendscho 1997; Edelson and Unanue 2000). Conversely, investigators have used cytokine-neutralizing monoclonal antibodies, and gene knockout mice for cytokines and cytokine receptors, to elucidate the roles of endogenous cytokines in anti-*Listeria* resistance. In particular, endogenous IFN-γ and TNF-α have been shown to play critical roles. *In vivo* neutralization of either of these cytokines has dire consequences for the infected host (Havell 1989; Kaufmann 1993). Work done with both rodents and cats suggests that a T-helper 1 cytokine response is protective and a T-helper 2 response is detrimental to anti-*Listeria* resistance (Edelson and Unanue 2000; Pedersen et al. 1998).

Prevention and Treatment

Experimental studies with rodents indicate that clearance of a sublethal *L. monocytogenes* infection renders the host highly resistant to rechallenge (Miki and Mackaness 1964). These animals will also display a strong delayed-type hypersensitivity response to listerial antigens, although this skin response can be dissociated from protective immunity (Mielke et al. 1988). This heightened acquired cellular resistance is maximal for 2 to 4 weeks and then wanes over the ensuing several months. During the period of maximal resistance, protection can be transferred to naive recipient mice by 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. It is generally agreed that active immunization requires the administration of viable *L. monocytogenes*; 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 a vector for conventional or DNA immunization against heterologous antigens has been shown in rodent studies (Mollenkopf et al. 2001)**.**

Immunization with live attenuated *L. monocytogenes* has been used outside the United States to protect sheep against naturally occurring or experimental listeriosis (Gudding et al. 1989**;** Vagsholm 1991**;** Linde et al. 1995). However, listeriosis remains a sporadic disease problem for cattle and sheep producers in the United States. Most outbreaks of listeriosis have been traced to use of poor-quality silage that contained increased numbers of *L. monocytogenes.* Ensuring use of good-quality, low pH silage, keeping bulk tank temperatures low, and practicing good hygiene to reduce fecal contamination should go a long way toward reducing *L. monocytogenes* contamination of milk, meat, and vegetables. Similar attention to scrupulous cleaning and hygiene must be maintained at dairy plants and food-production facilities. As a result, there would appear 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 *L. monocytogenes* 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).

Antibiotic treatment of food animal species with listeriosis may not be advisable considering the cost of therapy and losses associated with withdrawal times for the antibiotics used. Mastitis has proven refractory to treatment in several instances when it has been attempted. If latent udder infections occur, then significant numbers of listeriae might be shed in the milk when the cow experienced transient immunosuppressive events, such as parturition (Wesley et al. 1989). In cases of abortion, the dam may clear the infection without treatment. In human cases of listeriosis, the current treatment of choice is penicillin in combination with gentamicin. Sulfamethoxazole-trimethoprim, erythromycin, and tetracyclines have also proven effective in human listeriosis, whereas, cephalosporins are not recommended, even in combination with other antibiotics (Farber and Peterkin 1991; Schuchat et al. 1991).

CONCLUSIONS

Investigation of the molecular pathogenesis of 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 a paucity of published information on host defense mechanisms in cattle and sheep. Some of the observations made in these species (i.e., 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 understanding of the infectious dose of *L. monocytogenes* via the gastrointestinal tract, and 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.

REFERENCES

Anderson, G., L. Mascola, G. W. Rutherford, M. S. Rados, R. Hutcheson, P. Archer, P. Zenker, C. Harvey, and J. D. Smith. 1992. Foodborne listeriosis—United States, 1988–1990. Morb. Mortal. Wkly. Rep. 267:2446–2447.

Aoyagi, T., Y. Sato, S. Matsuura, and H. Wada. 2000. Listeriosis in a raccoon dog (*Nyctereutes procyonoides*) associated with canine distemper. J. Vet. Med. Sci. 62:639–641.

Asahi, O., T. Hosoda, and Y. Akiyama. 1957. Studies on the mechanism of infection of the brain with *Listeria monocytogenes*. Am. J. Vet. Res. 18:147–157.

Aureli, P., G.C. Fiorucci, D. Caroli, G. Marchiaro, O. Novara, L. Leonello, and S. Salmaso. 2000. An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. N. Engl. J. Med. 342:1236–1241.

Autio, T. T. Sater, M. Fredriksson-Ahomaa, M. Rahkio, J. Lunden, and H. Korkeala. 2000. *Listeria monocytogenes* contamination pattern in pig slaughterhouses. J. Food Prot. 63:1438–1442.

Bailey, J. S., D. L. Fletcher, and N. A. Cox. 1990. *Listeria monocytogenes* colonization of broiler chickens. Poult. Sci. 69:457–461.

Barbour, A. H., A. Rampling, and C. E. Hormache. 2001. Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. Infect. Immun. 69:4657–4660.

Barlow, R. M., and B. McGorum. 1985. Ovine listerial encephalitis: analysis, hypothesis and synthesis. Vet. Rec. 116:233–236.

Beckerle, M. C. 1998. Spatial control of actin filament assembly: lessons from *Listeria*. Cell. 95:741–748.

Bhunia, A. K. 1997. Antibodies to *Listeria monocytogenes*. Crit. Rev. Microbiol. 23:77–107.

Black, S. S., F. W. Austin, and E. McKinley. 1996. Isolation of *Yersinia pseudotuberculosis* and *Listeria monocytogenes* serotype 4 from a gray fox (*Urocyon cinereoargenteus*) with canine distemper. J. Wildl. Dis. 32:362–366.

Bourry, A., B. Poutrel, and J. Rocourt. 1995. Bovine mastitis caused by *Listeria monocytogenes*: Characteristics of natural and experimental infections. J. Med. Microbiol. 43:125–132.

Bunning, V. K., C. W. Donnelly, J. T. Peeler, E. H. Briggs, J. G. Bradshaw, R. G. Crawford, C. M. Beliveau, and J. T. Tierney. 1988. Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes. Appl. Envir. Microbiol. 54:364–370.

Cabanes, D., P. Dehoux, O. Dussurget, L. Frangeul, and P. Cossart. 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol. 10:238–245.

Charlton, K. M., and M. M. Garcia. 1977. Spontaneous listeric encephalitis and neuritis in sheep: light microscopic studies. Vet. Pathol. 14:297–313.

Conlan, J. W., and R. J. North. 1991. Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. J. Exp. Med. 174:741–744.

Cooper, G. L. 1989. Case report—an encephalitic form of listeriosis in broiler chickens. Avi. Dis. 33:182–185.

Czuprynski, C. J., and M. Haak-Frendscho. 1997. Non-specific resistance mechanisms to listeriosis: implications for experimental and naturally occurring infection. Immunol. Rev. 158:47–56.

Czuprynski, C. J., E. J. Noel, M. P. Doyle, and R. D. Schultz. 1989. Ingestion and killing of *Listeria monocytogenes* by blood and milk phagocytes from mastitic and normal cattle. J. Clin. Microbiol. 27:812–817.

Czuprynski, C. J., J. F. Brown, N. Maroushek, R. D. Wagner, and H. Steinberg. 1994. Administration of anti-granulocyte MAb RB6-8C5 depletes mice of neutrophils and impairs their resistance to *Listeria monocytogenes* infection. J. Immunol. 152:1836–1846.

Czuprynski, C. J., N. G. Faith, and H. Steinberg. 2002. Ability of the *Listeria monocytogenes* strain Scott A to cause systemic infection in mice infected by the intragastric route. Appl. Environ. Microbiol. 68:2893–900.

Czuprynski, C. J., P. M. Henson, and P. A. Campbell. 1984. Killing of *Listeria monocytogenes* by inflammatory neutrophils and mononuclear phagocytes from immune and nonimmune mice. J. Leukoc. Biol. 35:193–208.

Darji, A., D. Bruder, S. Zur Lage, B. Gerstel, T. Chakraborty, J. Wehland, and S. Weiss. 1998. The role of the bacterial membrane protein ActA in immunity and protection against *Listeria monocytogenes*. J. Immunol. 161:2414–2420.

Dean, G. A., J. A. Bernales, and N. C. Pedersen. 1998. Effect of feline immunodeficiency virus on cytokine response to *Listeria monocytogenes* in vivo. Vet. Immunol. Immunopathol. 65:125–138.

Decatur, A. L., and D. A. Portnoy. 2000. A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. Science 290:992–995.

Doyle, M. P., K. A. Glass, J. T. Beery, G. A. Garcia, D. J. Pollard, and R. D. Schultz. 1987. Survival of *Listeria monocytogenes* in milk during high-temperature, short-time pasteurization. Appl. Envir. Microbiol. 53:1433–1438.

Drevets, D. A. 1999. Dissemination of *Listeria monocytogenes* by infected phagocytes. Infect. Immun. 67:3512–3517.

Dunn, P. L., and R. J. North. 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infect. Immun. 59:2892–2900.

Edelson, B. T., and E. R. Unanue. 2000. Immunity to *Listeria* infection. Curr. Opin. Immunol. 12:425–431.

Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55:476–511.

Farber, J. M., E. Daley, F. Coates, N. Beausoleil, and J. Fournier. 1991. Feeding trials of *Listeria monocytogenes* with a nonhuman primate model. J. Clin. Microbiol. 29:2606–2608.

Farber, J. M., W. H. Ross, and J. Harwig. 1996. Health risk assessment of *Listeria monocytogenes* in Canada. Int. J. Food Microbiol. 130:145–156.

Fenlon, D. R. 1986. Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. Vet. Rec. 118:240–242.

Gitter, M., R. Bradley, and P. H. Blampied. 1980. *Listeria monocytogenes* infection in bovine mastitis. Vet. Rec. 107:390–393.

Glasser, P., et al. 200l. Comparative genomics of *Listeria* species. Science 294:849–852.

Glomski, I. J., M. M. Gedde, A. W. Tsang, J. A. Swanson, and D. A. Portnoy. 2002. The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. J. Cell Biol. 156:1029–1038.

Gray, M. L., and A. H. Killinger. 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. 30:309–382.

Greene, C. E., and J. F. Prescott. 1998. Streptococcal and other gram-positive bacterial infections. In Infectious diseases of the dog and cat. Philadelphia: W. B. Saunders Co., p. 213.

Gregory, S. H., and E. J. Wing. 2002. Neutrophil-Kupffer cell interaction: a critical component of host defenses to systemic bacterial infections. J. Leukoc. Biol. 72:239–248.

Gregory, S. H., A. J. Sagnimeni, and E. J. Wing. 1997. Internalin B promotes the replication of *Listeria monocytogenes* in mouse hepatocytes. Infect. Immun. 65:5137–5141.

Gudding, R., L. L. Nesse, and H. Gronstol. 1989. Immunization against infections caused by *Listeria monocytogenes* in sheep. Vet. Rec. 125:111–114.

Havell, E. A. 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J. Immunol. 143:2894–2899.

Hume, M. E., J. A. Byrd, L. H. Stanker, and R. L. Ziprin. 1998. Reduction of caecal *Listeria monocytogenes* in Leghorn chicks following treatment with a competitive exclusion culture (PREEMPT). Lett. Appl. Micriobiol. 26:432–436.

Hurd, S., et al. 2000. Multistate outbreak of listeriosis—United States, 2000. Morb. Mortal Wkly Rep. 49:1129–1130

Ireton, K., B. Payrasatre, and P. Cossart. 1999. The *Listeria monocytogenes* protein InlB is an agonist of mammalian phosphoinositide 3-kinase. J. Biol. Chem. 274:17025–17032.

Jungi, T. W., H. Pfister, H. Sager, R. Fatzer, M. Vandevelde, and A. Zurbriggen. 1997. Comparison of inducible nitric oxide synthase expression in the brains of *Listeria monocytogenes*-infected cattle, sheep, and goats and in macrophages stimulated in vitro. Infect. Immun. 65:5279–5288.

Kaufmann, S. H. 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. 11:129–163.

Kayal, S., A. Lilienbaum, O. Join-Lambert, X. Li, A. Israel, and P. Berche. 2002. Listeriolysin O secreted by *Listeria monocytogenes* induces NF-κB signaling by activating the IκB kinase complex. Mol. Microbiol. 44:1407–1419.

Kocks, E., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart. 1992. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. Cell 68:521–531.

Kohda, C. I. Kawamura, B. Hisash, T. Nomura, Y. Ito, T. Kimoto, I. Watanabe, and M. Mitsuyama. 2002. Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. Infect. Immun. 70:1334–1341.

Lecuit, M., S. Vandormaeil-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet and P. Cossart. 2002. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science 292:1722–1725.

Leman, A. D., B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (eds.). 1986. Diseases of swine. Ames: Iowa State University Press.

Linde, K., G. C. Fthenakis, R. Lippmann, J. Kinne, and A. Abraham. 1995. The efficacy of a live *Listeria monocytogenes* combined serotype 1/2a and serotype 4b vaccine. Vaccine 13:923–926.

Low, J. C., and C. P. Renton. 1985. Septicaemia, encephalitis and abortions in a housed flock of sheep caused by *Listeria monocytogenes* type 1/2. Vet. Rec. 116:147–150.

Mansell, A., N. Khelef, P. Cossart, and L. A. O'Neill. 2001. Internalin B activates nuclear factor-κB via Ras, phosphoinositide 3-kinase, and Akt. J. Biol. Chem. 276:43597–43603.

McClure, H. M., and L. M. Strozier. 1975. Perinatal listeric septicemia in a Celebese black ape. J. Am. Vet. Med. Assoc. 167:637–638.

Mielke, M. E. A., S. Ehlers, and H. Hahn. 1988. T-cell subsets in delayed-type hypersensitivity, protection and granuloma formation in primary and secondary *Listeria* infection in mice: superior role of Lyt-2+ cells in acquired immunity. Infect. Immun. 56:1920–1925.

Miki, K., and G. B. Mackaness. 1964. The passive transfer of acquired resistance to *Listeria monocytogenes*. J. Exp. Med. 129:93–108.

Mollenkopf, H., G. Dietrich, and S. H. Kaufmann. 2001. Intracellular bacteria as targets and carriers for vaccination. Biol. Chem. 382:521–532.

Moors, M. A., B. Levitt, P. Youngman, and D. A. Portnoy. 1999. Expression of listeriolysin O and ActA by intracellular and extracellular *Listeria monocytogenes*. Infect. Immun. 67:131–139.

Moriishi, K., S. Inoue, M. Koura, and F. Amano. 1999. Inhibition of listeriolysin O-induced hemolysis by bovine lactoferrin. Biol. Pharm. Bull. 22:1167–1172.

Notermans, S., J. Dufrenne, P. Teunis, and T. Chackraborty. 1998. Studies on the risk assessment of *Listeria monocytogenes*. J. Food Prot. 61:244–248.

Pedersen, N. C., G. A. Dean, J. Bernales, A. Sukura, and J. Higgins. 1998. *Listeria monocytogenes* and *Serratia marcescens* infections as models for Th1/Th2 immunity in laboratory cats. Vet. Immunol. Immunopathol. 63:83–103.

Portnoy, D. A., R. D. Schreiber, P. Connelly, and L. G. Tilney. 1989. γ-interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. J. Exp. Med. 170:2141–2146.

Redline, R. W., C. M. Shea, V. E. Papaioannou, and C. Y. Lu. 1988. Defective anti-listerial responses in deciduoma of pseudopregnant mice. Am. J. Pathol. 133:485–497.

Renzoni, A., P. Cossart, and S. Dramsi. 1999. PrfA, the transcriptional activator of virulence is upregulated during interaction *of Listeria monocytogenes* with mammalian cells and eukaryotic cell extracts. Mol. Microbiol. 34:552–561.

Sanchez, S., M. Studer, P. Currin, P. Barlett, and D. Bounous. 2001. *Listeria* keratitis in a horse. Vet. Ophthalmol. 4:217–219.

Schlüter, D. E., C. Domann, T. Buck, H. Hain, T. Hof, T. Chakraborty, and M. Deckert-Schlüter. 1998. Phosphatidylcholine-specific phospholipase C from *Listeria monocytogenes* is an important virulence factor in murine cerebral listeriosis. Infect. Immun. 66:5930–5938.

Schroeder, H., and I. B. van Rensburg. 1993. Generalised *Listeria monocytogenes* infection in a dog. J. S. Afr. Vet. Assoc. 64:133–136.

Schuchat, A., B. Swaminathan, and C. V. Broome. 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. 4:169–183.

Shetron-Rama, L. M., H. Marquis, H. G. A. Bouwer, and N. E. Freitag. 2002. Intracellular induction of *Listeria monocytogenes act*A expression. Infect. Immun. 70:1087–1096.

Shin, T., D. Weinstock, M. D. Castro, H. Acland, M. Walter, H. Y. Kim, and H. G. Purchase. 2000. Immunohistochemical study of constitutive neuronal and inducible nitric oxide synthase in the

central nervous system of goat with natural listeriosis. J. Vet. Sci. 1:77–80.

Sprong, R. C., M. F. Hulstein, and R. Van der Meer. 1999. High intake of milk fat inhibits intestinal colonization of *Listeria* but not of *Salmonella* in rats. J. Nutr. 129:1382–1389.

———. 2001. Bactericidal activities of milk lipids. Antimicrob. Agents Chemother. 45:1298–1301.

Tang, P., C. L. Sutherland, M. R. Gold, and B. B. Finlay. 1998. *Listeria monocytogenes* invasion of epithelial cells requires the MEK-1/ERK-2 mitogen-activated protein kinase pathway. Infect. Immun. 66:1106–1112.

Tappe, J. P., F. W. Chandler, W. K. Westrom, S. K. Liu, and E. P. Dolensek. 1984. Listeriosis in seven bushy-tailed jirds. J. Am. Vet. Med. Assoc. 185:1367–1370.

Tzora, A., G. C. Fthenakis, and K. Linde. 1998. The effects of inoculation of *Listeria monocytogenes* into the ovine mammary gland. Vet. Microbiol. 59:193–202.

Ueno, H. K. Yokota, T. Arai, Y. Muramatsu, H. Taniyama, T. Iida, and C. Morita. 1996. The prevalence of *Listeria monocytogenes* in the environment of dairy farms. Microbiol. Immunol. 40:121–124.

Vagsholm, J., L. L. Nesse, and R. Gudding. 1991. Economic analysis of vaccination applied to ovine listeriosis. Vet. Rec. 128:183–185.

Valenti, P., R. Greco, G. Pitari, P. Rossi, M. Ajello, G. Melino, and G. Antonini. 1999. Apoptosis of Caco-2 intestinal cells invaded by *Listeria monocytogenes*: protective effect of lactoferrin. Exp. Cell. Res. 250:197–202.

Vazquez-Boland, J. A., L. Dominguez, M. Blanco, J. Rocourt, J. F. Fernandez-Garayzabal, C. B. Gutierrez, R. I. Tascon, and E. F. Rodriguez-Ferri. 1992. Epidemiologic investigation of a silage-associated epizootic of ovine listeric encephalitis, using a new *Listeria*-selective enumeration medium and phage typing. Am. J. Vet. Res. 53:368–371.

Wang, C., L. F. Zhang, F. W. Austin, and C. R. Boyle. 1998. Characterization of *Listeria monocytogenes* isolated from channel catfish (*Ictalurus punctatus*). Am. J. Vet. Res. 59:1125–1128.

Weber, A., J. Potel, R. Schafer-Schmidt, A. Prell, and C. Datzmann. 1995. Studies on the occurrence of *Listeria monocytogenes* in fecal samples of domestic and companion animals. Zentrabl. Hyg. Umweltmed. 198:177–123.

Wesley, I. N., J. H. Bryner, M. J. Van Der Maaten, and M. Kehrli. 1989. Effects of dexamethasone on shedding of *Listeria monocytogenes* in dairy cattle. Am. J. Vet. Res. 50:2009–2013.

West, H. J., and M. Obwolo. 1987. Bilateral facial paralysis in a cow with listeriosis. Vet. Rec. 120:204–205.

Wiedmann, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough, and C. A. Batt. 1997a. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immun. 65:2707–2716.

Wiedmann, M., T. Arvik, J. L. Bruce, J. Neubauer, F. del Piero, M. C. Smith, J. Hurley, H. O. Mohammed, and C. A. Batt. 1997b. Investigation of a listeriosis epizootic in sheep in New York State. Am. J. Vet. Res. 58:733–737.

Yoshida, T. T. Sugimoto, M. Sato, and K. Hirai. 2000. Incidence of *Listeria monocytogenes* in wild animals in Japan. J. Vet. Med. Sci. 62:673–675.

10
Erysipelothrix rhusiopathiae

Y. Shimoji

Erysipelothrix rhusiopathiae, the causative agent of erysipelas, is a gram-positive, non-spore-forming, non-acid-fast bacterium. The cellular morphology of the organism is variable. Cells isolated directly from the blood or organs of acutely infected animals are slender and appear as straight or slightly curved rods occurring singly or in short chains. Organisms isolated from chronic lesions, such as vegetations on the heart valves, usually occur as long, entangled, unbranching filaments (Wood 1999).

E. rhusiopathiae causes a variety of diseases in many species of mammals and birds, including humans, and is of economic importance in the swine and turkey industries. It also causes polyarthritis in sheep, lambs, and calves. In humans, it causes erysipeloid, a cutaneous infection that occurs primarily as an occupational disease of persons engaged in handling and processing meat, poultry and fish, though in rare cases it may also cause endocarditis or acute septicemic disease (Wood 1999). The organism is ubiquitous in nature and has been isolated not only from many species of wild and domestic mammals and birds but also from reptiles, amphibians, and fish. The primary reservoir of the organism is generally believed to be domestic pigs, and high percentages of apparently healthy animals carry the organism in their tonsils and other lymphoid tissues of the alimentary canal. These asymptomatic animals can discharge the organism in their feces or oronasal secretions, creating a significant source of infection (Wood 1999).

E. rhusiopathiae is classified in the genus *Erysipelothrix* together with *E. tonsillarum* and two other unnamed species (Takahashi et al. 1992). *E. tonsillarum* is morphologically and biochemically indistinguishable from *E. rhusiopathiae* except that it ferments sucrose (Takahashi et al. 1992). It is also frequently recovered from the tonsils of apparently healthy pigs but experimentally does not cause disease in pigs or chickens (Takahashi et al. 1992, 1994).

BACTERIAL VIRULENCE FACTORS

Capsule

The most important virulence factor of *E. rhusiopathiae* is an acidic polysaccharide located on the cell surface (Shimoji 2000). The term "capsule" (Lachmann and Deicher 1986) has been used to refer to this component. The role of the capsule in virulence was examined in a study on transposon mutagenesis with Tn*916* (Shimoji et al. 1994). It was found that Tn*916* mutants that had lost the capsule were completely avirulent for mice; furthermore, one strain reverted to virulence following acquisition of ability to produce the capsule when the transposon was lost by spontaneous excision. Genetic analysis of one of the acapsular mutants found that Tn*916* inserted in a gene that potentially encodes a glycosyltransferase (Shimoji et al., unpublished data), which has been shown to be important in capsule biosynthesis in many bacteria. Immunogold electron microscopic analysis revealed that the capsule of *E. rhusiopathiae* is loosely bound to the cell surface and therefore appears as slime, or glycocalyx (Shimoji 2000).

Surface Proteins

Surface proteins of gram-positive bacteria play a pivotal role in virulence, often functioning as adhesins or antiphagocytic factors. Takahashi et al. (1987) have shown that virulent *E. rhusiopathiae* strains adhere more strongly to porcine kidney cells

in vitro than do avirulent strains, and that a heat-labile and trypsin-sensitive surface component is important in adherence. Nevertheless, the specific component responsible for this adherence has yet to be defined.

To date, only a few genes that encode surface proteins of *E. rhusiopathiae* have been cloned. Galán and Timoney (1990) cloned a 5.4-kb *Eco*RI DNA fragment containing a gene from the chromosome of a virulent strain. The gene product was found to be a protective antigen of the organism, and guinea pig antisera raised against recombinant phage clones were reactive with the 64- to 66-kDa proteins in detergent-solubilized surface antigens. It was shown that the protein was expressed less on strains of low or moderate virulence than on highly virulent strains. However, neither the DNA sequence of the gene nor the function of this protein has yet been described. SpaA protein, a major protective antigen, is also surface located (Makino et al. 1998; Shimoji et al. 1999). SpaA is very similar in both its structure and in the amino acid sequences of its C-terminal region to those of choline-binding proteins of *Streptococcus pneumoniae*, one of which has been shown to play a specific role as an adhesin; however, this role of SpaA remains to be elucidated. A 19-kDa protein is a putative lipoprotein located on the cell surface (Makino et al. 1999). This protein itself has no hemolytic activity, but when the gene is cloned in *Escherichia coli*, it facilitates the hemolytic activity of the host bacterium. However, neither the function nor the role of this protein in virulence is yet known.

E. rhusiopathiae contains two additional surface proteins, with molecular masses of 220 and 85 kDa. These proteins have been shown to participate in biofilm formation and may therefore play an important role in colonization in a host (Shimoji et al. 2003b).

Extracellular Enzymes

E. rhusiopathiae produces neuraminidase (sialidase), which releases terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides expressed on host cells. It has been shown that there is a correlation between the virulence of *E. rhusiopathiae* strains and the amount of neuraminidase produced, and that antibodies to this enzyme are protective in mice (Müller and Krasemann 1976). *E. rhusiopathiae* also produces hyaluronidase, a spreading factor that facilitates the dissemination of pathogens into tissues by breaking down hyaluronic acid, a high-molecular-mass polysaccharide found in the extracellular matrix, especially in that of soft connective tissues. The role of hyaluronidase in virulence was directly examined in a study on transposon mutagenesis with Tn*916* (Shimoji et al. 2002b). The data obtained in this study showed that six of the seven hyaluronidase-negative mutants tested were avirulent for mice, while the seventh, designated AST121, exhibited the same level of virulence for mice as its parent strain. Furthermore, none of the six avirulent mutants produced capsules, while the virulent AST121 still possessed the capsule, strongly indicating that the lack of virulence of the six mutants is associated with the loss of the capsule. Thus, hyaluronidase is not essential for the lethality of the infection in mice.

Superoxide dismutase and catalase produced by *E. rhusiopathiae* are further potential virulence factors. These enzymes quench oxidative metabolites produced by phagocytic cells and may therefore aid in protecting the organism from intracellular killing by the cells.

DISEASES

Swine erysipelas can be divided into three groups: acute, subacute, and chronic (Wood 1999). The acute form is associated with septicemia. It is characterized by its sudden onset, high temperature, rapid course, and high mortality. Accompanying symptoms are loss of appetite and general depression. In the subacute form, the early symptoms are similar to those of the acute form but somewhat milder. In both cases, skin lesions appear as red plaques, typically diamond shaped, of variable sizes and numbers. The chronic form of swine erysipelas follows the acute and subacute disease and is typically characterized by endocarditis and polyarthritis. The chronic arthritis resembles rheumatoid arthritis in many respects, making erysipelas polyarthritis an interesting model for studying the pathomechanisms of chronic inflammation.

In sheep and cattle, the disease usually takes the form of polyarthritis. In birds the infection is generally acute with high mortality and occurs most commonly in turkeys and only rarely in chickens. Male turkeys are more frequently affected than females, and the disease often manifests as sudden death but skin lesions may be apparent.

PATHOGENESIS

Bacterial Invasion

E. rhusiopathiae can gain entry to the body by a variety of routes. Natural infection in swine and per-

haps in other animals occurs commonly as a result of ingesting contaminated food or water. In swine, the bacteria present in their tonsils, and other lymphoid tissues of the alimentary canal are thought to gain entry to the deeper body tissues or bloodstream; however, the precise portal of entry into their host remains unclear (Wood 1999). In turkeys, the bacteria may gain entrance through wounds in the skin that develop during the fighting activities of the male birds.

In mice and pigeons, subcutaneously injected bacteria are usually observed within endothelial cells lining the veins and capillaries and in endothelial cells free in the blood stream (Tenbroeck 1920). Nakato et al. (1986a, 1987) noted that rats experimentally infected with a virulent *E. rhusiopathiae* strain developed arteritis as the result of bacterial invasion into endothelial cells. They found that *in vivo* distribution of bacterial invasion was always concomitant with that of desialated sites of the arterial regions and that *in vitro* adhesion of bacteria to aortic endothelial cells was inhibited by the addition of N-acetylneuramin-lactose, a substrate of bacterial neuraminidase. Thus, neuraminidase of *E. rhusiopathiae* plays an important role in bacterial attachment and subsequent invasion into host cells, and particularly into endothelial cells. This invasion into endothelial cells could contribute to the vascular disturbances, which are usually observed in the early septicemic stage of acute swine erysipelas.

E. rhusiopathiae appears to have invasive ability into other types of host cell as well. Franz et al. (1995) successfully recovered high numbers of viable *E. rhusiopathiae* bacteria from synovial cells and chondrocytes isolated from arthritic joints of pigs. The intracellular live bacteria or bacterial antigens in joint tissue are often responsible for chronic arthritis.

Bacterial Interactions with Phagocytic Cells

In the presence of normal serum, virulent capsulated *E. rhusiopathiae* strains are resistant to phagocytosis by polymorphonuclear leukocytes (PMNs) by virtue of the capsules, while acapsular mutants are avirulent and susceptible to phagocytosis by the cells under the same conditions; thus, the virulence of the bacteria correlates well with the antiphagocytic ability of the strains (Shimoji et al. 1994).

Phagocytosis of virulent *E. rhusiopathiae* bacteria is carried out primarily by mononuclear phagocytes, not PMNs. It has been shown that murine peritoneal macrophages can phagocytose virulent capsulated *E. rhusiopathiae* bacteria to some extent even in the presence of normal serum (Shimoji et al. 1996). This finding is consistent with other studies of mice and pigeons (Tenbroeck 1920), and of swine (Böhm and Suphasindhu 1980; Böhm et al. 1982); however, the reason for the difference between macrophages and PMNs in the ability to phagocytose the organism is unknown.

Early studies reported by Timoney (1969, 1970a) have provided evidence of the importance of intracellular survival within professional phagocytes for the pathogenicity of *E. rhusiopathiae*. It has also been shown that, in contrast to acapsular mutants, capsulated *E. rhusiopathiae* bacteria phagocytosed in the presence of normal serum can survive and replicate within macrophages (Shimoji et al. 1996). The intracellular replication of virulent *E. rhusiopathiae* bacteria within phagocytic cells is usually observed *in vivo* at inoculation sites after experimental challenge (fig. 10.1). Thus, the capsule of the organism also plays a critical role in intracellular replication within professional phagocytic cells. Shimoji (1996) showed that capsulated *E. rhusiopathiae* bacteria opsonized with normal serum did not induce oxidative burst responses in macrophages after phagocytosis, while acapsular mutants did. This is probably one of the mechanisms by which capsulated *E. rhusiopathiae* bacteria escape intracellular killing by phagocytic cells in a nonimmune host; however, it remains unknown why there is a difference in the induction of the oxidative burst between the capsulated and acapsular strains.

Complement Activation

C3b, the cleavage product of the third component of complement, can be generated by two independent mechanisms: the classical and alternative pathways. It has been shown that *E. rhusiopathiae* activates the complement system through an alternative pathway, that is, in the absence of specific antibodies (Dinter et al. 1976). Nakato et al. (1986b) found that rats experimentally infected with live *E. rhusiopathiae* bacteria showed platelet-bacteria aggregation generated by the complexes of the bacteria and C3b through the C3b receptor on the platelet, causing thrombocytopenia. Thus, activation of complement by the organism can cause thrombocytopenia and intravascular aggregations of bacteria could result in vascular disturbances.

Since C3b is of central importance, especially against invasive infections caused by capsulated bacteria, it is highly possible that after antibody-

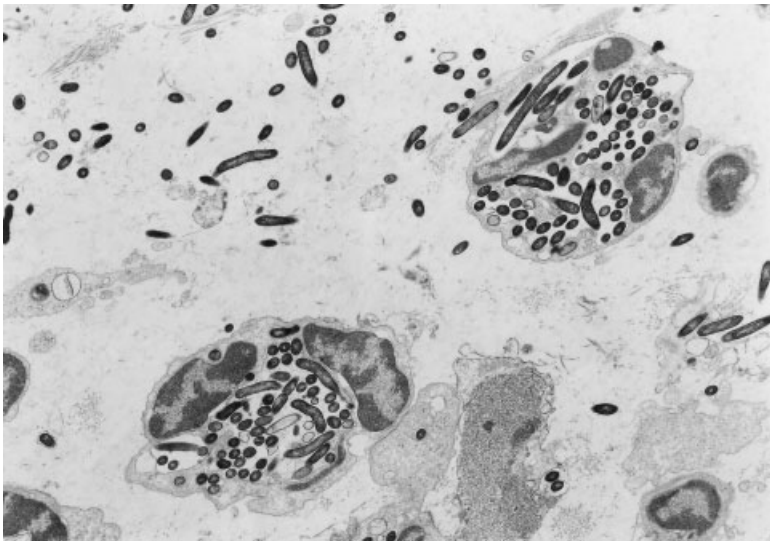


Figure 10.1. Thin section of a mouse skin lesion at 6 h of infection with *E. rhusiopathiae* strain Fujisawa-SmR. Note that within the phagocytic cells there are many morphologically intact organisms, some of which are undergoing division. (Electron micrograph prepared by Dr. Shogo Tanaka)

independent activation of C3, C3b deposits on the capsule of *E. rhusiopathiae* are used as opsonins for phagocytosis of the organism by phagocytic cells. In a study by Timoney (1970b), mice in which complement was greatly decreased by injection of heat-aggregated sheep γ-globulin died earlier than did normal control mice after lethal challenge. Timoney also showed that when challenge bacteria were pre-opsonized with fresh mouse serum, the decomplemented mice survived a lethal challenge, and heat treatment of the mouse serum at 56°C abolished its protective activity. Thus, in a nonimmune host, complement can protect by opsonization. However, phagocytosis via C3b (actually C3bi) may be insufficient to kill all ingested bacteria intracellularly because C3bi receptor-mediated phagocytosis is not accompanied by the oxidative burst response of the phagocytic cells in contrast to the Fc gamma receptor (FcγR)-mediated phagocytosis.

ACQUIRED IMMUNITY

Humoral Immunity

Disease caused by *E. rhusiopathiae* in swine and turkeys has been successfully controlled by the use of bacterins and attenuated vaccines (Wood 1999). In vaccinated animals, both humoral and cell-mediated immunity play an important role in host defense. The critical role of humoral immunity in the infection has long been suggested by the fact that treatment with antiserum has also been used successfully for disease control. The protective activity of antiserum against *E. rhusiopathiae* in mice is found only in the IgG fraction, and not in the IgM fraction (Yokomizo and Isayama 1972). Bacteria opsonized with immune serum are readily eliminated by PMNs (Shimoji et al. 1994; Sawada et al. 1988) and by macrophages (Shimoji et al. 1996). Thus, the protective activity of immune serum is, as shown above, most probably the opsonic activity of IgG antibodies in the FcγR-mediated phagocytosis in which oxidative burst and other important intracellular bactericidal mechanisms are generated. Immunization with capsular antigen of *E. rhusiopathiae* can extend the mean time to death in mice, but is unable to protect the animals completely against lethal challenge (Shimoji et al., unpublished data). A major protective antigen is the surface-located SpaA protein (Makino et al. 1998; Shimoji et al. 1999). Antisera raised against recombinant SpaA protect mice by passive immunization (Shimoji et al. 1999) and elicit protection in pigs by inducing opsonic antibodies (Imada et al. 1999). The 64- to 66-kDa protein in detergent-solubilized surface antigens and the 220-kDa surface protein

described above are also protective antigens. However, whether these proteins are capable of eliciting opsonic antibodies remains uninvestigated.

Cell-mediated Immunity

The role of cell-mediated immunity in protection has been demonstrated in studies using acapsular *E. rhusiopathiae* strains in mice and pigs (Shimoji et al. 1998, 2003a). These studies found that after immunization, spleen cells from mice and peripheral blood mononuclear cells from pigs proliferated significantly in response to *E. rhusiopathiae* antigens, showing that both arms of the immune system contribute to immunity to the infection. The specific bacterial antigen involved in inducing this cell-mediated immunity remains unidentified to date.

CONCLUSIONS

The pathogenicity of *E. rhusiopathiae* appears to be related primarily to the intracellular survival properties of the bacterium within phagocytic cells. Therefore, rationally attenuated acapsular *E. rhusiopathiae* mutants, which are unable to grow within macrophages, may be good candidates for live vaccines if they can stimulate effective immune responses. Recent research has shown that intranasal vaccination of pigs with a nonreverting acapsular *E. rhusiopathiae* mutant expressing a recombinant antigen of *Mycoplasma hyopneumoniae* elicits protective immunity not only against erysipelas but also against enzootic pneumonia (Shimoji et al. 2002a, 2003a). To date, effective bacterial vectors have not been developed for vaccination of animals. The potential of acapsular mutants for live delivery of heterologous antigens may have a significant impact on the future development of vaccines.

REFERENCES

Böhm K. H., R. Soliman, and W. Leibold. 1982. Interactions between erysipelas bacteria and swine macrophages with special reference to virulence. In Proceedings of the International Pig Veterinary Society Congress. R. R. Necoechea, C. Pijoan, A. Cesarin, and M. Guzman (eds.). Mexico City, p. 146.

Böhm K. H., and V. Suphasindhu. 1980. Investigations on phagocytosis of erysipelas bacteria by neutrophile leukocytes with special reference to virulence. In Proceedings of the International Pig Veterinary Society Congress. N. C. Nielsen, P. Hogh, and N. Bille (eds.). Copenhagen, p. 197.

Dinter, Z., H. Diderholm, and G. Rockborn. 1976. Complement-dependent hemolysis following hemagglutination by *Erysipelothrix rhusiopathiae*. Zentralbl. Bakteriol. Orig. 236:533–535.

Franz, B., M. E. Davies, and A. Horner. 1995. Localization of viable bacteria and bacterial antigens in arthritic joints of *Erysipelothrix rhusiopathiae*-infected pigs. FEMS Immunol. Med. Microbiol. 12:137–142.

Galán, J. E., and J. F. Timoney. 1990. Cloning and expression in *Escherichia coli* of a protective antigen of *Erysipelothrix rhusiopathiae*. Infect. Immun. 58:3116–3121.

Imada, Y., N. Goji , H. Ishikawa, M. Kishima, and T. Sekizaki. 1999. Truncated surface protective antigen (SpaA) of *Erysipelothrix rhusiopathiae* serotype 1a elicits protection against challenge with serotypes 1a and 2b in pigs. Infect. Immun. 67:4376–4382.

Lachmann, P. G., and H. Deicher. 1986. Solubilization and characterization of surface antigenic components of *Erysipelothrix rhusiopathiae* T28. Infect. Immun. 52:818–822.

Makino, S., K. Katsuta, and T. Shirahata. 1999. A novel protein of *Erysipelothrix rhusiopathiae* that confers haemolytic activity on *Escherichia coli*. Microbiology 145:1369–1374.

Makino, S. I., K. Yamamoto, S. Murakami, T. Shirahata, K. Uemura, T. Sawada, H. Wakamoto, and Y. Morita. 1998. Properties of repeat domain found in a novel protective antigen, SpaA, of *Erysipelothrix rhusiopathiae*. Microb. Pathog. 25:101–109.

Müller, H. E., and C. Krasemann. 1976. Immunity against *Erysipelothrix rhusiopathiae* infection by means of active immunization using homologous neuraminidase. Z. Immunitatsforsch. Exp. Klin. Immunol. 151:237–241.

Nakato, H., K. Shinomiya, and H. Mikawa. 1986a. Possible role of neuraminidase in the pathogenesis of arteritis and thrombocytopenia induced in rats by *Erysipelothrix rhusiopathiae*. Pathol. Res. Pract. 181:311–319.

———. 1986b. Effect of C3 depletion on the genesis of thrombocytopenia induced in rats by *Erysipelothrix rhusiopathiae*. Scand. J. Haematol. 37:18–24.

———. 1987. Adhesion of *Erysipelothrix rhusiopathiae* to cultured rat aortic endothelial cells. Role of bacterial neuraminidase in the induction of arteritis. Pathol. Res. Pract. 182:255–260.

Sawada, T., Y. Tamura, and T. Takahashi. 1988. Mechanism of protection induced in mice against *Erysipelothrix rhusiopathiae* infection by treatment

with porcine antiserum to the culture filtrate of an attenuated strain. Vet. Microbiol. 17:65–74.

Shimoji, Y. 2000. Pathogenicity of *Erysipelothrix rhusiopathiae*: virulence factors and protective immunity. Microbes Infect. 2:965–972.

Shimoji, Y., E. Oishi, T. Kitajima, Y. Muneta, S. Shimizu, and Y. Mori. 2002a. *Erysipelothrix rhusiopathiae* YS-1 as a live vaccine vehicle for heterologous protein expression and intranasal immunization of pigs. Infect. Immun. 70:226–232.

Shimoji, Y., E. Oishi, Y. Muneta, H. Nosaka, and Y. Mori. 2003a. Vaccine efficacy of the attenuated *Erysipelothrix rhusiopathiae* YS-19 expressing a recombinant protein of *Mycoplasma hyopneumoniae* P97 adhesin against mycoplasmal pneumonia of swine. Vaccine. 21:532–537.

Shimoji, Y., H. Asato, T. Sekizaki, Y. Mori, and Y. Yokomizo. 2002b. Hyaluronidase is not essential for the lethality of *Erysipelothrix rhusiopathiae* infection in mice. J. Vet. Med. Sci. 64:173–176.

Shimoji, Y., Y. Mori, and V. A. Fischetti. 1999. Immunological characterization of a protective antigen of *Erysipelothrix rhusiopathiae*: identification of the region responsible for protective immunity. Infect. Immun. 67:1646–1651.

Shimoji, Y., Y. Mori, T. Sekizaki, T. Shibahara, and Y. Yokomizo. 1998. Construction and vaccine potential of acapsular mutants of *Erysipelothrix rhusiopathiae*: use of excision of Tn*916* to inactivate a target gene. Infect. Immun. 66:3250–3254.

Shimoji, Y., Y. Ogawa, M. Osaki, and T. Sekizaki. 2002c. Novel *Erysipelothrix rhusiopathiae* surface proteins are involved in biofilm formation. In Proceedings of the 83rd annual meeting of the Conference of Research Workers in Animal Diseases. R. P. Ellis (ed.). Ames: Iowa State University Press.

Shimoji, Y., Y. Ogawa, M. Osaki, H. Maruyama, T. Mikami, and T. Sekizaki. 2003b. Adhesive surface proteins of *Erysipelothrix rhusiopathiae* bind to polystyrene, fibronectin, and type I and IV collagens. J. Bacteriol. 185:2739–2848.

Shimoji, Y., Y. Yokomizo, and M. Mori. 1996. Intracellular survival and replication of *Erysipelothrix rhusiopathiae* within murine macrophages: failure of induction of the oxidative burst of macrophages. Infect. Immun. 64:1789–1793.

Shimoji, Y., Y. Yokomizo, T. Sekizaki, Y. Mori, and M. Kubo. 1994. Presence of a capsule in *Erysipelothrix rhusiopathiae* and its relationship to virulence for mice. Infect. Immun. 62:2806–2810.

Takahashi, T., M. Takagi, R. Yamaoka, K. Ohishi, M. Norimatsu, Y. Tamura, and M. Nakamura. 1994. Comparison of the pathogenicity for chickens of *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum*. Avian Patho. 23:237–245.

Takahashi, T., N. Hirayama, T. Sawada, Y. Tamura, and M. Muramatsu. 1987. Correlation between adherence of *Erysipelothrix rhusiopathiae* strains of serovar 1a to tissue culture cells originated from porcine kidney and their pathogenicity in mice and swine. Vet. Microbiol. 13:57–64.

Takahashi, T., T. Fujisawa, Y. Tamura, S. Suzuki, M. Muramatsu, T. Sawada, Y. Benno, and T. Mitsuoka. 1992. DNA relatedness among *Erysipelothrix rhusiopathiae* strains representing all twenty-three serovars and *Erysipelothrix tonsillarum*. Int. J. Syst. Bacteriol. 42:469–473.

Tenbroeck, C. 1920. Studies on *Bacillus murisepticus*, or the rotlauf bacillus, isolated from swine in the United States. J. Exp. Med. 3:331–343.

Timoney, J. 1969. The inactivation of *Erysipelothrix rhusiopathiae* in macrophages from normal and immune mice. Res. Vet. Sci. 10:301–302.

———. 1970a. The inactivation of *Erysipelothrix rhusiopathiae* in pig buffy-coat leukocytes. Res. Vet. Sci. 11:189–190.

———. 1970b. The effect of decomplementation on *Erysipelothrix rhusiopathiae* infection in the mouse. Immunology. 19:561–567.

Wood, R. L. 1999. Erysipelas. In Diseases of swine. A. D. Leman et al. (ed.). Ames: Iowa State University Press, pp. 419–430.

Yokomizo, Y., and Y. Isayama. 1972. Antibody activities of IgM and IgG fractions from rabbit anti-*Erysipelothrix rhusiopathiae* sera. Res. Vet. Sci. 13:294–296.

11
Neurotoxigenic Clostridia

R. H. Whitlock

More than a hundred years ago, botulism and tetanus were associated with the effects of potent neurotoxins, and knowledge accumulated since that time has enabled immunoprophylactic approaches to prevention and, more recently, use of botulinum neurotoxins (BoNTs) as therapeutic agents.

BOTULISM

Pathogenesis of botulism is mediated by botulinum neurotoxin (BoNT) (Linial 1995), which affects all mammals, birds, and fish (Smith 1977). The seven types of BoNT (table 11.1; Smith 1979) have unique geographic distribution and species susceptibility patterns (Smith 1977; Smith and Milligan 1979; Hatheway 1990; Hatheway 1995; CDC 1998; Shapiro et al. 1998).

CHARACTERISTICS OF *CLOSTRIDIUM BOTULINUM*

Clostridium botulinum is a gram-positive, straight-to-curved, large rod, although staining may be weak or negative in cultures older than 24 hours. Lipase activity appears as a film around colonies on egg yolk agar and is characteristic of all strains except those producing BoNT-G. Cells often carry oval, subterminal spores, which are among the most resistant in nature; they remain viable for more than 30 years in liquid media and exhibit only a gradual decrease in viability after multiple freeze-thaw cycles.

Table 11.1. Host Predilection of Various Antigenic Types of Botulinum Toxin

Types of *C. botulinum*	Hosts
A	Cattle, humans
B	Horses, cattle, humans
C	Birds, cattle
D	Cattle
E	Fish, fish-eating birds, humans
F	Humans
G	Humans

Botulism spores are ubiquitous in soil in most areas of the United States (Smith 1979). Spores of type A organisms are common in soil west of the Rocky Mountains, where nearly one-fifth of soil samples are culture positive (Smith 1979). They have rarely been associated with botulism in cattle; cases have been reported in California, Utah, Idaho, Oregon, and Ohio, but almost never in the mid-Atlantic region (Whitlock and Williams 1999).

Spores of type B organisms are common in soil in the mid-Atlantic states (Smith 1979) and are responsible for more than 85% of cases of botulism in cattle and horses in North America (Whitlock and Williams 1999; Kelch et al. 2000). The source of contamination is often not identified in sporadic equine botulism, but when several horses are involved, it is typically forage, as in outbreaks in California (hay cubes; Kinde et al. 1991), Ohio (contaminated wheat; Whitlock 1997), North Carolina (baled alfalfa hay; Wichtel and Whitlock 1991), England (baled hay; Ricketts et al. 1984), Sweden (large-bale ensilage; Franzen et al. 1992), and Australia (oat chaff; Kelly et al. 1984). These cases rarely involve contamination of grain by decomposing animal carcasses (Whitlock and Williams 1999). High forage pH encourages germination of spores, and silage at a high pH is a common source of botulism for horses (Divers et al. 1986). Ryelage is more frequently associated with

botulism than other types of forage in cattle, and hay stored in plastic bags has become a common factor in outbreaks in the United States and England.

Botulism associated with feed contaminated by a decomposing carcass is most often type C or D (Enfors et al. 1975). Type C botulism in horses is invariably associated with a decomposing animal carcass (Galey et al. 2000) or situations where ravens or crows feeding on a decomposing carcass transport BoNT to feed containers (Schoenbaum et al. 2000; Whitlock et al 1997). Type C intoxication of cattle is also usually associated with a decomposing carcass (Craven 1964) or soil contamination of feed (Heath et al. 1990). Feeding of poultry litter containing chicken carcasses has frequently been associated with outbreaks of botulism in beef cattle, including major outbreaks in beef cattle in Israel (Savoir 1978) and Australia (Trueman 1992).

Type D botulism occurs commonly in South America and South Africa and has been linked to pica, in which phosphorus-deficient cattle consume bones of colleagues dead of botulism (Dobereiner et al. 1992). Feeding of poultry litter to cattle in Europe, Israel, and Australia has been associated with type D botulism, often in massive outbreaks (Appleyard and Mollison 1985; Dobereiner et al. 1992; McLoughlin et al. 1988; Neill et al. 1989; Trueman 1992). Type D botulism is rare in the United States (Whitlock and Messick 1986).

Type E botulism occurs in humans following consumption of fish, but apparently does not affect cattle or other domestic species. Most human cases in North America occur in the Pacific Northwest, with the incidence in Alaska estimated at 20–50 human cases annually (CDC 1998; Shapiro et al. 1998). Type E *C. botulinum* is found frequently around Lake Michigan, and this parallels the frequency of type E isolates from fish in the Great Lakes (Sugiyama 1970).

Types F and G botulism have not been reported in domestic animals.

PATHOGENESIS OF BOTULISM

Spores are relatively ubiquitous, and they remain dormant-yet-viable for years, even in harsh environmental conditions (Smith 1979). Germination in environments with suitable nutrients and pH leads to BoNT production.

Botulism intoxication may occur by ingestion of preformed BoNT or may be toxicoinfectious (dissemination of toxin from an infected wound or from a focus of *C. botulinum* multiplication in the gastrointestinal tract) (Hatheway 1995; Swerczek 1980). Horses are much more susceptible than cattle to BoNT, perhaps due to degradation of preformed toxin by rumen microbes in cattle (Divers et al. 1986; Allison et al. 1976). Horses absorb more toxin before it reaches the colon, where microbial degradation occurs in equids.

Wound botulism occurs when spores germinate in a wound and produce toxin (Bernard et al. 1987). In horses, this occurs most often at castration sites (Bernard et al. 1987), umbilical hernia repairs, or deep puncture wounds from injections of counterirritants (Mitten et al. 1994). Wound botulism also occurs in calves castrated by banding (J. Glenn Songer, personal communication) and has been reported secondary to illicit drug use in humans (MacDonald et al. 1985) and in addicts of black tar heroin who administer the drug by "skin-popping."

Toxicoinfectious botulism in foals, often called "shaker foal syndrome" (Swerczek 1980), follows absorption of toxin from the gastrointestinal tract. This also occurs in human infants and is called "floppy baby syndrome." It may be responsible for some fatalities in sudden infant death syndrome (Arnon and Shin 1979; Arnon 1986).

Disease in foals occurs up to eight months of age (Swerczek 1980), and toxin can be detected in feces of approximately 30% of shaker foals during the acute clinical phase (Whitlock 1997). Normal intestinal flora of adult horses, humans, and other animals largely inhibit intraintestinal growth of *C. botulinum* (Arnon et al. 1978; Arnon and Shin 1979). However, in cattle, deaths may continue for an extended period after diagnosis of botulism and removal of contaminated feed. It is tempting to speculate that the organism can establish residence even in the mature bovine gastrointestinal tract and produce sufficient toxin to cause fatal intoxication.

Plasma concentrations of toxin usually remain below the detection limit of the most sensitive toxin test, the mouse bioassay. In rare situations, toxin is detected in serum or plasma of acutely affected cattle and horses. Birds are more resistant to botulism, and toxin is frequently detected in birds with acute disease.

The higher the dose of BoNT at the neuromuscular junction, the more rapidly progressive the clinical signs and the poorer the prognosis for survival. Experimentally administered small doses of BoNT may not give rise to clinical signs for 5–10 days, while massive doses (on the order of 10^8 IP MLD_{50} units) administered to cattle lead to recumbency and

death in 18–24 h. In field cases, cattle exposed to moderate amounts of BoNT exhibit evidence of weakness for 24–48 h and become recumbent for 2–3 days before death. Cattle that are physically active are more likely to be severely affected by lower doses of toxin. In mild or subclinical cases, the course ranges from 2–30 days, depending on the dose of toxin.

In horses, smaller amounts of toxin (~ 10^3 IP MLD_{50} units) result in onset of clinical signs over 3–10 days and less-severe signs (Whitlock and Williams 1999). Mildly affected horses may have only transient dysphagia and recover with minimal treatment. Larger doses of toxin cause peracute, rapidly progressive illness, with animals becoming recumbent 8–12 h after onset of signs. Massive concentrations of toxin ingested with forage may yield clinical signs within 12–24 h of ingestion.

Gross or histological lesions are not usually associated with botulism. Inhalation pneumonia may occur in some cases, due to an abnormal deglutition reflex. Botulism cases in general should have relatively empty gastrointestinal tracts due to dysphagia. Absence of significant lesions following a period of progressive weakness leading to recumbency in an afebrile, relatively bright-and-alert group of subjects should increase the index of suspicion for botulism.

TETANUS

Characteristics of *Clostridium tetani*

Clostridium tetani is a slender, motile, Gram-positive, sporulating rod. Organisms from older cultures and in smears from wounds may stain Gram-negative. No capsule is produced and the organism does not ferment sugars or produce lecithinase or lipase. The outstanding morphological feature of *C. tetani* is the terminal, spherical spore.

Clostridium tetani is a moderately fastidious anaerobe, and will grow on the surface of agar media only under strongly reducing conditions. Optimal growth temperature is 37°C. Colonies on blood agar are slightly raised, semitransparent, and gray, often with an irregular margin and surrounded by a narrow halo of hemolysis.

Spores of *C. tetani* are ubiquitous in the environment, although their frequent occurrence in horse manure appears to be a fable perpetuated for decades. Spores are concentrated in some geographic areas and present at much lower levels in other regions, so clinical cases may occur commonly on specific farms but rarely on others, even with similar husbandry practices.

PATHOGENESIS OF TETANUS

Portals of entry for *C. tetani* are contaminated traumatic wounds in horses; castration band-induced lesions in sheep, goats, and cattle; and tail-docking wounds and dehorning sites in adult cattle. Use of elastrator bands for castration or tail docking is a risk factor in lambs and calves. Tetanus in adult cows may begin from contaminated, periparturient, uterine prolapses. Proliferation in the reticulorumen may yield sufficient amounts of toxin to produce clinical tetanus without evidence of contaminated wounds. Horses are particularly susceptible to tetanus, and in these animals it is most often associated with contaminated wounds. Necrosis is often not extensive, and may be dismissed by the examining veterinarian as insignificant.

Tetanus spores may remain dormant in healed human wounds, with latent periods up to 10 years. Germination of spores in necrotic tissue often occurs in the presence of facultative bacteria that use oxygen, creating a microenvironment with reduced eH and allowing growth of *C. tetani*. Tetanolysin is an oxygen-labile hemolysin that inhibits chemotaxis of phagocytes and may also increase local tissue injury, enhancing bacterial multiplication and production of tetanospasmin (TeNT).

NATURE AND ACTION OF BOTULINUM AND TETANUS TOXINS

BoNTs cause flaccid paralysis by binding to and entering peripheral cholinergic nerve terminals and blocking acetylcholine release. Paralysis is reversible without treatment in mild cases or with respiratory support in those that are more severe. TeNT, on the other hand, blocks neurotransmitter release at inhibitory interneurons of the spinal cord, resulting in spastic paralysis (van Heyningen 1968) and, ordinarily, a much less promising prognosis than botulism. In spite of causing disparate clinical symptoms, BoNT and TeNT have similar structures and both affect neuroexocytosis (Burgen et al. 1949; Brooks et al. 1955).

BoNT and TeNT are synthesized without signal sequences and are released upon bacterial lysis as a polypeptide protoxin of 150 kDa. Proteolysis activates the molecule (Das Gupta 1994), leaving heavy (100 kDa) and light (L, 50 kDa) chains associated via noncovalent interactions and a disulfide bond (Schiavo et al. 1990).

BoNTs A and B and TeNT have a C-terminal 50-kDa receptor binding domain (Halpern and Loftus 1993; Coen et al. 1997; Lalli et al. 1999) composed of two subdomains. Deletion of the N-terminal portion of this domain has no effect on membrane binding, whereas deletion of as few as ten residues from the C-terminus abrogates TeNT binding to spinal cord neurons (Halpern and Loftus 1993). The 50 kDa N-terminal portion of the H-chain mediates translocation of the 50-kDa L-chain, which is a zinc-dependent metalloprotease, to the cytosol of susceptible cells (Schiavo et al. 1992a, 1992b; Hohne-Zell et al. 1994).

BoNTs and TeNT in body fluids bind to the presynaptic membrane of cholinergic terminals. Binding sites for BoNTs A and B at the rat neuromuscular junction probably number in the hundreds per square meter (Black and Dolly 1986), while a neuroblastoma-glioma line cell has about 450 TeNT receptors (Wellhoner and Neville 1987). Host cell sialogangliosides and other molecules are involved in binding (Halpern and Neale 1995). Low and high affinity receptors exist, and both glycolipids and proteins are essential for high affinity binding (Bakry et al. 1991; Halpern and Loftus 1993).

BoNTs block neuroexocytosis at peripheral terminals, whereas TeNT does so at CNS synapses of the spinal cord (Wellhoner 1992). TeNT enters a peripheral motorneuron and moves, within vesicles, retrograde along the axon (Schwab et al. 1979). When vesicles reach dendritic terminals in the cell body, they release TeNT into the intersynaptic space, where it equilibrates between pre- and postsynaptic membranes and enters the inhibitory interneurons by synaptic vesicle endocytosis. Trafficking of BoNT, on the other hand, utilizes receptors that guide toxin-containing vesicles that acidify within the neuromuscular junction.

Nerve intoxication by L-chains of TeNT and BoNTs occurs only after toxin exposure to low pH (Simpson 1983, 1993; Simpson et al. 1994; Williamson and Neale 1994; Matteoli et al. 1996) and an accompanying conformational change exposing hydrophobic surface patches on the L-chain and mediating insertion into the hydrocarbon core of the lipid bilayer (Montecucco 1986; Montecucco and Schiavo 1993). Ion channels form in planar lipid bilayers (Hoch et al. 1985; Blaustein et al. 1987; Shone et al. 1987; Rauch et al. 1990), and these channels participate in transmembrane translocation of the L-chain into the nerve terminal cytosol (Boquet and Duflot 1982).

Table 11.2. Substrate Specificity Among Clostridial Neurotoxins

Substrate	Cleaved by
VAMP	TeNT; BoNTs B, D, F, G
SNAP-25	BoNTs A, C, E
Syntaxin	BoNT C

Cytoplasmic targets of the L-chain metalloprotease are three so-called SNARE proteins (table 11.2). TeNT and BoNT B cause the disparate symptoms of tetanus and botulism while cleaving VAMP at the same location (Schiavo et al. 1992c), so it is apparent that different sites of intoxication underlie the differing clinical outcomes.

Synaptic vesicles in BoNT-intoxicated striated muscle accumulate on the inner leaflet of the plasma membrane (Neale et al. 1989), as they can no longer discharge neurotransmitter into the synaptic space. Nerve and muscle remain connected and motor axon populations are maintained. Motor end plates enlarge and outgrowths infiltrate the muscle. Over time, muscle regains its normal size, sprouts degenerate, and the original end-plate regains function with acetylcholinesterase and acetylcholine receptors concentrated at the junctions (Borodic et al. 1994). Time to recovery varies with the type of nerve terminal and type of BoNT. The mammalian neuromuscular junction is typically repaired in 2–4 months, but more than 1 year is required for restoration of human autonomic nervous system function (Naumann et al. 1999). BoNT A and BoNT C have the most long-lasting effects, and recovery from neuromuscular junction paralysis by other types is more rapid (Ludlow et al. 1992; Eleopra et al. 1997, 1998). The half-life of the L-chain in the cytosol (estimated to be weeks [Habig et al. 1986; Marxen and Bigalke 1991; Bartels et al. 1994]) and turnover of SNARE proteins (Raciborska and Charlton 1999) contribute to these differences.

IMMUNITY TO BOTULISM AND TETANUS

In a typical herd outbreak of botulism, many cattle will have minimal clinical signs, such as a weak tongue, decreased jaw tone, and mild dysphagia, without recumbency. These animals develop an antibody response to BoNT 3–4 weeks following recovery (Thomas 1991; Jubb et al. 1993; Main and Gregory 1996).

A toxoid vaccine for *C. botulinum* type B is available, and three doses at 1-month intervals are required to successfully immunize horses (Lewis et al. 1981; Thomas et al. 1988). Three immunizations at 10–12-day intervals may provide protective antibody after 3 weeks, if necessary for emergency situations, such as in an outbreak (Crane et al. 1991). Revaccination of mares 4–6 weeks before foaling is recommended, as is annual revaccination of all horses in endemic areas. Colostrum from vaccinated mares boostered 6–8 weeks before foaling should contain adequate antibody to protect the foal for 8–12 weeks (Lewis 1991). Foals vaccinated with toxoid in the first few days of life develop antibodies, even in the presence of passive antibody from colostrum (Lewis 1991). Horses in North America are immunized with a type C toxoid approved for use in mink. This vaccine is used routinely in areas such as the southwestern United States, where type C botulism is endemic.

No botulism vaccine is approved for cattle in the United States. A type C and D toxoid is available in Australia and has been used to prevent outbreaks of botulism in sheep and cattle.

Immunization with tetanus toxoid provides excellent protection for horses and humans. Annual revaccination of horses is recommended (Green et al. 1994). Passive immunization with tetanus antitoxin is recommended, in addition to active immunization with toxoid, in neonatal foals and other species. Preexisting passive antibody does not interfere with toxoid immunization.

CONCLUSION

Progress in understanding the nature and mode of action of clostridial neurotoxins has been exciting and has opened new areas for study in both infectious disease and neurobiology. Perhaps the greatest obstacle to effectively dealing with neurotoxic clostridia in domestic animals is diagnosis of botulism. As noted, the most sensitive assay currently available is the mouse protection test, and it is likely that false negatives are common. Development and application of highly sensitive and serotype-specific assays, based at least in part upon the metalloprotease activity of the toxins, will provide the opportunity for greater understanding of the pathogenesis, epidemiology, and natural history of these intoxications.

REFERENCES

Allison, M. J., S. E. Maloy, and R. R. Matson. 1976. Inactivation of *Clostridium botulinum* toxin by ruminal microbes from cattle and sheep. Appl. Environ. Microbiol. 32:685–688.

Appleyard, W. T., and A. Mollison. 1985. Suspected bovine botulism associated with broiler litter waste. Vet. Rec. 116:522.

Arnon, S. S. 1986. Infant botulism: anticipating the second decade. J. Infect. Dis. 154:201–206.

Arnon, S. S., and J. Shin. 1979. The clinical spectrum of infant botulism. Rev. Inf. Dis. 1:614–621.

Arnon, S. S., T. F. Midra, K. Damus, and R. M. Wood. 1978. Chin J: Intestinal infection and toxin production by *Clostridium botulinum* as one cause of sudden infant death syndrome. Lancet i:1273–1277.

Bakry, N., Y. Kamata, R. Sorensen, and L. L. Simson. 1991. Tetanus toxin and neuronal membranes: the relationship between binding and neurotoxicity. J. Pharmacol. Exp. Ther. 258:613–619.

Bartels, F., H. Bergel, H. Bigalke, J. Frevert, J. Halpern, and J. Middlebrook. 1994. Specific antibodies against the Zn2+-binding domain of clostridial neurotoxins restore exocytosis in chromaffin cells treated with tetanus or botulinum A neurotoxin. J. Biol. Chem. 269:8122–8127.

Bernard, W., T. J. Divers, R. H. Whitlock, J. Messick, and E. Tulleners. 1987. Botulism as a sequel to open castration in a horse. J. Am. Vet. Med. Assoc. 191:73–74.

Black, J. D., and J. O. Dolly. 1986. Interaction of 125I-labeled botulinum neurotoxins with nerve terminals. Part II: Autoradiographic evidence for its uptake into motor nerves by acceptor-mediated endocytosis. J. Cell Biol. 103:535–544.

Blaustein, R. O., W. J. Germann, A. Finkelstein, A., and B. R. Das Gupta. 1987. The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers. FEBS Lett. 226:115–120.

Boquet, P., and E. Duflot. 1982. Tetanus toxin fragment forms channels in lipid vesicles at low pH. Proc. Natl. Acad. Sci. USA 79:7614–7618.

Borodic, G. E., R. J. Ferrante, L. B. Perace, and K. Alderson. 1994. Pharmacology and histology of the therapeutic application of botulinum toxin. In *Therapy with Botulinum Toxin*, edited by J. Jankovic and M. Hallett, pp. 119–157. New York: Marcel Dekker.

Brooks, V. B., D. R. Curtis, and J. C. Eccles. 1955. Mode of action of tetanus toxin. Nature 175:120–121.

Burgen, A. S. V., F. Dickens, and L. J. Zatman. 1949. The action of botulinum toxin on the neuromuscular junction. J. Physiol. (Lond.) 109:10–24.

Centers for Disease Control and Prevention. 1998. Botulism in the United States, 1899–1996.

Handbook for epidemiologists, clinicians, and laboratory workers. Atlanta, GA: Centers for Disease Control and Prevention.

Coen, L., R. Osta, M. Maury, and P. Brulet. 1997. Construction of hybrid proteins that migrate retrogradely and transsynaptically into the central nervous system. Proc. Natl. Acad. Sci. U S A 94: 9400–9405.

Crane, S. A., R. H. Whitlock, C. Buckley, and B. Forney. 1991. *Clostridium botulinum* type-B toxoid for vaccination of adult horses, pregnant mares and foals: a study of vaccination protocols. In Proceedings 37th Conv. Am. Assoc. Equine Pract., p. 611.

Craven, C. P. 1964. Control of a botulism-like disease in North Queensland. Aust. Vet. J. 40:127–130.

Das Gupta, B. R. 1994. Structures of botulinum neurotoxin, its functional domains, and perspectives on the crystalline type A toxin. In Therapy with botulinum toxin. J. Jankovic and M. Hallett (eds.). New York: Marcel Dekker, pp. 15–39.

Divers, T. J., R. C. Bartholomew, J. B. Messick, R. H. Whitlock, and R. W. Sweeney. 1986. *Clostridium botulinum* Type B toxicosis in a herd of cattle and a group of mules. J. Am. Vet. Med. Assoc. 188:382–386.

Dobereiner, J., C. H. Tokarnia, and J. I. S. Langenegger Dutra. 1992. Epizootic botulism of cattle in Brazil. Dtsch. Tierarztl. Wschr. 99:188–190.

Eleopra, R., V. Tugnoli, O. Rossetto, C. Montecucco, and D. De Grandis. 1997. Botulinum neurotoxin serotype C: a novel effective botulinum toxin therapy in human. Neurosci. Lett. 224:91–94.

Eleopra, R., V. Tugnoli, O. Rossetto, D. De Grandis, and C. Montecucco. 1998. Botulinum neurotoxin serotype A and E in human: evidence of a different temporal profile in the neuromuscular block induced. Neurosci. Lett. 224:91–94.

Enfors, E., A. Gunnarsson, B. Hurvell, and N. Ringarp. 1975. Outbreak of botulism caused by *Clostridium botulinum* Type C in cattle. Svensk. Vet. 27:333–339.

Franzen, P., A. Gustafsson, and A. Gunnardsson. 1992. Botulism in horses associated with feeding big bale silage. Svensk Veterinar tidning. 44:555–559.

Galey, F. D., R. Terra, R. Walker, J. Adaska, M. A. Etchebarne, B. Puschner, E. Fisher, R. H. Whitlock, T. Rocke, D. Willoughby, and E. Tor. 2000. Type-C botulism in dairy cattle from feed contaminated with a dead cat. J. Vet. Diagn. Invest. 12:204–209.

Green, S. L., C. B. Little, J. D. Baird, R. R. Tremblay, and L. L. Smith-Maxie. 1994. Tetanus in the horse: a review of 20 cases (1970 to 1990). J. Vet. Intern. Med. 8:128–132.

Habig, W. H., H. Bigalke, G. K. Bergey, E. A. Neale, M. C. Hardegree, and P. G. Nelson. 1986. Tetanus toxin in dissociated spinal cord cultures: long-term characterization of form and action. J. Neurochem. 47:930–937.

Halpern, J. L., and A. Loftus. 1993. Characterization of the receptor-binding domain of tetanus toxin. J. Biol. Chem. 268: 11188–11192.

Halpern, J. L., and E. A. Neale. 1995. Neurospecific binding, internalization, and retrograde axonal transport. Curr. Top. Microbiol. Immunol. 195:221–241.

Hatheway, C. L. 1990. Toxigenic clostridia. Clin. Microbiol. Rev. 3:66–98.

———. 1995. Botulism: the present status of the disease. Curr. Topics Microbio. Immun. 195:55–75.

Heath, S. E., R. J. Bell, M. Chirino-Trejo, J. C. L. Schuh, and R. J. Harland. 1990. Feed trough dirt as a source of *Clostridium botulinum* type C intoxication in a group of farm horses. Can. Vet. J. 31:13–19.

Hoch, D. H., M. Romero-Mira, B. E. Ehrlich, A. Finkelstein, B. R. Das Gupta, and L. L. Simpson. 1985. Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: relevance to translocation of proteins across membranes. Proc. Natl. Acad. Sci. U S A 82:1692–1696.

Hohne-Zell, B., A. Ecker, U. Weller, and M. Gratzl. 1994. Synaptobrevin cleavage by the tetanus toxin light chain is linked to the inhibition of exocytosis in chromaffin cells. FEBS Lett. 355:131–134.

Jubb, T. F., T. M. Ellis, and A. R. Gregory. 1993. Diagnosis of botulism in cattle using ELISA to detect antibody to botulinum toxins. Aust. Vet. J. 70:226–227.

Kelch, W. J., L. A. Kerr, J. K. Pringle, B. W. Rohrbach, and R. H. Whitlock. 2000. Fatal *Clostridium botulinum* toxicosis in eleven Holstein cattle fed round bale barley haylage. J. Vet. Diagn. Invest. 12:453–455.

Kelly, A. R., R. J. Jones, J. C. Gillick, and L. D. Sims. 1984. Outbreak of botulism in horses. Eq. Vet. J. 16:519–521.

Kinde, H., R. L. Betty, A. Ardans, F. D. Galey, B. M. Daft, R. L. Walker, M. W. Eklund, and J. W. Byrd. 1991. *Clostridium botulinum* type C intoxication associated with consumption of processed hay cubes in horses. J. Am. Vet. Med. Assoc. 199:742–746.

Lalli, G., J. Herreros, S.L. Osborne, C. Montecucco, O. Rossetto, and G. Schiavo. 1999. Functional characterization of tetanus and botulinum neurotoxins binding domains. J. Cell. Sci. 112:2715–2724.

Lewis, G. E., S. Kulinski, E. H. Fallon, J. P. Klyza, and J. T. Bryans. 1981. Evaluation of a *Clostridium botulinum* toxoid, type B, for the prevention of

shaker foal syndrome. Proc. Am. Assoc. Equine Pract. 27:233–237.

Linial, M. 1995. Bacterial neurotoxins—a thousand years later. Isreal J. Med. Sci. 31:591–595.

Ludlow, C. L., M. Hallett, K. Rhew, R. Cole, T. Shimizu, G. Sakaguchi, J. A. Bagley, G. M. Schulz, S. G. Yin, and J. Koda. 1992. Therapeutic use of type F botulinum toxin. N. Engl. J. Med. 326: 349–350.

MacDonald, K. L., G. W. Rutherford, S. M. Friedman, J. R. Dietz, B. R. Kaye, G. F. McKinley, J. H. Tenney, and M. L. Cohen. 1985. Botulism and botulism-like illness in chronic drug abusers. Ann. Intern. Med. 102:616–618.

Main, D. C., and A. R. Gregory. 1996. Serological diagnosis of botulism in dairy cattle. Aust. Vet. J. 73:77–78.

Marxen, P., and H. Bigalke. 1991. The chromaffin cell: a suitable model for investigating the actions and the metabolism of tetanus and botulinum A neurotoxin. Naunyn Schemiedebergs Arch. Pharmacol. 343:12–29.

Matteoli, M., C. Verderio, O. Rossetto, N. Iezzi, N., S. Coco, G. Schiavo, and C. Montecucco. 1996. Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. Proc. Natl. Acad. Sci. U S A 93: 13310–13315.

McLoughlin, M. F., S. G. McIlroy, and S. D. Neill. 1988. A major outbreak of botulism in cattle being fed ensiled poultry litter. Vet. Rec. 122:579–581

Mitten, L. A., K. W. Hinchchiff, S. J. Holcombe, and S. M. Reed. 1994. Mechanical ventilation and management of botulism secondary to an injection abscess in an adult horse. Equine Vet. J. 26:420–423.

Montecucco, C. 1986. How do tetanus and botulinum toxins bind to neuronal membranes? Trends Biochem. Sci. 11:315–317.

Montecucco, C., and G. Schiavo. 1993. Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem. Sci. 18:324–327.

Naumann, M., W. H. Jost, and K. V. Toyka. 1999. Botulinum toxin in the treatment of neurological disorders of the autonomic nervous system. Arch. Neurol. 56:914–916.

Neale, E. A., W. H. Habig, B. K. Schrier, G. K. Bergey, L. M. Bowers, and J. Koh. 1989. Application of tetanus toxin for structure-function studies in neuronal cell cultures. In Eighth international conference on tetanus. G. Nistico, B. Bizzini, B. Bytchencko, and R. Triau (eds.). Rome: Pythagora Press, pp. 66–70.

Neill, S. D., M. F. McLoughlin, and S. G. McIlroy. 1989. Type C botulism in cattle being fed ensiled poultry litter. Vet. Rec. 124:558–560.

Raciborska, D. A., and M. P. Charlton. 1999. Retention of cleaved synaptosome-associated protein of 25 kDa (SNAP-25) in neuromuscular junctions: a new hypothesis to explain persistence of botulinum A poisoning. Can. J. Physiol. Pharmacol. 77:679–688.

Rauch, G., F. Gambale, and M. Montal. 1990. Tetanus toxin channel in phosphatidylserine planar bilayers: conductance states and pH dependence. Eur. J. Biochem. 18:79–83.

Ricketts, S. W., T. R. C. Greet, P. J. Glyn, C. D. R Ginnett, E. P. McAllister, J. McCaig, P. H. Skinner, P. M. Webbon, D. L. Frape, G. R. Smith, and L. G. Murray. 1984. Thirteen cases of botulism in horses fed big bale silage. Eq. Vet. J. 16:515–518.

Savoir, D. 1978. Mass outbreaks of botulism in ruminants associated with ingestion of feed containing poultry waste. III. Epidemiologic findings. Refuah. Vet. 35:105–108.

Schiavo, G., B. Poulain, O. Rossetto, F. Benfenati, L. Tauc, and C. Montecucco. 1992a. Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc. EMBO J. 11: 3577–3583.

Schiavo, G., E. Papini, G Genna, and C. Montecucco. 1990. An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. Infect. Immun. 58:4136–4141.

Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B. R. Das Gupta, and C. Montecucco. 1992b. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature 359:832–835.

Schiavo, G., O. Rossetto, A. Santucci, B. R. Das Gupta, and C. Montecucco. 1992c. Botulinum neurotoxins are zinc proteins. J. Biol. Chem. 267: 23479–23483.

Schoenbaum, M. A., S. M. Hall, R. D. Glock, K. Grant, A. L. Jenny, T. J. Schiefer, P. S. Sciglibaglio, and R. H. Whitlock. 2000. An outbreak of type C botulism in 12 horses and a mule. J. Am. Vet. Med. Assoc. 217:365–368.

Schwab, M.E., K. Suda, and H. Thoenen. 1979. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. J. Cell. Biol. 82:798–810.

Shapiro, R. L., C. Hatheway, and D. L. Swerdlow. 1998. Botulism in the United States: a clinical and epidemiologic review. Ann. Int. Med. 129:221–228.

Shone, C. C., P. Hambleton, and J. Melling. 1987. A 50-kDa fragment from the NH_2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles. Eur. J. Biochem. 167:175–180.

Simpson, L. L. 1983. Ammonium chloride and methylamine hydrochloride antagonize clostridial neurotoxins. J. Pharmacol. Exp. Ther. 225:546–552.

———. 1993. Botulinum neurotoxin and tetanus toxin. San Diego: Academic Press.

Simpson, L. L., J. L. Coffield, and N. Bakry. 1994. Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. J. Pharmacol. Exp. Ther. 269:256–269.

Smith, L. D. S., 1977. Botulism in animals. In Botulism: the organism, its toxins, the disease. Springfield, IL: C. C. Thomas, p. 236.

———. 1979. *Clostridium botulinum*: Characteristics and occurrence. Rev. Inf. Dis. 1:637–641.

Smith, G. R., and R. A. Milligan. 1979. *Clostridium botulinum* in soil on the site of the former metropolitan (Caledonian) cattle market, London. J Hyg Camb 83:237–241.

Sugiyama, H., T. L. Bott, and E. M. Foster. 1970. *Clostridium botulinum* type E in an island bay (Green Bay of Lake Michigan). In Toxic microorganisms. M. Herzberg (ed.). Washington, DC: U.S. Dept. of Interior.

Swerczek, T. W. 1980. Toxicoinfectious botulism foals and adult horses. J. Am. Vet. Med. Assoc. 176:348–350.

Thomas, R. J. 1991. Detection of *Clostridium botulinum* types C and D toxin by ELISA. Aust. Vet. J. 68:111–113.

Thomas, R. J., D. V. Rosenthal, and R. J. Rogers 1988. A *Clostridium botulinum* type B vaccine for prevention of shaker foal syndrome. Aust. Vet. J. 65:79–80.

Trueman, K. F., R. E. Bock, R. J. Thomas, J. D. Taylor, P. A. Green, H. M. Roeger, and P. J. Ketterer. 1992. Suspected botulism in three intensively managed Australian cattle herds. Vet. Record 130:398–400.

van Heyningen, W. E. 1968. Tetanus. Sci. Am. 218: 69–77.

Wellhoner, H. H. 1992. Tetanus and botulinum neurotoxins. In Handbook of experimental pharmacology, vol. 102. H. Herchen and F. Hucho (eds.). Berlin: Springer-Verlag, pp.357–417.

Wellhoner, H. H., and D. M. Neville, Jr. 1987. Tetanus toxin binds with high affinity to neuroblastoma: glioma hybrid cells NG 108 15 and impairs their stimulated acetylcholine release. J. Biol. Chem. 262:17374–17378.

Whitlock, R. H. 1997. Botulism. Vet. Clin. North Am. Equine Pract. 13:107–128

Whitlock, R. H., and J. B. Messick. 1986. Botulism in cattle and horses. In Proceedings of the 4th Ann. Am. College Vet. Internal Med., Washington, DC, pp. 57–62.

Whitlock, R. H., and J. M. Williams. 1999. Botulism toxicosis of cattle. Proc. Bovine Practitioner 32:45–53.

Whitlock, R. H., E. J. Sandigo, S. C. Trock, T. Francisco, T. Schiefer, D. Saari, and J. P. Tilsted. Type C Botulism in New Mexico horses: an outbreak. In 40th annual proceedings American Association of Veterinary Laboratory Diagnosticians, October 17–24, 1997, Louisville, KY, abstract p. 61.

Wichtel, J. J., and R. H. Whitlock 1991. Botulism associated with feeding alfalfa hay to horses. J. Am. Vet. Med. Assoc. 199:471–472.

Williamson, L. C., and E. A. Neale. 1994. Bafilomycin A1 inhibits the action of tetanus toxin in spinal cord neurons in cell culture. J. Neurochem. 63:2342–2345.

12
Histotoxic Clostridia

J. G. Songer

Numerous histotoxic clostridia affect humans and domestic animals, but a limited group causes most of the infections. Common themes of these infections are acquisition of the infecting organism from soil or an endogenous source (such as the intestinal tract), entry to tissue following trauma (in many cases), local multiplication, extensive local and systemic tissue damage, and rapid death. The hallmark is enthusiastic toxinogenesis. Control by vaccination of animals has decreased the incidence and the visibility of some clostridial diseases, but renewed interest in mechanisms of pathogenesis has yielded new information, particularly about modes of toxin action.

VIRULENCE FACTORS AND PATHOGENESIS

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 widely known typing system divides the species into five toxinogenic types (Hatheway 1990; Rood and Cole 1991; chap. 13), and the general assumption is that most cases of myonecrosis are caused by type A strains. However, toxin typing of myonecrosis strains is not widely practiced, either by traditional *in vivo* neutralization tests or PCR genotyping; thus, occasional cases may be caused by strains of other toxin types. We have no information on the nature of clinical disease that might result from infections with strains other than type A.

Thus, a discussion of *C. perfringens*-induced myonecrosis is limited to infections with strains of type A (Hatheway 1990). Typically, spores germinate and vegetative cells multiply in ischemic tissues and the infection spreads to healthy muscle, often leading to mortality. As with the other histotoxic clostridia, toxins are thought to be the major virulence factors. Endomyometritis, sometimes called "pink lady syndrome," can follow cesarean delivery or bungled obstetrical procedures; early

Table 12.1. Clostridia as Agents of Myonecrosis

Organism	Major toxins	Diseases	Species affected
C. perfringens type A	Alpha, theta	Gas gangrene, myonecrosis	All warm-blooded
C. septicum	Alpha	Abomasitis, malignant edema	Sheep, cattle
C. chauvoei	Alpha, beta	Blackleg	Sheep, cattle
C. novyi types A and B	Alpha, beta	Wound infections ("bighead"), infectious necrotic hepatitis	Sheep, goats
C. novyi type D	Beta	Bacillary hemoglobinuria	Cattle
C. sordellii	Hemolytic, lethal	Myonecrosis	Sheep, cattle

recognition and aggressive management may prevent fulminant and ultimately fatal disease (Halpin and Molinari 2002).

Alpha toxin may play both local and systemic roles in clostridial myonecrosis, possibly, in the latter, by effects on platelets and phagocytes. Virulence of mutants of *pfoA* (the gene for theta toxin) and *cpa* (the gene for alpha toxin) revealed synergistic effects of the two toxins in a mouse model. Infection with the double mutant resulted in minimal pathology, and the isogenic strain complemented with genes for both toxins produced lesions that were unquestionably more severe than when CPA production alone was reconstituted. Differences were most apparent in the rapid spread of the infection and in the rate at which mice became moribund. The results support the hypothesis that synergy between CPA and PFO is important in the pathology of gas gangrene (Awad et al. 1995, 2001). Both toxins also influence the occurrence of the characteristic vascular leukostasis of clostridial myonecrosis (Ellemor et al. 1999). Inability to produce collagenase has no apparent effect on virulence in the mouse myonecrosis model (Awad et al. 2000).

The precise roles of CPA and PFO are less clear. Diacylglycerol is produced in rabbit neutrophil membranes by exposure to CPA and, as a consequence, adhesion of the cells to fibrinogen and fibronectin and production of O_2^{--} (Ochi et al. 2002). There is also evidence for a deleterious effect of toxin on venous capillary endothelial cells, leading to expression of proinflammatory mediators and adhesion molecules (Stevens 2000; Bryant et al. 2000).

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. Such vaccines are widely available elsewhere in the world and are apparently efficacious. 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 LD_{50} of *C. perfringens* (Titball et al. 1993; Williamson and Titball 1993).

CLOSTRIDIUM SEPTICUM

Clostridium septicum is commonly found in soil and in the feces of domestic animals (Princewill et al. 1984; Princewill 1985). It is a frequent postmortem invader from the gut of domestic animals, particularly ruminants, and may enter in one of the life stages of liver flukes. Iatrogenic infections (Harwood 1984; Mullaney et al. 1984) are more common in horses than in other species, and umbilical infections are not uncommon in sheep. Ovine or bovine braxy follows invasion by *C. septicum* of abomasal mucosa damaged by ingestion of frozen or poor-quality feed (Saunders 1986; Schamber et al. 1986). Abomasal and proximal small intestine walls become edematous, hemorrhagic, and sometimes necrotic (Ellis et al. 1983), and signs of toxemia are common.

Principally, the organism causes malignant edema in domestic animals and humans, 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 clinical features such as rapid onset, pain, tachycardia, and bullus formation, followed by hypotension and acute renal failure; palpation and radiography reveal gas in soft tissues (Burke and Opeskin 1999).

Hemorrhage, edema, and necrosis develop rapidly as the infection spreads along muscular fascial planes. Early on, tissues are painful and warm, with pitting edema, but later they become crepitant and cold. The clinical course is often less than 24 h. The source of the organism is gastrointestinal in more than 50% of human patients, and the mortality rate varies from 33 to 58%, depending upon the method of management.

Toxic or potentially toxic products of *C. septicum* include alpha toxin (oxygen-stable hemolysin), beta toxin (DNase, leukocidin), gamma toxin (hyaluronidase), delta toxin (oxygen-labile hemolysin), a neuraminidase and hemagglutinin, a chitinase, and sialidase (Ballard et al. 1992; Zenz et al. 1993). The effects of the purified toxin mimic features of animal and human diseases caused by *C. septicum* (Tweten 2001). Alpha toxin is secreted as a 46.5-kDa protoxin, which has 72% amino acid sequence similarity with the primary structure of the *Aeromonas hydrophila* aerolysin (Ballard et al. 1995). There are common features between primary structures of aerolysin and alpha toxin, excepting the counterpart of the amino terminal domain of mature aerolysin (~70 amino acids), which is absent from alpha toxin (Parker et al. 1994; Ballard et al. 1995). Both toxins have similar modes of action. Activated alpha toxin has specific activity of approximately 1.5×10^6 hemolytic units per mg, and the 50% mouse lethal dose is about 10 μg per kg (Ballard et al. 1992, 1993).

Alpha toxin 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 and colloidal-osmotic lysis (Ballard et al. 1993; Sellman and Tweten 1997; Sellman et al. 1997). *In vitro* activation can be by cleavage with trypsin (Ballard et al. 1993), but furin and other eukaryotic proteases activate toxin on the cell surface *in vivo* (Gordon et al. 1997). It is likely that alpha toxin binds to glycosylphosphatidylinositol-anchored protein receptors (Gordon et al. 1999). A role for potential virulence attributes other than alpha toxin has not been proven (Hatheway 1990; Riddell et al. 1993).

The brief clinical course dictates a preference for prevention rather than treatment, and death following *C. septicum* challenge is delayed in immunized mice. Antibody responses to somatic and toxin antigens (Hjerpe 1990) yield lifelong immunity to malignant edema in domestic animals (Green et al. 1987), although differences in immunogenicity by vaccine and by host species have been reported (Green et al. 1987; Morgan et al. 1988). In feedlot cattle, death losses were reduced by approximately 50% in vaccinates, with a significant cost benefit to vaccination (Knott et al. 1985).

Clostridium chauvoei

Clostridium chauvoei, a close relative of *C. septicum* (Kuhnert et al. 1996; Burke and Opeskin 1999), causes blackleg, an emphysematous, necrotizing myositis (table 12.1), which, in sheep, most often resembles malignant edema or gas gangrene. 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 alpha toxin, which is necrotizing, hemolytic, and lethal, and beta toxin, a DNase (Ramachandran 1969), remain undefined. Flagella expression is associated with virulence, and phase variation occurs in motility and flagellation (Tamura et al. 1995)**;** flagella are apparently protective antigens (Verpoort et al. 1966; Kojima et al. 2000). Anti-idiotypic antibodies imaging *C. chauvoei* flagella immunized mice against challenge, suggesting the possible efficacy of this as an approach to prevention of blackleg (Kijima-Tanaka et al. 1994). The *fliC* gene has been cloned and characterized, but recombinant flagellin protein failed to protect mice against challenge (Kojima et al. 2000). Equine hyperimmune serum and penicillin can be used for therapy and prophylaxis.

Clostridium novyi

Clostridium novyi type C is avirulent, but strains of type A cause gas gangrene in humans and wound infections in animals. The hallmark lesion is edema, and "bighead" of young rams is illustrative; rapidly spreading edema of the head, neck, and cranial thorax follows invasion of subcutaneous tissues damaged by fighting (Sterne and Batty 1975). Infectious necrotic hepatitis ("black disease") of sheep and cattle results from *C. novyi* type B infection. Dormant spores germinate in liver tissue, often damaged by fluke migration, and systemic effects with acute or peracute death follow dissemination of alpha toxin (Elder and Miles 1957)**.** Its cardio-, neuro-, histo-, and hepatotoxic effects apparently produce edema, serosal effusion, and focal hepatic necrosis. 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 type B in the liver at or about the time of parturition. *Clostridium novyi* type D, often referred to as *C. haemolyticum*, causes bacillary hemoglobinuria of cattle and other ruminants (Bender et al. 1999).

Differential production of alpha and beta toxins is the major phenotypic differentiating factor. However, *C. novyi* types B, C, and D may have arisen from a single phylogenetic origin (Sasaki et al. 2001); 16S rDNA sequences of type B and D strains are identical, and are nearly identical to sequences from types C (> 99.9%) and A (98.7%); on the other hand, type B and D sequences are more like those from *C. botulinum* types C and D than that of *C. novyi* type A.

In addition to gas gangrene, *C. novyi* type A has been recently recognized with alarming frequency, as has *C. botulinum*, as a cause of septicemia in drug addicts who had injected themselves intramuscularly (McGuigan et al. 2002). Among 60 cases, the median age was 30 years and most injected acidified heroin extravascularly. The case fatality rate was near 90% (McGuigan et al. 2002).

C. novyi alpha toxin belongs to the family of large clostridial cytotoxins that modify small GTP-binding proteins, affecting the cytoskeleton. Full

enzyme activity of the intact toxin resides on an approximately 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).

There is no effective treatment for *C. novyi* infections, but prophylaxis with bacterin:toxoids or toxoids can be achieved. Second generation veterinary vaccines may be based upon native or recombinant alpha toxoids (Amimoto et al. 1998).

CLOSTRIDIUM SORDELLII

Clostridium sordellii has inherent importance in veterinary medicine and recently raised visibility as a cause of fatalities in human postsurgical patients. Information on the source is incomplete, but deaths were almost exclusively in patients following knee replacement. It is also a rare cause of postpartum endometritis and, even more rarely, of spontaneous endometritis. Onset is typically sudden, with flulike symptoms and progressive refractory hypotension. Edema begins locally and spreads rapidly. Laboratory findings are typically marked leukocytosis and elevated hematocrit. Death follows rapidly in most cases (Rorbye et al. 2000).

C. 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 *C. sordellii* (Al-Mashat and Taylor 1983).

Two toxins, one lethal and one hemolytic, are antigenically and pathophysiologically similar to *C. difficile* toxins B and A, respectively. *C. sordellii* lethal toxin (TcsL) glucosylates Ras, Rac, and Ral, differing from other large clostridial toxins in its modification of Ras, which does not cycle from cytosol to membrane (Qa'Dan et al. 2001).

Guinea pigs immunized with toxoids prepared from purified lethal toxin and partially purified hemolytic toxin are protected against spore challenge, and results of trials suggest that both toxins must be represented in the vaccine (Amimoto et al. 2001).

CONCLUSION

Control by vaccination has decreased incidence, and perhaps also the visibility, of clostridial myonecrosis in domestic animals. Renewed interest in mechanisms of pathogenesis has yielded new information about clostridia, and particularly about the mode of action of their toxins, whether arising from study of disease problems in humans or domestic animals. Genetic systems in clostridia are relatively primitive, but rapid progress in some systems holds the promise of substantial advances in the immediate future. The rapid expansion of genome sequencing efforts will be a major factor in our understanding of the interaction of histotoxic clostridia with hosts.

The veterinary biologics industry is seeking a new paradigm for preparation and delivery of immunoprophylactic products in the face of continuing concerns about undesirable postvaccination effects. Delivery of recombinant proteins by conventional means or by *in vivo* expression from attenuated bacterial delivery systems is a focus of industry efforts.

REFERENCES

Al-Mashat, R. R., and D. J. Taylor. 1983. Production of diarrhoea and enteric lesions in calves by the oral inoculation of pure cultures of *Clostridium sordellii*. Vet. Rec. 112:141–146.

Amimoto, K., E. Oishi, H. Yasuhar, O. Sasak, S. Katayama, T. Kitajima, A. Izumida, and T. Hirahara. 2001. Protective effects of *Clostridium sordellii* LT and HT toxoids against challenge with spores in guinea pigs. J. Vet. Med. Sci. 63:879–883.

Amimoto, K., O. Sasaki, M. Isogai, T. Kitajima, E. Oishi, N. Okada, and H. Yasuhara. 1998. The protective effect of *Clostridium novyi* type B alpha-toxoid against challenge with spores in guinea pigs. J. Vet. Med. Sci. 60:681–685.

Awad, M. M., A. E. Bryant, D. L. Stevens, and J. I. Rood. 1995. Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. Mol. Microbiol. 15:191–202.

Awad, M. M., D. M. Ellemor, A. E. Bryant, O. Matsushita, R. L. Boyd, D. L. Stevens, J. J. Emmins, and J. I. Rood. 2000. Construction and virulence testing of a collagenase mutant of *Clostridium perfringens*. Microb. Pathog. 28:107–117.

Awad, M. M., D. M. Ellemor, R. L. Boyd, J. J. Emmins, and J. I. Rood. 2001. Synergistic effects of alpha-toxin and perfringolysin O in *Clostridium perfringens*-mediated gas gangrene. Infect. Immun. 69:7904–7910.

Ballard, J., A. Bryant, D. Stevens, and R. K. Tweten. 1992. Purification and characterization of the lethal toxin (alpha-toxin) of *Clostridium septicum*. Infect. Immun. 60:784–790.

Ballard, J., J. Crabtree, B. A. Roe, and R. K. Tweten. 1995. The primary structure of *Clostridium septicum* alpha-toxin exhibits similarity with that of *Aeromonas hydrophila* aerolysin. Infect. Immun. 63:340–344.

Ballard, J., Y. Sokolov, W. L. Yuan, B. L. Kagan, and R. K. Tweten. 1993. Activation and mechanism of *Clostridium septicum* alpha toxin. Mol. Microbiol. 10:627–634.

Bender, L. C., P. B. Hall, M. M. Garner, and J. L. Oaks. 1999. Bacillary hemoglobinuria in a free-ranging elk calf. J. Zoo. Wild. Med. 30:293–296.

Bryant, A. E., R. Y. Chen, Y. Nagata, Y. Wang, C. H. Lee, S. Finegold, P. H. Guth, and D. L. Stevens. 2000. Clostridial gas gangrene. II. Phospholipase C-induced activation of platelet gpIIbIIIa mediates vascular occlusion and myonecrosis in *Clostridium perfringens* gas gangrene. J. Infect. Dis. 182:808–815.

Burke, M. P., and K. Opeskin. 1999. Nontraumatic clostridial myonecrosis. Am. J. Forensic Med. Pathol. 20:158–162.

Busch, C., K. Schomig, F. Hofmann, and K. Aktories. 2000. Characterization of the catalytic domain of *Clostridium novyi* alpha-toxin. Infect. Immun. 68:6378–6383.

Chen, Y. M., H. C. Lee, C. M. Chang, Y. C. Chuang, and W. C. Ko. 2001. Clostridium bacteremia: emphasis on the poor prognosis in cirrhotic patients. J. Microbiol. Immunol. Infect. 34:113–118.

Elder, J. M., and A. A. Miles. 1957. The action of the lethal toxins of gas gangrene clostridia on capillary permeability. J. Pathol. Bacteriol. 74:133–145.

Ellemor, D. M., R. N. Baird, M. M. Awad, R. L. Boyd, J. I. Rood, and J. J. Emmins. 1999. Use of genetically manipulated strains of *Clostridium perfringens* reveals that both alpha-toxin and theta-toxin are required for vascular leukostasis to occur in experimental gas gangrene. Infect. Immun. 67:4902–4907.

Ellis, T. M., J. B. Rowe, and J. M. Lloyd. 1983. Acute abomasitis due to *Clostridium septicum* infection in experimental sheep. Aust. Vet. J. 60:308–309.

Gordon, V. M., K. L. Nelson, J. T. Buckley, V. L. Stevens, R. K. Tweten, P. C. Elwood, and S. H. Leppla. 1999. *Clostridium septicum* alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. J. Biol. Chem. 274:27274–27280.

Gordon, V. M., R. Benz, K. Fujii, S. H. Leppla, and R. K. Tweten. 1997. *Clostridium septicum* alpha-toxin is proteolytically activated by furin. Infect. Immun. 65:4130–134.

Green, D. S., M. J. Green, M. H. Hillyer, and K. L. Morgan. 1987. Injection site reactions and antibody responses in sheep and goats after the use of multivalent clostridial vaccines. Vet. Rec. 120:435–439.

Halpin, T. F., and J. A. Molinari. 2002. Diagnosis and management of *Clostridium perfringens* sepsis and uterine gas gangrene. Obstet. Gynecol. Surv. 57:53–57.

Harwood, D. G. 1984. Apparent iatrogenic clostridial myositis in cattle. Vet. Rec. 115:412.

Hatheway, C. L. 1990. Toxigenic clostridia. Clin. Microbiol. Rev. 3:66–98.

Hjerpe, C. A. 1990. Clostridial disease vaccines. Vet. Clin. North. Amer. Food Animal Pract. 6:222–234.

Kijima-Tanaka, M., Y. Tamura, S. Suzuki, N. Nagamine, and M. Nakamura. 1994. Antigenic mimicry of *Clostridium chauvoei* flagella by polyclonal anti-idiotypic antibodies. J. Med. Microbiol. 40:70–75.

Knott G. K. L., B. G. Erwin, and L. G. Classick. 1985. Benefits of a clostridial vaccination program in feedlot cattle. Vet Med 80:95–97.

Kojima, A., I. Uchida, T. Sekizaki, Y. Sasaki, Y. Ogikubo, M. Kijima, and Y. Tamura. 2000. Cloning and expression of a gene encoding the flagellin of *Clostridium chauvoei*. Vet. Microbiol. 76:359–372.

Kuhnert, P., S. E. Capaul, J. Nicolet, and J. Frey. 1996. Phylogenetic positions of *Clostridium chauvoei* and *Clostridium septicum* based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 46:1174–1176.

Manteca, C., G. Daube, V. Pirson, B. Limbourg, A. Kaeckenbeeck, and J. G. Mainil. 2001. Bacterial intestinal flora associated with enterotoxaemia in Belgian Blue calves. Vet. Microbiol. 81:21–32.

McGuigan, C. C., G. M. Penrice, L. Gruer, S. Ahmed, D. Goldberg, M. Black, J. E. Salmon, and J. Hood. 2002. Lethal outbreak of infection with *Clostridium novyi* type A and other spore-forming organisms in Scottish injecting drug users. J. Med. Microbiol. 51:971–977.

Morgan, K. L., D. S. Green, N. J. Green, and M. H. Hillyer. 1988. The injection site reaction and antibody response in sheep and goats following the use of multivalent clostridial vaccines. In Proceedings of the workshop on goat production in the humid tropics, University of Ife, 1987. O. B. Smith and H. G. Osman (eds.), pp. 117–124.

Mullaney, T. P., C. M. Brown, and R. Flint-Taylor. 1984. Clostridial myositis in horses following intramuscular administration of ivermectin. Proc. Annu. Meet. Am. Assoc. Vet. Lab. Diagn. 27:171–177.

Ochi, S., T. Miyawaki, H. Matsuda, M. Oda, M. Nagahama, and J. Sakurai. 2002. *Clostridium perfringens* alpha-toxin induces rabbit neutrophil adhesion. Microbiology 148:237–245.

Parker, M. W., J. T. Buckley, J. P. M. Postma, A. D. Tucker, K. Leonard, F. Pattus, and D. Tsernoglou.

1994. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. Nature 367:292–295.

Princewill, T. J. T. 1985. Sources of clostridial infections in Nigerian livestock and poultry. Bulletin of Animal Health and Production in Africa 33:323–326.

Princewill, T. J. T., M. I. Agba, and S. O. Jemitola. 1984. Isolation of *Clostridium* species from paddock soil. Bull. An. Hlth. Produc. Africa 32:263–268.

Qa'Dan, M., L. M. Spyres, and J. D. Ballard. 2001. pH-enhanced cytopathic effects of *Clostridium sordellii* lethal toxin. Infect. Immun. 69:5487–5493.

Ramachandran, S. 1969. Haemolytic activities of *Cl. chauvoei*. Indian Vet. J. 46:754–68.

Riddell, S. W., B. L. Miles, B. L. Skitt, and S. D. Allen. 1993. Histopathologic effects of culture filtrates of *Clostridium septicum* in rabbit intestinal loops. Clin. Infect. Dis. 16 Suppl. 4:S211–213.

Rood, J. I., and S. T. Cole. 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. 55:621–648.

Rorbye, C., I. S. Petersen, and L. Nilas. 2000. Postpartum *Clostridium sordellii* infection associated with fatal toxic shock syndrome. Acta Obstet. Gynecol. Scand. 79:1134–1135.

Sasaki, Y. N. Takikawa, A. Kojima, M. Norimatsu, S. Suzuki, and Y. Tamura. 2001. Phylogenetic positions of *Clostridium novyi* and *Clostridium haemolyticum* based on 16S rDNA sequences. Int. J. Syst. Evol. Microbiol. 51:901–904.

Saunders, G. 1986. Diagnosing braxy in calves and lambs. Vet. Med. 81:1050–1052.

Schamber, G. J., I. E. Berg, and J. R. Molesworth. 1986. Braxy or bradsot-like abomasitis caused by *Clostridium septicum* in a calf. Can. Vet. J. 27:194.

Sellman, B. R., and R. K. Tweten. 1997. The propeptide of *Clostridium septicum* alpha toxin functions as an intramolecular chaperone and is a potent inhibitor of alpha toxin-dependent cytolysis. Mol. Microbiol. 25:429–440.

Sellman, B. R., B. L. Kagan, and R. K. Tweten. 1997. Generation of a membrane-bound, oligomerized pre-pore complex is necessary for pore formation by *Clostridium septicum* alpha toxin. Mol. Microbiol. 23:551–558.

Selzer, J., F. Hofmann, G. Rex, M. Wilm, M. Mann, I. Just, and K. Aktories. 1996. *Clostridium novyi* alpha-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. J. Biol. Chem. 271:25173–25177.

Sterne, M., and I. Batty. 1975. Pathogenic clostridia. London: Butterworths.

Stevens, D. L. 2000. The pathogenesis of clostridial myonecrosis. Int. J. Med. Microbiol. 290:497–502.

Tamura, Y., M. Kijima-Tanaka, A. Aoki, Y. Ogikubo, and T. Takahashi. 1995. Reversible expression of motility and flagella in *Clostridium chauvoei* and their relationship to virulence. Microbiology 141:605–610.

Titball, R. W., A. M. Fearn, and E. D. Williamson. 1993. Biological and immunological properties of the C-terminal domain of the alpha-toxin of *Clostridium perfringens*. FEMS Microbiol. Lett. 110:45–50.

Tweten, R. K. 2001. *Clostridium perfringens* beta toxin and *Clostridium septicum* alpha toxin: their mechanisms and possible role in pathogenesis. Vet. Microbiol. 82:1–9.

Verpoort, J. A., F. J. Joubert, and B. C. Jansen. 1966. Studies on the soluble antigen and haemolysin of *Clostridium chauvoei* strain 64. S. Afr. J. Agric. Sci. 9:153–172.

Williamson, E. D., and R. W. Titball. 1993. A genetically engineered vaccine against the alpha-toxin of *Clostridium perfringens* protects mice against experimental gas gangrene. Vaccine 11:1253–1258.

Zenz, K. I., P. Roggentin, and R. Schauer. 1993. Isolation and properties of the natural and the recombinant sialidase from *Clostridium septicum.* Glycoconj. J. 10:50–56.

13
Enteric Clostridia

J. G. Songer

Clostridia are widely recognized enteric pathogens of domestic animals. They have an impressive array of virulence attributes, and infections occur in myriad forms in a surfeit of hosts. Commercial immunoprophylactic products for clostridial diseases are effective and often are the mainstay of the product line of biologics companies. In spite of this, clostridial enteric infections remain a common presentation to veterinary diagnostic laboratories. First among these, in severity, economic impact, and overall incidence, are infections by the various toxinotypes of *C. perfringens. Clostridium difficile* has emerged as an important pathogen of horses, pigs, and perhaps other species, and other clostridia (*C. septicum*, *C. spiroforme*, and *C. colinum*) are common pathogens in some geographic areas (tables 13.1 and 13.2).

CLOSTRIDIUM PERFRINGENS

CHARACTERISTICS

Clostridium perfringens is recovered in large numbers from the intestinal tract of animals, but some toxinotypes are consistently associated with disease (table 13.1). The organism produces many toxic or potentially toxic molecules (Hatheway 1990), but most of these products lack a definitive role in pathogenesis. All isolates from animals produce alpha toxin (CPA), and more than 98% produce theta toxin, a cholesterol-dependent cytolysin also known as perfringolysin O (Songer 2003). Production of the so-called major toxins (CPA, beta [CPB], epsilon [ETX], and iota [ITX]) divides the species into five toxinotypes (tables 13.1 and 3).

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

Table 13.2. Enteric Infections by Clostridia Other Than *C. perfringens*

Organism	Virulence factor(s)	Diseases
C. septicum	Alpha (pore former)	Abomasitis (braxy) in sheep, calves
C. difficile	A (enterotoxin) B (cytotoxin)	Antibiotic-associated typhlocolitis (humans, rodents); equine neonatal hemorrhagic enterocolitis; porcine neonatal typhlocolitis
C. spiroforme	Iota toxin (ADP-ribosylating)	Rabbit diarrhea
C. colinum	None confirmed	Fowl ulcerative enteritis

Table 13.3. Activities of the Major Toxins of *C. perfringens*

Toxin	Activity
Alpha	Multifunctional phospholipase, universal production by animal isolates, membrane phospholipid hydrolysis, cytotoxic or lytic; hemolytic, necrotizing, lethal
Beta	Mucosal necrosis, CNS signs, homology with *S. aureus* alpha and gamma toxins and leukocidin, necrotizing, lethal; *cpb* is plasmid-borne.
Epsilon	Prototoxin activated by proteolysis, necrotizing, lethal; Category B Select Agent; *etx* is plasmid-borne.
Iota	Two components (ITXA, ITXB): ITXB mediates entry to cytoplasm; ITXA ADP-ribosylates actin, dermonecrotic, lethal; related to toxins of *C. spiroforme*, *C. botulinum*, *C. difficile*
Enterotoxin*	Production coregulated with sporulation, proteolytic activation, N-terminus with cytotoxicity, C-terminus with receptor activity; *cpe* (chromosomal or plasmid-borne) present in strains of all types; silent in type E

* Not by definition a "major toxin," but important in clostridial enteric disease.

The %G + C of type strain ATCC 13124 is nearly identical to that of strain 13, but ATCC 13124 contains no plasmids and its chromosome is > 225 kbp larger. ATCC 13124 contains 3,305 protein-encoding open reading frames (ORFs), 645 more than encoded by strain 13 (Shimizu et al. 2002). Both sequenced strains contain many putative and confirmed virulence genes (Shimizu et al. 2002).

Virulence Factors and Pathogenesis

Potent toxins produced by *C. perfringens* are dominant virulence attributes in the gut (table 13.1; Hatheway 1990; Songer 1996). Strains of type A are the most widespread toxinotype in the intestines of warm-blooded animals and in the environment. They are well-known causes of wound contamination, anaerobic cellulitis, and gas gangrene in humans (Awad et al. 1995).

Necrotic enteritis (NE) of domestic poultry is associated mainly with type A (occasionally with type C) strains of *C. perfringens* (Prescott 1979). Increased prevalence has accompanied the introduction of highly effective anticoccidial vaccines and withdrawal of antimicrobial growth promotants. Mild NE results in decreased rates of gain, but inappetence, anorexia, and diarrhea are often part of a short clinical course culminating in death. Necrosis encompasses the jejunal and ileal mucosa (Ratz et al. 1989). Type A strains are common in the intestinal tract of chicks, and soil, dust, and contaminated feed and litter have been incriminated as sources of infection. High-fiber litter predisposes to NE, as does concurrent infection with coccidia (Al-Sheikhly and Al-Saieg 1980) and composition of the diet (Branton et al. 1987).

Type A clostridial enteritis in suckling and feeder pigs often takes the form of necrotizing enterocolitis with mild villous atrophy (Johannsen et al. 1993 a, b). Jejunal and ileal lesions may be heavily colonized with *C. perfringens*. Disease has been reproduced by oral inoculation of gnotobiotic

colostrum-deprived and conventional weaner pigs (Johannsen et al. 1993a,b).

Equine neonatal hemorrhagic diarrhea due to type A infection is characterized by extensive subserosal hemorrhage, diffuse mucosal necrosis, and hemorrhage of lamina propria and submucosa. Clinical presentation ranges from watery-to-mucoid diarrhea to peracute death with hemorrhagic mucosal necrosis. Gram-positive bacilli are associated with necrotic tissue, but apparently do not invade normal tissue (Bueschel et al. 1998).

Type A *C. perfringens* is commonly associated with abomasal ulceration and tympany in beef calves (Mills et al. 1990). Calves with acute onset of abdominal tympany, colic, or death are often suspected of having abomasal displacement or intestinal obstruction (Roeder et al. 1987)**,** but postmortem findings frequently include abomasal distention and abomasitis, hemorrhage, and ulceration; type A *C. perfringens* is isolated in large numbers from affected tissues. Lamb enterotoxemia (yellow lamb disease) occurs sporadically in the Pacific Northwest of the United States. Affected animals are anemic and icteric, eventually developing hemoglobinuria and dying after a brief clinical course.

The involvement of type A strains in enterotoxemia and sudden death in dairy and feedlot cattle is controversial. The most impressive postmortem finding is "redgut," in which large segments of the small intestine are reddish-purple and filled with blood. Large numbers of *C. perfringens* can be isolated from the jejunum (and often from liver) obtained antemortem. A clostridial etiology for such problems is not universally accepted and a good working hypothesis may be that type A organisms multiply anarchically in the face of damage initiated by some other agent.

A central role for CPA has been demonstrated in pathogenesis of muscle disease (Awad et al. 1995), and alpha antitoxin protects against such infections (Williamson and Titball 1993). On the other hand, little is known of the effects of CPA on the intestine. Enterotoxemia in lambs and calves is compatible with the action of a hemolytic toxin in circulation, causing intravascular hemolysis and capillary damage, inflammation, platelet aggregation, shock, and cardiac effects (Stevens et al. 1988)**.** CPA in intestinal contents of chicks with necrotic enteritis may contribute to intestinal mucosal necrosis (Fukata et al. 1991).

In neonatal pigs, type A strains cause enteropathy after substantial multiplication, which is sometimes accompanied by adherence, in the gut. However, CPA administered alone to piglets caused mild enteritis and villous edema, with minimal damage to the epithelium and blood vessels and no ultrastructural changes in villi, lymphatics, or other tissues (Johannsen et al. 1993a,b).

All strains of type A *C. perfringens* produce CPA, and variations in quantity of toxin produced have been documented. There was little sequence variation among >20 *cpa* genes from isolates of bovine origin (Tsutsui et al. 1995, Songer 2003). Specific activity of purified CPA did not vary, but *cpa* transcript levels varied up to 40-fold. Lack of apparent differences in *cpa* promoter regions suggests that differences in CPA production are likely due to variations in some extragenic region (Tsutsui et al. 1995).

A recently described putative virulence attribute is the beta2 toxin (CPB2) (Gibert et al. 1997; Herholz et al. 1999; Garmory et al. 2000; Bueschel et al. 2003). CPB2 has no significant homology with CPB, but was nonetheless so named (Gibert et al. 1997). The initial report suggested that CPB2 is cytotoxic and lethal for mice, but others have been unable to reproduce these findings (Tweten 2002; Songer 2003). Nonetheless, *cpb2* has been associated with typical and atypical typhlocolitis in horses (~50% of affected animals) (Herholz et al. 1999). PCR screening of more then 3,000 field isolates revealed *cpb2* in 37.2% (Bueschel et al. 2003). Only about 10% of isolates from normal pigs contained *cpb2*, whereas over 85% from porcine enteritis and over 90% from porcine neonatal enteritis were positive. In contrast, *cpb2* was found in 47.3% of isolates from calves with enteritis or abomastitis and in only 21.4% of isolates from all bovine enteritis cases. The majority of *cpb2*-positive porcine isolates (96.9%) produced CPB2, but isolates from other species were less-commonly positive (50.0%). Sequence variations in *cpb2* (Billington 2003) and consequent differences in CPB2 may have caused false negatives (Bueschel et al. 2003). 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 at least a marker of virulence.

Enterotoxigenic strains of type A *C. perfringens* may be involved in the etiology of diarrheal conditions in animals. Enterotoxigenic strains apparently cause hospital-acquired enteropathy in dogs (Kruth et al. 1989; Marks et al. 2002), with mild depression, anorexia, and soft-to-watery diarrhea, sometimes with blood and mucus. Diarrheic dogs are often heavily colonized, and CPE is detected much

more commonly in the feces of these dogs than in the feces of normal dogs (Kruth et al. l989).

Enterotoxigenic *C. perfringens* has also been implicated in diarrheal disease in pigs (Collins et al. 1989; Estrada-Correa and Taylor 1989). Postmortem findings include superficial mucosal necrosis and villous atrophy (Collins et al. 1989). Pigs with enteritis had spore counts up to 5 x 10^6 CFU per gram of intestinal contents (Estrada-Correa and Taylor 1989). Experimental infection causes signs ranging from creamy diarrhea and emaciation with low mortality to profuse, bloodstained diarrhea, enteritis, and death (Olubunmi and Taylor 1985). Clinical disease gives rise to serum antibodies to CPE (Estrada-Correa and Taylor 1989), but antibodies derived from parenteral inoculation do not protect (Hanna et al. 1989).

CPE is produced as a 35-kDa protein following sporulation and vegetative cell lysis in the gastrointestinal tract. It inserts into the plasma membrane and complexes with membrane proteins (Wnek and McClane 1989) causing plasma membrane damage and a rapid decrease in intracellular concentrations of ions and small molecules (McDonel 1979). Mapping of CPE has revealed distinct epitopes involved in receptor binding and insertion and cytotoxicity (Hanna et al. 1989).

Strains of type B *C. perfringens* cause dysentery in newborn lambs (table 13.1). Disease occurs in many locations worldwide, but it is relatively rare in North America. Lamb dysentery usually develops early in life, with infection acquired from the dam or her environment. Nutrient excess in the small intestine is common in lambs suckling heavily lactating dams, and the organism multiplies in this environment. Inappetance, abdominal pain, and bloody diarrhea are accompanied by recumbency, coma, and death, with a case fatality rate approaching 100%. Lesions include extensive hemorrhage and ulceration of the small intestine. Disease (called "pine") in older lambs manifests as chronic abdominal pain without diarrhea. Type B strains may also cause hemorrhagic enteritis in goats, calves, and foals. Pathogenesis is poorly understood but is likely due to additive or synergistic effects of CPB and ETX.

Strains of type C *C. perfringens* infect many domestic species worldwide (Songer 1996) (table 13.1). Newborn animals are most susceptible, although in older animals disease may follow alteration of flora by sudden dietary changes. Peracute disease affecting young piglets manifests as diarrhea and dysentery, with blood and necrotic debris in feces (Niilo 1988), and emphysema and comprehensive hemorrhagic necrosis of mucosa, submucosa, and muscularis mucosa (Ohnuna et al. 1992; Okazaki et al. 1993). Case fatality rates are 50–100%, often with a clinical course of less than 24 h. Older piglets have a less-acute clinical course, with nonbloody diarrhea and jejunal mucosal necrosis (Niilo 1988). Sows are probably the source of infection, but low numbers in sow feces may not be detected (Ohnuna et al. 1992). A similar disease occurs in neonatal calves, lambs, foals, and goats. Young ewes are sometimes affected by an enterotoxemia colorfully named "struck"; rapid death often leaves the impression that the animal has been struck by lightning. Gastrointestinal mucosal damage is followed by multiplication of type C organisms in the abomasum and small intestine, causing mucosal necrosis, usually without dysentery or diarrhea but with evidence of toxemia.

CPB plays the central role in pathogenesis of infections due to type C *C. perfringens*, and its role has been demonstrated indirectly, by challenge after vaccination with CPB toxoid (Lawrence et al. 1990). In pigs, *C. perfringens* adheres to jejunal mucosa, although damage to microvilli, mitochondria, and terminal capillaries occurs as the organism approaches the mucosa (Johannsen et al. 1993b). This process begins at the apices of jejunal villi, but progressive, widespread mucosal necrosis follows, with death and desquamation of enterocytes (Kubo and Watase 1985). Death is probably due to toxemia (Niilo 1988); acute deaths are common, without diarrhea. CPB activity in the jejunum requires curtailed protease activity, which accompanies early neonatal pancreatic secretion deficiency or ingestion of protease inhibitors in colostrum (Niilo 1988). CPB causes increased capillary permeability, perhaps facilitating uptake into the circulation and promoting systemic effects. Intravenous administration of CPB causes arterial constriction, increased blood pressure, and decreased heart rate. Amelioration of blood pressure effects by adrenal medullectomy or treatment with guanethidine suggests that CPB affects the autonomic nervous system (Sakurai et al. 1984).

Similarities of *cpb* to genes for alpha and gamma hemolysins of *S. aureus* (Hunter et al. 1993) suggest that CPB might be a spore-forming toxin. It 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), and these channels are selective for monovalent cations; thus, CPB might function as a neurotoxin, depolarizing membranes of excitable cells. On the other hand, disease cannot be reproduced by toxin alone (Niilo 1988) but rather requires inoculation with type C cultures and a protease inhibitor (Niilo 1986).

Strains of type D cause enterotoxemia (sudden death, overeating), which affects calves, goats, horses, and adult cattle, but is most prevalent in young lambs (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, 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 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 that 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).

ETX is one of the most potent bacterial toxins, with a mouse lethal dose of 100 ng/kg (Gill 1982). The 32.5-kDa inactive prototoxin (E-PTX) is converted to the >1,000-fold more toxic form following proteolytic cleavage by host or microbial proteases (Bhown and Habeeb 1977; Worthington and Mülders 1977). Removal of 13 amino acid N-terminal (Bhown and Habeeb 1977) and 22 amino acid C-terminal fragments is required for activation (Miyata et al. 2002).

Small amounts of ETX in the gut of normal animals are innocuous, but persistence of high concentrations leads to increased permeability and absorption into the circulation. 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. Focal encephalomalacia, with bilaterally symmetrical brain lesions, is a chronic neurological manifestation of enterotoxemia. The common name "pulpy kidney" derives from a hallmark ovine lesion.

ETX binds with high affinity to rat brain synaptosomes and the receptor may be a sialoglycoprotein (Nagahama and Sakurai, 1992; Payne et al. 1997). Focal-to-diffuse CNS degeneration, necrosis, and bilateral macroscopic foci of encephalomalacia are common in affected animals (Finnie 1984). Vascular endothelial tight junctions degenerate, with swelling and rupture of perivascular astrocyte processes. Increased capillary permeability (Finnie 1984), rapid extravasation of fluid (Finnie and Hajduk 1992), and elevated intracerebral pressure follow. Disruption of the blood brain barrier is evidenced by leakage of radiolabeled polyvinylpyrrolidone or serum albumin into the brain (Worthington and Mülders 1975).

Permeability effects, evidenced by edema in affected animals, suggest that ETX is a permease (Worthington and Mülders 1975). Cytotoxicity for MDCK cells is temperature and pH-dependent (Payne et al. 1994), but is unaffected in cells treated with sodium azide or agents that block endosome acidification, 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 and develop morphologic changes consistent with intoxication (Petit et al. 1997; Petit et al. 2001). Cells subsequently swell, develop membrane blebs, and lyse. Binding of ETX to MDCK and rat synaptosomal membranes is associated with the formation of a stable high-molecular-weight complex (Petit et al. 1997; Nagahama et al. 1998), apparently composed of ETX in a heptameric form (Miyata et al. 2001, 2002).

Located on a plasmid (Cole and Canard 1997), *etx* appears to be associated with IS*1151* (Johnson 1997), suggesting that it may be mobile. The *etxB* (from a type B strain) (Hunter et al. 1992) and *etxD* (from a type D strain) differ 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; Thanabalu and Porter 1996). Site-directed mutagenesis of His106 and His149 results in decreased ETX toxicity (Oyston et al. 1998).

ETX stimulates specific antitoxic immunity, and disease control is by vaccination with toxoids (Kennedy et al. 1977). Neutralizing and anti-idiotypic

monoclonal antibodies have been produced (Percival et al. 1990), suggesting that neutralization can be achieved at a single epitope. However, vaccination is not completely protective; anti-ETX titers in vaccinated goats and sheep are similar and prevent toxemic deaths in both species, but there is insufficient protection against caprine enterocolitis (Blackwell et al. 1991).

ETX is a candidate biological weapon; its mouse IV lethal dose of 100 ng/kg is greater than that of botulinum toxin (1–2 ng/kg IV) but less than that of ricin (2.7 mg/kg IV) (Gill 1982), prompting its inclusion as a Category B Select Agent.

Strains of type E *C. perfringens* are distinguished from other toxinotypes by their production of iota toxin (ITX), which is composed of two noncovalently associated components and ADP-ribosylates actin at Arg-177 (Perelle et al. 1993). Iota enterotoxemia in calves and lambs was first reported over 50 years ago, and subsequent reports have described hemorrhagic, necrotic enteritis of calves and detection of type E and ITX in ovine or bovine intestines at postmortem. Putative type E infection of rabbits may have actually been caused by *C. spiroforme* (Borriello and Carman 1985).

In North America, type E strains are associated solely with often-fatal hemorrhagic enteritis in neonatal calves (Billington et al. 1998). Necropsy findings include abomasal and small intestinal hyperemia and edema, with multifocal mucosal hemorrhage, acute inflammation, and edema of the submucosae. The morbidity rate in affected herds is typically about 10%, with a case fatality rate greater than 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. Commercial toxoids offer no protection against type E infections.

Silent *cpe* sequences are highly conserved in type E isolates; they contain nonsense and frameshift mutations and lack an initiation codon, promoters, and ribosome binding site. These strains are not clonal, and the silent type E *cpe* sequences are always located near *itxA* and *itxB* on episomal DNA; it is tempting to speculate that these conserved, silent *cpe* sequences were recently transferred horizontally on an episome carrying the ITX genes, to multiple type A isolates (Billington et al. 1998).

Immunity

Equine hyperimmune antiserum administered early in the clinical course of infections by types B, C, and D strains protects animals for up to three weeks (Ripley and Gush 1983). Active immunoprophylaxis is of paramount importance, and commercial toxoid vaccines are widely used. Vaccination of sows results in greater than tenfold reductions in mortality (Ripley and Gush 1983). Ewes and cows may be vaccinated against infection by types B, C, or D strains depending on the geographic locale, and lambs and calves are then passively protected against dysentery (Kennedy et al. 1977).

CLOSTRIDIUM SEPTICUM

Clostridium septicum causes malignant edema, but also enteric infections (Songer 1996; table 13.2). Most notable among these is braxy, in which the organism establishes in the abomasum and produces a fatal bacteremia. The pathogenetic mechanism may involve ingestion of frozen feed (Schamber et al. 1986), which impairs mucosal function (Ellis et al. 1983). Abomasal walls and the proximal small intestine become edematous, hemorrhagic, and sometimes necrotic, and other organs show only degenerative changes (Ellis et al. 1983). There are numerous reports of braxy in calves, and humans with hemolytic uremic syndrome due to *E. coli* O157:H7 may become superinfected by *C. septicum*, developing highly fatal necrotizing enterocolitis or gangrene (Barnham and Weightman 1998). Characteristics of this organism and its virulence factors and pathogenesis are discussed in chapter 12.

CLOSTRIDIUM DIFFICILE

Introduction

The importance of *C. difficile* in humans has prompted detailed study of the organism, its natural history, and its mechanisms of pathogenesis (Borriello and Wilcox 1998). Today, human *C. difficile*-associated disease (CDAD) is responsible for approximately 25% of all cases of antibiotic-associated diarrhea (Bartlett 1992), and clindamycin, ampicillin, amoxicillin, the cephalosporins, and many other antimicrobials are associated with development of CDAD (Johnson et al. 1999). Spores germinate in the colon, and vegetative cells multiply rapidly, filling empty niches and producing toxins (Kelly et al. 1994). Disease may present as diarrhea, colitis, pseudomembranous colitis, or fulminant colitis. This organism has also become well known as a cause of disease in domestic and laboratory animals.

Characteristics

Clostridium difficile has been isolated from many sources, including marine sediment, soil, feces of

normal humans and many domestic and companion animals (Songer 1996), and rarely from septicemias and pyogenic infections (Hirsh et al. 1979). Spores are the means for transmission of *C. difficile*, and they persist for years. Contamination in hospitals is especially significant (Beier et al. 1994), and *C. difficile* can be isolated from environmental surfaces and from the hands, clothing, and equipment of health care workers.

Virulence Factors and Pathogenesis

CDAD occurs in hamsters and guinea pigs after antibiotic treatment (Libby et al. 1982). It is an important cause of diarrhea and fatal necrotizing enterocolitis in horses, with profuse watery diarrhea and dehydration in neonatal foals (Jones et al. 1988). 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, with fatal outcome. *Clostridium difficile* has also become quite important as a cause of nosocomial, often-antimicrobial-associated diarrhea in adult horses.

CDAD has emerged as a cause of enteritis in neonatal pigs. Among piglets 1–7 days old submitted for diagnosis of enteritis, uncomplicated CDAD was found in 35% and mixed infections in a further 20–25% (Songer et al. 2000; Songer 2001). Prevalence in a production system (n = 10 herds) was 47.6% on a per litter basis, and nine of ten herds were infected (Songer 2003).

The case definition for porcine CDAD includes piglets 1–7 days of age, presenting with a history of early onset scours. Litters from gilts and sows are affected, and respiratory distress and sudden death (with hydrothorax and/or ascites) are sometimes reported. Gross pathology usually includes moderate-to-severe mesocolonic edema. Large intestines are frequently filled with pasty-to-watery yellowish feces. Extensive sampling in CDAD-affected herds has revealed that in an infected barn or room, about 67% of litters and 35% of individual pigs are toxin positive (Songer 2003). Piglets without enteric signs may be TcdA/B positive, and defining CDAD solely on the basis of enteric effects may be inadvisable (Waters et al. 1998; Yaeger et al. 2002).

Scattered foci of suppuration are found in the colonic lamina propria. Colonic serosal and mesenteric edema and infiltrations of mononuclear inflammatory cells and neutrophils in the edematous areas are common. There may be segmental erosion of the colonic mucosal epithelium, and exudation of neutrophils and fibrin into the lumen gives rise to the so-called volcano lesions (Songer et al. 2000). Epithelial necrosis becomes diffuse, with ulceration and pseudomembrane formation. CDAD has been reproduced by administration of pure cultures of *C. difficile* (Songer 2003).

Clostridium difficile produces monomeric toxins A (TcdA, 308 kDa, an enterotoxin) and B (TcdB, 270 kDa, a cytotoxin), which are 45% identical at the amino acid level; similarity to the hemolytic and lethal toxins of *C. sordellii* was recognized as early as 1979 (Allo et al. 1979). TcdA and TcdB belong to the structurally homologous family of large clostridial cytotoxins (von Eichel-Streiber et al. 1996; Aktories 1997). They share an N-terminal enzymatic domain and a C-terminal domain containing the so-called clostridial repetitive oligopeptides (CROPs), which are involved in binding to target cells (Aktories 1997; Moncrief et al. 1997). CDAD may be caused by $TcdA^{-}TcdB^{+}$ strains, but tissue damage in typical cases results from the action of both toxins (Kelly et al. 1994; Riegler et al. 1995). The *tcdA* and *tcdB* genes are located about 1 kb apart on a 19 kbp pathogenicity island (Braun et al. 1996).

TcdA and TcdB monoglucosylate, and thereby inactivate, Rho subfamily proteins, low MW GTP-binding proteins involved in regulation of the F-actin cytoskeleton (von Eichel-Streiber et al. 1996). Disaggregation of polymerized actin is followed by opening of tight junctions and cell death. TcdA/B also cause release of proinflammatory mediators and cytokines and activation of the enteric nervous system leading to PMN chemotaxis and fluid secretion (Johnson et al. 1999).

Most knowledge of CDAD pathogenesis derives from study of the human disease, and little is known of the impact of virulence attributes in pigs and other domestic animal species.

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 anti-TcdA antibodies correlate with resolution of clinical disease (Warny et al. 1994). Active immunization against TcdA prevents clinical disease (Ketley et al. 1987; Kim et al. 1987) and anti-TcdA antibodies protect passively (Allo et al. 1979; Leung et al. 1991). Thus, systemic anti-Tcd IgG and IgA, as well as mucosal IgA, are involved in protection against CDAD.

CLOSTRIDIUM SPIROFORME

Clostridium spiroforme is the cause of iota enterotoxemia of rabbits and other laboratory rodents (Carman and Borriello 1984) (table 13.2). 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 (Borriello et al. 1986).

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.

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 are commonly involved in initiation of disease. Diarrhea with perineal staining develops quickly, and death is common. The cecum becomes immensely dilated, with watery contents, and epithelial necrosis is accompanied by pronounced inflammation of the lamina propria. Affected animals have iotalike toxin and high numbers of spores in cecal contents.

ADP-ribosyltransferases (toxin component Ia) of 43 to 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).

CONCLUSION

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 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.

REFERENCES

Aktories, K. 1997. Rho proteins: targets for bacterial toxins. Trends Microbiol. 5:282–288.

Allo, M., J. Silva, R. Fekety, G. D. Rifkin, and H. Waskin. 1979. Prevention of clindamycin-induced colitis in hamsters by *Clostridium sordellii* antitoxin. Gastroenterol. 76:351–355.

Al-Sheikhly, F., and A. Al-Saieg. 1980. Role of coccidia in the occurrence of necrotic enteritis of chickens. Avian Dis. 24:324–333.

Awad, M. M., A. E. Bryant, D. L. Stevens, and J. I. Rood. 1995. Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. Mol. Microbiol. 15:191–202.

Barnham, M., and N. Weightman. 1998. *Clostridium septicum* infection and hemolytic uremic syndrome. Emerg. Infect. Dis. 4:321–324.

Bartlett, J. G. 1992. Antibiotic-associated diarrhea. Clin. Infect. Dis. 15:573–581.

Beier, R., G. Amtsberg, and M. Peters. 1994. Bacteriological investigation of the occurrence and significance of *Clostridium difficile* in horses. Pferdeheilkunde 10:3–7.

Bhown, A. S., and A. F. S. A. Habeeb 1977. Structural studies of epsilon-prototoxin of *Clostridium perfringens* type D. Localisation of the site of tryptic scission necessary for activation to epsilon-toxin. Biochem. Biophys. Res. Commun. 78:889–896.

Billington, S. J. 2003. Unpublished data.

Billington, S. J., E. U. Wieckowski, M. R. Sarker, D. M. Bueschel, J. Glenn Songer, and B. A. McClane. 1998. *Clostridium perfringens* type E animal

enteritis isolates with highly conserved, silent enterotoxin gene sequences. Infect. Immun. 66:4531–4536.

Blackwell, T. E., D. G. Butler, J. F Prescott, and B. P. Wilcock. 1991. Differences in signs and lesions in sheep and goats with enterotoxemia induced by intraduodenal infusion of *Clostridium perfringens* type D. Am. J. Vet. Res. 52:1147–1152.

Borriello, S. P., and M. H. Wilcox. 1998. *Clostridium difficile* infections of the gut: the unanswered questions. J. Antimicrobial Chemotherapy 41 Suppl C: 67–69

Borriello, S. P., and R. J. Carman. 1985. Clostridial diseases of the gastrointestinal tract in animals. In Clostridia in gastrointestinal disease. P. Borriello (ed.). Boca Raton, FL: CRC Press, Inc.

Borriello, S. P., H. A. Davies, and R. J. Carman. 1986. Cellular morphology of *Clostridium spiroforme*. Vet. Microbiol. 11:191–195.

Branton, S. L., F. N. Reece, and W. M. Hagler, Jr. 1987. Influence of a wheat diet on mortality of broiler chickens associated with necrotic enteritis. Poult. Sci. 66:1326–1330.

Braun, V., T. Hundsberger, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber. 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. Gene 181:29–38.

Bueschel, D. M , B. H. Jost, S. J. Billington, H. T. Trinh, and J. G. Songer. 2003. Prevalence of *cpb2*, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. Vet. Microbiol. 94:121–129.

Bueschel, D. M., R. Walker, L. Woods, J. Kokai-Kun, B. McClane, and J. Glenn Songer. 1998. Enterotoxigenic *Clostridium perfringens* type A necrotic enteritis in a foal. J. Amer. Vet. Med. Assoc. 213:1305–1307.

Carman, R. J., and S. P. Borriello. 1984. Infectious nature of *Clostridium spiroforme*-mediated rabbit enterotoxaemia. Vet. Microbiol. 9:497–502.

Cole, S. T., and B. Canard. 1997. Structure, organisation and evolution of the genome of *Clostridium perfringens*. In The clostridia: molecular biology and pathogenesis. J. I. Rood, B. McClane, J. G. Songer, and R. W. Titball (eds.). London: Academic Press, pp. 49–63.

Collins, J. E., M. E. Bergeland, D. Bouley, A. L. Ducommun, D. H. Francis, and P. Yeske. 1989. Diarrhea associated with *Clostridium perfringens* type A enterotoxin in neonatal pigs. J. Vet. Diagn. Invest. 1:351–353.

Ellis, T. M., A. R. Gregory, and G. D. Logue. 1991. Evaluation of a toxoid for protection of rabbits against enterotoxaemia experimentally induced by trypsin-activated supernatant of *Clostridium spiroforme*. Vet. Microbiol. 28:93–102.

Ellis, T. M., J. B. Rowe, and J. M. Lloyd. 1983. Acute abomasitis due to *Clostridium septicum* infection in experimental sheep. Aust. Vet. J. 60:308–309.

Estrada-Correa, A. E., and D. J. Taylor. 1989. Porcine *Clostridium perfringens* type A spores, enterotoxin, and antibody to enterotoxin. Vet. Rec. 124:606–610.

Finnie, J. W. 1984. Histopathological changes in the brain of mice given *Clostridium perfringens* type D epsilon toxin. J. Comp. Pathol. 94:363–370.

Finnie, J. W., and P. Hajduk. 1992. An immunohistochemical study of plasma albumin extravasation in the brain of mice after the administration of *Clostridium perfringens* type D epsilon toxin. Aust. Vet. J. 69:261–262.

Fukata, T., Y. Hadate, E. Baba, and A. Arakawa. 1991. Influence of bacteria on *Clostridium perfringens* infections in young chickens. Avian Dis. 35:224–247.

Garmory, H. S., N. Chanter, N. P. French, D. M. Bueschel, J. G. Songer, and R. W. Titball. 2000. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiol. Infect. 124:61–67.

Gibert, M., C. Jolivet-Renaud, and M. R. Popoff. 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene 203: 65–73.

Gill, D. M. 1982. Bacterial toxins: a table of lethal amounts. Microbiol. Rev. 46:86–94.

Hanna, P. C., A. P. Wnek, and B. A. McClane. 1989. Molecular cloning of the 3' half of the *Clostridium perfringens* enterotoxin gene and demonstration that this region encodes receptor-binding activity. J. Bacteriol. 171:6815–6820.

Hatheway, C. L. 1990. Toxigenic clostridia. Clin. Microbiol. Rev. 3:66–98.

Havard, H. L., S. E. C. Hunter, and R. W. Titball. 1992. Comparison of the nucleotide sequence and development of a PCR test for the epsilon toxin gene of *Clostridium perfringens* type B and type D. FEMS Microbiol. Lett. 97:77–82.

Herholz, C., R. Miserez, J. Nicolet, J. Frey, M. Popoff, M. Gibert, H. Gerber, and R. Straub. 1999. Prevalence of beta2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. J. Clin. Microbiol. 37:358–61.

Hirsh, D. C., E. L. Biberstein, and S. S. Jang. 1979. Obligate anaerobes in clinical veterinary practice. J. Clin. Microbiol. 10:188–191.

Hunter, S. E. C., I. N. Clarke, and R. W. Titball. 1992. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon toxin gene and its expression in *Escherichia coli*. Infect. Immun. 60:102–110.

Hunter, S. E. C., J. E. Brown, P. C. F. Oyston, J. Sakurai, and R. W. Titball. 1993. Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma-toxin, and leukocidin of *Staphylococcus aureus*. Infect. Immun. 61:3958–3965.

Johannsen, U., P. Arnold, B. Köhler, and H. J. Selbitz. 1993a. Studies into experimental *Clostridium perfringens* type A enterotoxaemia of suckled piglets: Experimental provocation of the disease by *Clostridium perfringens* type A intoxication and infection. Monatsh für Veterinaermed 48:129–136.

Johannsen, U., S. Menger, P. Arnold, B. Köhler, and H. J. Selbitz. 1993b. Experimental *Clostridium perfringens* type A enterotoxaemia in unweaned piglets. Monatsh für Veterinarmed. 48:267–273.

Johnson, E. A. 1997. Extrachromosomal virulence determinants in the clostridia. In The clostridia: molecular biology and pathogenesis. J. I. Rood, B. A. McClane, J. G. Songer, and R. W. Titball (eds.). London: Academic Press, pp. 35–48.

Johnson, S., M. H. Samore, and K. A. Farrow. 1999. Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. N. Engl. J. Med. 341:1645–1651.

Jones, R. L., W. S. Adney, A. F. Alexander, R. K. Shideler, and J. L. Traub-Dargatz. 1988. Hemorrhagic necrotizing enterocolitis associated with *Clostridium difficile* infection in four foals. J. Am. Vet. Med. Assoc. 193:76–79.

Katz, L., J. T. Lamont, J. S. Trier, E. B. Sonnenblick, S. W. Rothman, S. A. Broitman, and S. Reith. 1978. Experimental clindamycin-associated colitis in rabbits. Gastroenterology 74:246.

Kelly, C. P., C. Pothoulakis, and J. T. LaMont. 1994. *Clostridium difficile* colitis. N. Engl. J. Med. 330:257–262.

Kelly, C. P., C. Pothoulakis, J. Orellana, and J. T. LaMont. 1992. Human colonic aspirates containing immunoglobulin A antibody to *Clostridium difficile* toxin A inhibit toxin A-receptor binding. Gastroenterology 102:35–40.

Kennedy, K. K., S. J. Norris, W. H. Beckenhauer, and R. G. White. 1977. Vaccination of cattle and sheep with combined *Clostridium perfringens* type C and type D toxoid. Am. J. Vet. Res. 38:1515–1518.

Ketley, J. M., T. J. Mitchell, D. C. Candy, D. W. Burdon, and J. Stephen. 1987. The effects of *Clostridium difficile* crude toxins and toxin A on ileal and colonic loops in immune and non-immune rabbits. J. Med. Microbiol. 24:41–52

Kim, P. H., J. P. Iaconis, and R. D. Rolfe. 1987. Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect. Immun. 55:2984–2992.

Kink, J. A., and J. A. Williams. 1998. Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. Infect. Immun. 66:2018–2025.

Kruth, S. A., J. F. Prescott, M. K. Welch, and M. H. Brodsky. 1989. Nosocomial diarrhea associated with enterotoxigenic *Clostridium perfringens* infection in dogs. J. Am. Vet. Med. Assoc. 195:331–334.

Kubo, M., and H. Watase. 1985. Electron microscopy of *Clostridium perfringens* in the intestine of neonatal pigs with necrotic enteritis. Japan J. Vet. Sci. 47:497–501.

Lawrence, G. W., D. Lehmann, G. Anian, C. A. Coakley, G. Saleu, M. J. Barker, and M. W. Davis. 1990. Impact of active immunisation against enteritis necroticans in Papua, New Guinea. Lancet 336: 1165–1167.

Leung, D. Y., C. P. Kelly, and M. Boguniewicz. 1991. Treatment with intravenously administered gamma globulin of chronic relapsing colitis induced by *Clostridium difficile* toxin. J. Pediatr. 118:633–637.

Libby, J. M., B. S. Jortner, and T. D. Wilkins. 1982. Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. 36:822–829.

Liu, J. W., A. G. Porter, B. Y. Wee, and T. Thanabalu. 1996. New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. Appl. Environ. Microbiol. 62:2174–2176.

Marks, S. L., E. J. Kather, P. H. Kass, and A. C. Melli. 2002. Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. J. Vet. Intern. Med. 16:533–540.

McDonel, J. L. 1979. The molecular mode of action of *Clostridium perfringens* enterotoxin. Am. J. Clin. Nutr. 32:210–218.

Mills, K. W., J. L. Johnson, R. L. Jensen, L. F. Woodard, and A. R. Doster. 1990. Laboratory findings associated with abomasal ulcers/tympany in range calves. J. Vet. Diagn. Invest. 2:208–212.

Miyata, S., J. Minami, E. Tamai, O. Matsushita, S. Shimamoto, and A. Okabe. 2002. *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of MDCK cells and rat synaptosomes. J. Biol. Chem. 277:39463–39468.

Miyata, S., O. Matsushita, J. Minami, S. Katayama, S. Shimamoto, and A. Okabe. 2001. Cleavage of a C-terminal peptide is essential for heptamerization

of *Clostridium perfringens* epsilon-toxin in the synaptosomal membrane. J. Biol. Chem. 276:13778–13783.

Moncrief, J. S., L. A. Barroso, and T. D. Wilkins. 1997. Positive regulation of *Clostridium difficile* toxins. Infect. Immun. 65:1105–1108.

Nagahama, M., and J. Sakurai. 1992. High-affinity binding of *Clostridium perfringens* epsilon-toxin to rat brain. Infect. Immun. 60:1237–1240.

Nagahama, M., S. Ochi, and J. Sakurai. 1998. Assembly of *Clostridium perfringens* epsilon-toxin on MDCK cell membrane. J. Nat. Toxins 7:291–302.

Niilo, L. 1986. Experimental production of hemorrhagic enterotoxemia by *Clostridium perfringens* type C in maturing lambs. Can. J. Vet. Res. 50:32–35.

———. 1988. *Clostridium perfringens* type C enterotoxemia. Can. Vet. J. 29:658–664.

Okazaki, Y., M. Inage, I. Iwabuchi, T. Suzuki, N. Okada, K. Watanabe, and T. Yamamoto. 1993. Mass outbreak of necrotic enteritis in newborn piglets caused by *Clostridium perfringens* type C on a pig farm. J. Japan. Vet. Med. Assoc. 46:214–217.

Olubunmi, P. A., and D. J. Taylor. 1985. *Clostridium perfringens* type A in enteric diseases of pigs. Trop. Vet. 3:28–33.

Oyston, P. C. F., D. W. Payne, H. L. Havard, E. D. Williamson, and R. W. Titball. 1998. Production of a non-toxic site-directed mutant of *Clostridium perfringens* epsilon-toxin which induces protective immunity in mice. Microbiology 144:333–341.

Payne, D. L., E. D. Williamson, and R. W. Titball. 1997. The *Clostridium perfringens* epsilon-toxin. Rev. Med. Microbiol. 8:S28–S30.

Payne, D. W., E. D. Williamson, H. Havard, N. Modi, and J. Brown. 1994. Evaluation of a new cytotoxicity assay for *Clostridium perfringens* type D epsilon toxin. FEMS Microbiol. Lett. 116:161–167.

Percival, D. A., A. D. Shuttleworth, E. D. Williamson, and D. C. Kelly. 1990. Anti-idiotype antibody-induced protection against *Clostridium perfringens* type D. Infect. Immun. 58:2487–2492.

Perelle, S., M. Gibert, P. Boquet, and M. R. Popoff. 1993. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. 61:5147–5156.

Petit, L., E. Maier, M. Gibert, M. R. Popoff, and R. Benz. 2001. *Clostridium perfringens* epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid bilayers. J. Biol. Chem. 276:15736–15740.

Petit, L., M. Gibert, D. Gillet, C. Laurent-Winter, P. Boquet, and M. R. Popoff. 1997. *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. J. Bacteriol. 179:6480–6487.

Popoff, M. R., and P. Boquet. 1988. *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. Biochem. Biophys. Res. Commun. 152:1361–1368.

Prescott, J. F. 1979. The prevention of experimentally-induced necrotic enteritis in chickens by avoparcin. Avian Dis. 23:1072–1074.

Ratz, F., E. Szabo, and E. Szentmiklossy. 1989. Observations on the pathomorphology of necrotic enteritis in chickens. Magyar Allatorvosok Lapja 44:133–140.

Riegler, M., C. Pothoulakis, and G. Hamilton. 1995. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. J. Clin. Invest. 95:2004–2011.

Ripley, P. H., and A. F. Gush. 1983. Immunization schedule for the prevention of infectious necrotic enteritis caused by *Clostridium perfringens* type C in piglets. Vet. Rec. 112:201–202.

Roeder, B. L., M. M. Chengappa, T. G. Nagaraja, T. B. Avery, and G. A. Kennedy. 1987. Isolation of *Clostridium perfringens* from neonatal calves with ruminal and abomasal tympany, abomasitis, and abomasal ulceration. J. Am. Vet. Med. Assoc. 190:1550–1555.

Sakurai, J., Y. Fujii, K. Dezaki, and K. Endo. 1984. Effect of *Clostridium perfringens* beta toxin on blood pressure of rats. Microbiol. Immunol. 28: 23–31.

Schamber, G. J., I. E. Berg, and J. R. Molesworth. 1986. Braxy or bradsot-like abomasitis caused by *Clostridium septicum* in a calf. Can. Vet. J. 27:194.

Shatursky, O., R. Bayles, M. Rogers, B. H. Jost, J. G. Songer, and R. K. Tweten. 2000. *Clostridium perfringens* beta-toxin forms potential-dependent, cation selective channels in lipid bilayers. Infect. Immun. 68: 5546–5551.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. U S A. 99:996–1001.

Shortt, S. J., R. W. Titball, and C. D. Lindsay. 2000. An assessment of the *in vitro* toxicology of *Clostridium perfringens* type D epsilon-toxin in human and animal cells. Hum. Exp. Toxicol. 19:108–116.

Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9: 216–234.

———. 2001. A practical approach to clostridial infections. In emerging infections and molecular pathogenesis. Ann. Mtg. Amer. Coll. Vet. Pathol. Salt Lake City, UT, December 2–5.

———. 2003. Unpublished data.

Songer, J. G., K. W. Post, D. J. Larson, B. Helen Jost, and R. D. Glock. 2000. Enteric infection of neonatal swine with *Clostridium difficile*. Swine Health Produc. 8: 185–189.

Steinthorsdottir, V., H. Halldorsson, and O. S. Andresson. 2000. *Clostridium perfringens* beta-toxin forms multimeric transmembrane pores in human endothelial cells. Microb. Pathol. 28:45–50.

Stevens, D. L., B. E. Troyer, D. T. Merrick, J. E. Mitten, and R. D. Olson. 1988. Lethal effects and cardiovascular effects of purified a and q toxins. J. Infect. Dis. 157:272–279.

Thanabalu, T., and Porter, A. G. 1996. A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. Gene 170:85–89.

Tsutsui, K., J. Minami, O. Matsushita, S. Katayama, Y. Taniguchi, S. Nakamura, M. Nishioka, and A. Okabe. 1995. Phylogenetic analysis of phospholipase C genes from *Clostridium perfringens* types A to E and *Clostridium novyi*. J. Bacteriol. 177:7164–7170.

Tweten, R. K. 2002. Personal communication

Uzal, F. A., and W. R. Kelly. 1997. Effects of the intravenous administration of *Clostridium perfringens* type D epsilon toxin on young goats and lambs. J. Comp. Pathol. 116:63–71.

von Eichel-Streiber, C., P. Boquet, M. Sauerborn, and M. Thelestam. 1996. Large clostridial cytotoxins: a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol. 4:375–382.

Warny, M., J. P. Vaerman, V. Avesani, and M. Delmee. 1994. Human antibody response to *Clostridium difficile* toxin A in relation to clinical course of infection. Infect. Immun. 62:384–389.

Waters, E. H., J. P. Orr, E. G. Clark, and C. M. Schaufele. 1998. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. J. Vet. Diagn. Invest. 10:104–108.

Williamson, E. D., and R. W. Titball. 1993. A genetically engineered vaccine against the alpha-toxin of *Clostridium perfringens* protects mice against experimental gas gangrene. Vaccine 11:1253–1258.

Wnek, A. P., and B. A. McClane. 1989. Identification of a 50,000 mr protein from rabbit brush border membranes that binds *Clostridium perfringens* enterotoxin. Biochem. Biophys. Res. Commun. 112:1094–1105.

Worthington, R. W., and M. S. G. Mülders. 1975. Effect of *Clostridium perfringens* epsilon toxin on the blood brain barrier of mice. Onderstepoort J. Vet. Res. 42:25–27.

———. 1977. Physical changes in the epsilon prototoxin molecule of *Clostridium perfringens* during enzymatic activation. Infect. Immun. 18:549–551.

Yaeger, M., N. Funk, and L. Hoffman. 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 14:281–287.

14
Salmonella

S. J. Libby, T. A. Halsey, C. Altier, J. Potter, and C. L. Gyles

Salmonella is a zoonotic pathogen that is capable of colonizing most vertebrates. Infection due to *Salmonella* in humans and domestic animals remains a serious, worldwide problem, with outcomes that range from asymptomatic carriage to the more serious systemic infection that may result in high mortality. Infection in animals is of importance because of the direct economic consequences of disease attributable to mortality and morbidity. Of even greater importance are the human health consequences of salmonellosis acquired by direct or indirect contact with animals, which constitute a vast reservoir of these organisms. Increasing levels of drug resistance in *Salmonella* recovered from animals is also of considerable concern because of the implications for treatment of animal and human disease. These concerns have resulted in intense efforts in many countries to substantially reduce the level of infection in animals.

CLASSIFICATION AND NOMENCLATURE

The genus *Salmonella* encompasses a large taxonomic group, with over 2,463 recognized serotypes (Popoff and Le Minor 1997; Popoff et al. 2000). Classification of *Salmonella* has been traditionally based on the Kauffmann-White scheme in which assignment to serotypes is based on H (flagellar) and O (somatic) antigens (Kauffmann 1966). A capsular polysaccharide (the Vi antigen) is present on *Salmonella* Typhi and a few other serotypes of *Salmonella,* including *S.* Dublin.

Newer methodologies have given a clearer picture of the relationships among groups of *Salmonella* (Brenner et al. 2000). Genetic distance measured by multilocus enzyme electrophoresis and DNA-DNA hybridization studies showed that all groups of *Salmonella*, except one, belonged to the same species (LeMinor and Popoff 1987; Reeves et al. 1989). These findings have resulted in identification of two species of *Salmonella*, namely *S. enterica* and *S. bongori*, with all but 20 of the 2,463 serotypes of *Salmonella* belonging to the species *S. enterica*. The species *S. enterica* is divided into six subspecies, called *enterica, salamae, arizonae, diarizonae, houtenae*, and *indica*. Almost 60% of the serotypes of *Salmonella* belong to *S. enterica* subspecies *enterica* and live in avian and mammalian hosts. It is this subspecies that accounts for almost all the serotypes that cause disease in domestic animals and humans. Fewer than 50 serotypes account for the vast majority of cases of disease in animals and humans. The other *Salmonella* live in cold-blooded vertebrates (mostly reptiles) and the environment. An important difference between *S. enterica* and *S. bongori* serotypes is that the latter lack the pathogenicity island SPI-2.

Genomic analyses are contributing to a clearer understanding of relationships among the various serotypes (Chan et al. 2003). Chan and coworkers (2003) hybridized genomic DNA of 24 *Salmonella* strains, consisting of 12 *S. enterica* serovars and 2 *S. bongori* strains, to a microarray of the 4,169 ORFs of *S.* Typhimurium strain SL1344. They determined that 54% of the ORFs were shared by all of the serovars and considered these to be core genes. In addition to genes that were involved in typical cell functions, operons for flagella, certain fimbriae, hydrogen sulfide production, and LPS biosynthesis were conserved. Certain virulence-related genes, such as those of the SPI-1 pathogenicity island, were also present in the core genes. It is interesting that elements of the SPI-2 PAI were detected in the

S. bongori strains. The data placed the Arizona isolate, with 73.5 to 77.5% of the SL1344 genome hybridizing with it, in a more distant relationship to subspecies I, than *S. bongori* with 83% of the SL1344 genome hydridizing.

Isolates may be identified at the subserotype level by phage typing, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), biotyping, drug resistance, and plasmid profile analysis. Phage typing is very valuable but is limited to a few serotypes for which typing schemes have been developed, including serotypes Typhimurium, Typhi, Enteritidis, Heidelberg, and Schottmuelleri. Identification at this level is important for epidemiologic studies and for tracing sources of disease outbreaks.

Salmonella nomenclature is often confusing because it lacks uniformity (Brenner et al. 2000). Unresolved issues include the name of the type species and the system of naming members of the genus. Given the new classification, it seemed logical that the name of the type species be *S. enterica.* However, attempts to have the type species officially changed from *S. choleraesuis* have been unsuccessful. Nonetheless, many organizations, including the CDC in Atlanta, Georgia, and the American Society for Microbiology (ASM), have adopted the two-species concept with *S. enterica* as the type species and with the following nomenclature. The individual serotype names are retained but are not italicized. For example, *Salmonella enterica* serotype Typhimurium is used in place of *Salmonella typhimurium* for the first citation, then *Salmonella* serotype Typhimurium (or *Salmonella* Typhimurium) is used in subsequent references.

BIOCHEMICAL CHARACTERISTICS AND BACTERIAL ISOLATION

Salmonella are facultatively anaerobic, non-spore-forming, gram-negative rods that are members of the *Enterobacteriaceae* family. Most *Salmonella* isolates grow optimally at 37°C and all but two serotypes (Pullorum and Gallinarum) are motile by means of peritrichous flagella. *Salmonella* are non–lactose fermenting, with the exception of *S. enterica* subspecies *arizonae* and *diarizonae*. Typically, *Salmonella* are negative in the indole, Voges-Proskauer, and urease tests and positive in the methyl red, citrate utilization, H_2S production, lysine decarboxylase, and ornithine decarboxylase tests. The two species of *Salmonella* and the six subspecies of *S. enterica* may be differentiated on the basis of biochemical tests.

Recovery of *Salmonella* from fecal or environmental samples is often required to diagnose disease or to identify sources of infection for animals. In either case, the sample usually has large numbers of non-*Salmonella* bacteria, which make it difficult to isolate colonies of *Salmonella* organisms by direct plating. It is therefore common practice not only to plate such samples directly on selective and differential agar media, but also to enrich for *Salmonella* in selective broth media prior to plating on selective and differential agar media. Once potential *Salmonella* colonies are identified, a battery of biochemical tests is used to determine whether the isolate is *Salmonella*. Polymerase chain reaction (PCR) amplification methods that target genes specific to *Salmonella* are also available for identification of this genus. The *Salmonella* isolate is then sent to a specialized laboratory to have the serotype determined.

HOST SPECIFICITY

Host adaptation occurs in *Salmonella* at both the serotype level and at the level of phage type within a serotype. Clear associations have been demonstrated between *S. enterica* serovars Typhi, Paratyphi A, and Sendai and humans; *S.* Dublin and cattle; *S.* Choleraesuis and swine; *S.* Pullorum and Gallinarum and poultry; *S.* Abortusequi and horses; *S.* Abortusovis and sheep; and *S.* Arizona and reptiles. Interestingly, genomic comparisons of *Salmonella* serovars showed that serovars Typhi, Paratyphi A, and Sendai clustered together, suggesting that they share genetic features (Chan et al. 2003). *S.* Typhi appears to have an exclusive association with humans, but the other serotypes may cause disease in hosts other than those to which they have adapted. For example, even serotype Gallinarum may sometimes cause disease in mammalian host, and *S.* Dublin, *S.* Choleraesuis, and *S.* Arizona are associated with disease in humans and other hosts to which the serotypes are not adapted. The vast majority of serotypes of *Salmonella enterica* show no host adaptation. *S.* Typhimurium and *S.* Enteritidis are frequently isolated from a variety of vertebrates with and without clinical disease and may be considered the least host-adapted serotypes. Typically, host-adapted serotypes cause severe systemic disease in adult as well as young hosts, whereas the unadapted serotypes are associated with enteric disease primarily in young hosts. It is

interesting that *S.* Typhimurium, which causes a predominantly gastrointestinal illness in a wide range of hosts, causes a typhoidlike disease in mice. *S.* Abortusovis causes only a mild disease in adult sheep.

Host adaptation may occur among subtypes of a serotype (Rabsch et al. 2002). Examination of the association of various phage types of *S.* Typhimurium with animal hosts showed that certain phage types had a very narrow host range, others had a narrow host range, and the majority had a broad host range. Phage types DT2 and DT99 were adapted to pigeons in which they caused severe disease. These phage types were rarely recovered from other host species, and pigeons were rarely infected with other phage types of *S.* Typhimurium. Phage types DT8 and DT46 were isolated primarily from ducks, and phage type DT40 mostly from wild birds, but these animal hosts also become infected with a wide range of other phage types of *S.* Typhimurium. Most phage types of *S.* Typhimurium, however, affected humans and a large number of animal species. It was suggested that host adaptation may be caused by phage-mediated transfer of virulence genes that give rise to host specificity (Rabsch et al. 2002).

Baumler et al. (1998) observed that the non-host-adapted serotypes such as Typhimurium and Enteritidis are not well equipped to contend with the immune systems of mature hosts and tend to cause disease primarily in young animals. These researchers also noted that host-adapted serotypes such as Typhi, Choleraesuis, Dublin, and Pullorum/Gallinarum typically cause severe systemic disease rather than the milder enteric disease caused by the non-host-adapted serotypes. These authors and others (Kingsley and Baumler 2002) have suggested that linkage between transmissibility and high virulence may have provided evolutionary pressure that selected for high virulence during the development of host adaptation. The transmissibility factor came from the chronic carrier state that arose frequently following systemic infection. *S.* Abortusovis appears to depend on infection of the fetus and contamination of the environment at birth, rather than virulence for the adult sheep, for transmission.

Fimbrial operons of *S.* Typhi were examined to determine whether there is a fimbrial basis for host species specificity of this serotype (Townsend et al. 2001). However, the authors could not identify a fimbrial operon that was uniquely present in *S.* Typhi.

Liu and colleagues (2002) reported an interesting study in which they mapped the genome of *S.* Pullorum and compared it with the genome of *S.* Typhimurium. The *S.* Pullorum genome showed major rearrangements compared with *S.* Typhimurium. Many deletions and insertions were identified; these included an insertion of 157-kb, which the authors postulated has thrown the genome out of balance. They noted variations in the genomes of various strains of *S.* Pullorum and suggested that the serotype is attempting to rebalance its genome. They also suggested that host specificity may be due to deletion of genes that may be present in broad-host-range *Salmonella*.

Bacteriophages may also contribute to host species adaptation by *Salmonella*. Pelludat and coworkers (2003) remarked that converting phages like the lambdoid GIFSY phages and the P2-like SopEϕ may contribute to host adaptation and emergence of new epidemic strains through reassortment of repertoires of virulence factors. The SopEϕ phage encodes the SopE protein, which is secreted by the SPI-1 type III secretion (TTSS) system and which is an important virulence factor. Lysogenic conversion by this phage appears to have been an important aspect of the emergence of the epidemic serovar Typhimurium strain DT49/DT204. There is evidence that *sopE* has been transferred between a variety of P2-like bacteriophages, and it is likely that exchange of virulence factors among *Salmonella* may be enhanced by the movement of virulence factors between phages (Pelludat et al. 2003).

Survival in macrophages may be associated with host adaptation. Thus, *S.* Typhimurium survived better in murine compared with human macrophages, and *S.* Typhi survived better in human compared with murine macrophages (Knodler and Finlay 2001).

DISEASES CAUSED BY *SALMONELLA*

Sources of Infection

Salmonella can inhabit the gastrointestinal tract of a wide variety of vertebrates, often causing no apparent ill effects. The asymptomatic carrier animal, which may be domestic or wild mammal or bird, commonly excretes *Salmonella* and serves as an important source of herd or flock contamination. Humans rarely are carriers of nontyphoidal *Salmonella*, but 1–4% of humans who develop typhoid fever develop chronic carriage and shed the organisms for the rest of their lives. Contaminated feed, water, and environment are also common

sources of infection for animals. Feeds of animal origin, such as fish meal, bone meal, meat meal, and milk, are all potential sources of the organism. Rodents and wild birds present a severe challenge to biocontainment schemes that seek to keep *Salmonella* out of animal-rearing facilities. The ability of the organism to survive in manure, soil, and the environment for many months facilitates transmission to animals. Animals become infected by ingestion or inhalation of the bacteria. *S.* Typhimurium and *S.* Enteritidis are among the serotypes most frequently isolated from infected human, animal, or environmental sources.

A major effort to control *Salmonella* infection in production facilities is currently being attempted around the world. In several countries, the HAACP (Hazard Analysis and Critical Control Point) methodology is being used at meat-processing facilities to control pathogen introduction into the food supply. In Denmark, there has been a national surveillance and control program for *Salmonella* in pigs since 1995. Surveillance is based on an enzyme-linked immunosorbance assay (ELISA) applied to meat-juice samples. The program involves all stages of production and has been credited with reducing the prevalence of *Salmonella* in Danish pork from 3.5% in 1993 to 0.7% in 2000 (Nielsen et al. 2001). Human cases of pork-associated salmonellosis declined from 1,144 to 166 over the same time period.

Animal Diseases Caused by *Salmonella*

It is convenient to consider two major forms of disease, namely, enteritis and septicemia, but affected animals may show one or both of these. *Salmonella* infection can also result in abortion as a result of systemic infection. In some regions, for example, *S.* Abortusovis is an important cause of abortion in sheep, usually in the absence of other clinical symptoms. *S.* Dublin is also associated with ovine abortion, but enteritis is also observed.

Animals that are acutely infected with *Salmonella* develop depression, diarrhea, fever, and inappetence. Most affected animals do not die from the infection; rather, they develop symptoms that eventually resolve. Those animals that do succumb to *Salmonella* infection will generally die of systemic infection or from dehydration and electrolyte loss due to profound diarrhea. The outcome of the disease will depend in part on the serotype that is the cause of the infection. *S.* Dublin infection in calves and *S.* Choleraesuis infection in piglets often result in a very severe invasive disease with high mortality. In contrast, *S.* Typhimurium infection often results in high morbidity but low mortality. The age of the animal is another important factor, as young animals tend to develop more severe disease compared with adults. Factors that compromise the immune defense of the host or alter its intestinal flora, such as transport, disease, surgery, immunosuppressive drugs, and oral antibiotics, increase susceptibility to salmonellosis.

Infection of swine with *Salmonella* has been the subject of intense research and surveillance, as pork and pork products frequently serve as sources of human *Salmonella* infection. Many serotypes have been isolated from swine. In the United States, the most frequent serotypes are Typhimurium, Copenhagen, Derby, Newport, Agona, and Choleraesuis var kunzendorf. In Europe, *S*. Typhimurium is the dominant serotype but *S.* Derby is also encountered frequently.

S. Choleraesuis has been implicated as a swine-adapted serotype, and infection with this serotype results in a high mortality and low morbidity. Experimental infection of pigs with *S.* Choleraesuis, *S.* Typhimurium, and *S.* dublin showed that systemic disease was produced by *S.* Choleraesuis, compared with enteric disease by *S.* Typhimurium and no disease by *S.* Dublin (Watson et al. 2000). Infection with *S.* Choleraesuis often causes a fatal systemic disease in young pigs but not in adults. Other serotypes may result in long-term asymptomatic carriage, with carriers becoming a source of herd infection. The carrier animals are often culture negative for *Salmonella* on the farm but develop severe enteritis when stressed during shipping. Stress-induced *Salmonella* enteritis creates a major problem through the contamination of shipping equipment and holding areas resulting in preslaughter transmission of *Salmonella* to noninfected animals.

Salmonella infection in cattle is often non–life threatening and results in animals that shed the bacteria for variable lengths of time. The most prevalent serotypes recovered from cattle are *S.* Typhimurium and *S.* Dublin, but many other serotypes have been isolated. *S.* Dublin in cattle was thought to be confined to the western United States but now is frequently isolated in the northeastern United States (McDonough et al. 1999). Adult dairy cattle that are infected serve as carriers for many years and are a potential source for contamination of milk. These carrier animals become a source of continued herd infection. Carriage tends to be more prolonged following infection with *S.* Dublin compared with *S.* Typhimurium.

Infection of calves usually occurs after the first 1–2 weeks of life and results in more serious disease than occurs in adults. Calves that are nursing or being maintained on milk replacer are frequently infected but show variable levels of disease symptoms. *S.* Typhimurium causes primarily an enteric disease in calves, whereas *S.* Dublin causes a primarily septicemic disease, sometimes with localizations that manifest as polyarthritis, osteomyelitis, pneumonia, or meningoencephalitis. The infected calves may die due to dehydration as a result of severe diarrhea or they may develop lethal pneumonia. It is interesting that most calves are able to resolve the infection but do become carrier animals. Reports of *S.* Dublin and *S.* Muenster causing abortion in dairy cattle are not infrequent. It is often difficult to determine the source of *Salmonella* contamination—whether the herd acquired infection directly from a carrier animal already present, from introduced animals, fomites, or a contaminated environment.

In poultry, *S.* Pullorum and *S.* Gallinarum infections were a serious problem in the early 1900s and prevented the development of a significant poultry production industry at that time. Fowl typhoid caused by *S.* Gallinarum caused a serious economic problem in poultry production facilities world wide. When appropriate testing and control methods were developed to control the spread of this serotype, the poultry industry finally became established. *S.* Pullorum and *S.* Gallinarum continue to be important causes of septicemic disease in young chickens and in chickens and turkeys, respectively, in many countries. Despite elimination of infections with Pullorum and Gallinarum in several regions of the world, *Salmonella* in poultry remains a primary source of human infection through contaminated eggs, chicken and turkey meat, and processed food from chickens and turkeys. Chickens and turkeys are frequently colonized with one or more serotypes of *Salmonella* including Typhimurium, Enteritidis, Heidelberg, Infantis, Montevideo, and Anatum.

Chickens 1–2 days old are susceptible to experimental infection with *Salmonella* and usually succumb to systemic infection. Chickens older than 4 weeks rarely develop salmonellosis but remain infected and excrete *Salmonella*. *S.* Enteritidis phage type 4 causes a lethal infection in chickens. The molecular basis for the particular virulence of this phage group is unknown. In turkeys, infection with *S. enterica* subspecies *arizonae* is a substantial problem. Vertical transmission of a few serotypes, notably *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg, occurs. Eggs have been an important source of *S.* Enteritidis infections in humans (Rabsch et al. 2001), and special programs to reduce eggborne salmonellosis have met with some degree of success.

Attempts to control *Salmonella* infection and carriage by vaccination and antibiotic treatment of chickens have not produced significant results. An alternative approach has been the use of competitive exclusion, which was first proposed by Nurmi (Rantala and Nurmi 1973). In this method, newly hatched chickens are given a mixture of adult chicken cecal bacterial flora to decrease the time required for normal gastrointestinal colonization. By decreasing the time for the normal flora establishment, the likelihood of pathogen colonization is decreased. The mechanism is not completely understood, but the possible bases for the protective effect of the adult flora include occupation of sites on the intestinal surface where pathogens could bind, production of antibacterial compounds, and competition for nutrients.

Salmonella infection in horses is not common but it is an important infectious cause of diarrhea in horses because of morbidity, mortality, and persistence in groups of valuable animals (Spier 1993; Murray 1996). Infection with *Salmonella* has become a significant problem in veterinary teaching hospitals, particularly in horses that have undergone surgery and/or been treated with antibiotics. Not surprisingly, the teaching hospital environment is frequently contaminated with *Salmonella,* and horses that suffer stresses through transportation, illness, anesthesia, and surgery are likely to develop salmonellosis following exposure to relatively low numbers of bacteria. Clusters of cases appear to represent primarily nosocomial infections rather than activation of latent infections, although *Salmonella* are repeatedly introduced into the hospital setting by carrier animals. Private veterinary clinics that treat horses are also at risk for outbreaks of salmonellosis, but the frequency is less than in the teaching hospitals, likely because of having lower numbers of animals and fewer severely ill patients.

In any setting, foals tend to be more susceptible than adults and often develop septicemic disease. Adult horses often develop diarrhea accompanied by fever. *S.* Typhimurium is the serotype most frequently recovered from equine salmonellosis but a wide variety of other serovars, including Agona, Krefeld, Anatum, and Saint-paul, have also been implicated in disease. Multiple drug resistance is a common feature of *Salmonella* recovered from

horses in the veterinary teaching hospital. Infection with *S.* Abortusequi is rare but it often results in abortion.

Clinical salmonellosis occurs infrequently in cats and dogs, but there is a variable frequency of *Salmonella* infection or carriage in cats and dogs, with the frequency being greatly dependent on where they live. Working dogs around cattle may transiently excrete *Salmonella,* having acquired the infection from the environment. Surveillance of dogs and cats around swine and chicken operations has demonstrated carriage but few or no symptoms. In racing greyhounds, *Salmonella* infection is common. The source of infection has been traced to poor-quality meat and other food (Chengappa et al. 1993).

Salmonella infection in turtles, snakes, and lizards has been the source of several outbreaks of human infection associated with reptile shows (Friedman et al. 1998). Children handled these animals and subsequently became seriously ill. Most states in the United States ban these shows and prohibit the sale of certain reptiles. Reptiles rarely demonstrate any symptoms of *Salmonella* infection but can become lifelong carriers.

VIRULENCE FACTORS

Several virulence factors have been identified in *Salmonella*. The major ones will be described prior to a discussion of pathogenesis.

SALMONELLA PATHOGENICITY ISLAND-1 (SPI-1)

Salmonella diverged from its closest relative, *E. coli*, approximately one hundred million years ago (Ochman and Wilson 1987), and there are large clusters of conserved virulence genes in the chromosome of pathogenic *Salmonella* spp. that are absent from the analogous region of nonpathogenic spp., such as *E. coli* K12. These regions of chromosomal DNA are referred to as SPIs (*Salmonella* Pathogenicity Islands)(Blum et al. 1994). The GC content of these islands is much lower than that of the remaining chromosomal DNA sequence, suggesting that the SPIs were obtained through horizontal transfer. The insertion sites of many of these pathogenicity islands are in or near tRNA genes, which are known to serve as anchor points for temperate phages (Hou 1999). Five pathogenicity islands have been identified in *Salmonella* Typhimurium and *S.* Typhi (Groisman and Ochman 1997; Wong et al. 1998; Wood et al. 1998).

SPI-1 encodes a TTSS, which is required for the uptake of *Salmonella* by intestinal epithelial cells (Mills et al. 1995; Galan 1996, 2001). This island comprises a 40-kb region of DNA that encodes a secretion apparatus, transcriptional regulators, and secreted effector proteins. SPI-1 is found in all *Salmonella,* is located at centisome 63 on the *Salmonella* chromosome map, and contains the *inv/spa* cluster (fig. 14.1). Over 29 genes encoded within SPI-1 are involved in the assembly of the TTSS apparatus (Collazo and Galan 1997a, 1997b).

Environmental stimuli similar to those found in the intestinal tract are known to regulate SPI-1 invasion genes (Galan and Curtiss 1990; Francis et al. 1992; Lee et al. 1992). Regulation of SPI-1 is also controlled by several proteins, including HilA and InvF, which are encoded within SPI-1 itself, and others that are encoded outside SPI-1 (Bajaj et al. 1996; Altier et al. 2000a; Murray and Lee 2000). HilA is a transcriptional regulator whose expression is controlled by HilC and HilD (Schechter et al. 1999; Lucas and Lee 2001). InvF regulates the expression of secreted SPI-1 effector proteins both dependently and independently of HilA (Eichelberg and Galan 1999; Lucas et al. 2000). The PhoP/PhoQ system represses the invasion genes, whereas the BarA/SirA system positively regulates them (Behlau and Miller 1993; Pegues et al. 1995; Ahmer et al. 1999; Altier et al. 2000a). Recent studies have also determined that invasion gene expression is regulated by CsrA/CsrB (Altier et al. 2000b).

The effector proteins encoded within SPI-1 are translocated into the host cell cytoplasm through the secretion apparatus (fig.14.1). In particular, AvrA, SipABCD, SopE, SopE2, SopB, and SopD are translocated into the host enterocyte by the secretion machinery encoded by SPI-1. These effector proteins orchestrate the cytosol changes that result in uptake of *Salmonella.* In the murine model of salmonellosis, SPI-1 mutant *Salmonella* are avirulent by the oral route of infection but are virulent by the intraperitoneal or intravenous route, indicating that SPI-1 is required for intestinal invasion but not for systemic infection (Murray and Lee 2000). AvrA is anti-inflammatory by its inhibition of activation of NFκB transcription and also enhances apoptosis in epithelial cells (Collier-Hyams et al. 2002). SipA binds to the host cell's actin cytoskeleton preventing actin rearrangements and inducing membrane ruffling (Zhou et al. 1999). SipA also promotes inflammation by inducing the production of PMN chemoattractants; it may not even need to enter the epithelial cells to be effective (Lee et al. 2000). SipB activates caspase-1, thereby inducing apoptosis within epithelial cells (Hersh et al. 1999). SipB,

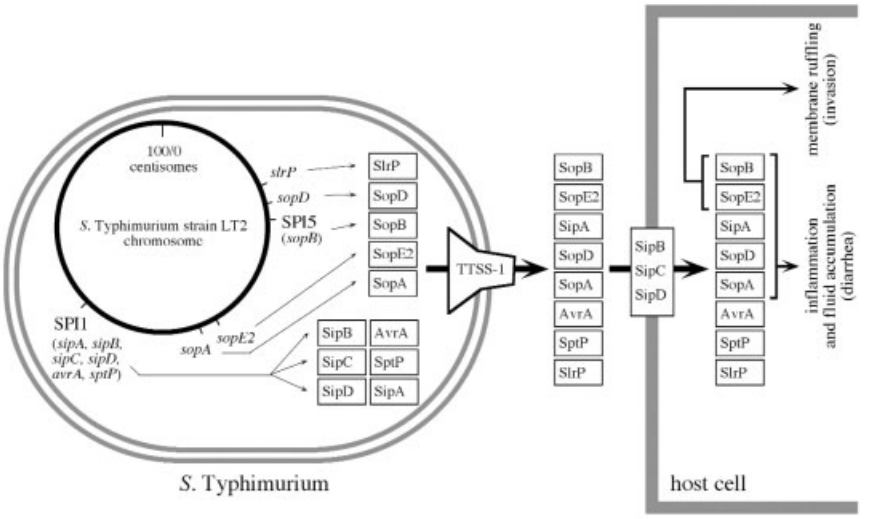


Figure 14.1. SPI-1-encoded gene products of *Salmonella* Typhimurium and their roles in diarrhea in calves. The SPI-1-encoded TTSS includes a translocation complex formed by SipB, SipC, and SipD. Effector proteins encoded by genes in SPI-1 and SPI-5 are transported into the host-cell cytosol. SipA and the Sop proteins are responsible for inflammation and diarrhea as well as membrane ruffling.(From Zhang et al. 2003)

SipC, and SipD are required for entry of other effector proteins into the host cell.

SopE and SopE2 are responsible for activating Rho GTPases including cdc42 and Rac, which influence cytoskeletal rearrangements (Hardt et al. 1998; Bakshi et al. 2000; Stender et al. 2000). SopB protein, which is encoded on SPI-5 but injected by the SPI-1 TTSS, encodes an inositol phosphate phosphatase that induces secretory diarrhea by blocking chloride channel closure (Norris et al. 1998). SopB also promotes inflammation. SopD is important in induction of the diarrheal response in calves and likely in humans as well, but its mechanism of action has not been identified (Zhang et al. 2003; Prager et al. 2003).

Salmonella Pathogenicity Island-2 (SPI-2)

Pathogenicity island SPI-2 is essential for intramacrophage survival (Hensel et al. 1995; Ochman et al. 1996; Cirillo et al. 1998; Vazquez-Torres and Fang 2001). The SPI-2 gene cluster is located at centisome 30.7 on the *Salmonella* chromosome and comprises 40 kilobases that contain 44 ORFs, many of which encode the two-component regulatory system SsrA/SsrB and a TTSS apparatus. Interestingly, only a 25 kb portion (which encodes SsrAB and the TTSS system) is necessary for virulence (Hansen-Wester and Hensel 2001). SPI-2 mutants are profoundly attenuated for virulence whether administered orally or intraperitoneally and cannot proliferate in the spleen or liver of infected mice, suggesting that SPI-2 is required for the systemic phase of infection (Shea et al. 1999).

In vitro, SPI-2 gene expression is induced in response to low levels of magnesium or calcium or to phosphate starvation (Valdivia and Falkow 1997; Cirillo et al. 1998; Deiwick et al. 1998). Regulators of SPI-2 include SsrA/SsrB and EnvZ/OmpR, which regulates SsrA/SsrB and presumably responds to low osmolarity in the phagolysosome (Garcia Vescovi et al. 1996; Cirillo et al. 1998; Deiwick et al. 1999; Lee et al. 2000). Elegant studies by Feng et al. (2003) have shown that phospho-OmpR activates the SsrA/B locus by direct interaction at their promoters. The predicted structure of SsrA is that of a phosho-relay type sensor kinase similar to BarA or ArcA while SsrB has homology to UvrY of *E. coli* and SirA of *Salmonella* (Deiwick et al. 1999). Hensel et al. (1998) and Cirillo et al. (1998) have demonstrated that the SPI-2 genes are required for survival in host phagocytes. Subsequent research by Vazquez-Torres et al. (2000) suggests that SPI-2 may interfere with the

trafficking of the NADPH oxidase to *Salmonella*-containing vacuoles thereby preventing phagocyte-dependent oxidative killing. There was a tenfold reduction of colocalized phagosome-associated NADPH oxidase in wild-type *Salmonella*-infected macrophages as compared to an SPI-2 mutant-infected macrophage. This suggests that the secreted protein products from the SPI-2 locus deflect trafficking of the vesicles containing NADPH oxidase to *Salmonella*-containing phagosomes, thereby preventing *Salmonella* exposure to toxic reactive oxygen species. The SPI-2-encoded SpiC protein that is secreted into the macrophage cytosol is required for prevention of endosomal or lysosomal fusion to *Salmonella*-containing phagosomes (Uchiya et al. 1999; Vazquez-Torres and Fang 2001). SPI-2-deficient mutants of *S.* Typhimurium, which are a millionfold attenuated in normal mice, are lethal for mice that are deficient in the ability to produce NADPH oxidase.

Salmonella Pathogenicity Island-3 (SPI-3)

Salmonella spp. encode a third pathogenicity island designated SPI-3. SPI-3 is a 17-kb region located at centisome 82 on the *Salmonella* chromosome and encodes the *mgtCB* operon that is required for both growth in Mg^{+2}-limiting conditions and intramacrophage survival (Blanc-Potard and Groisman 1997; Smith et al. 1998). This region encodes four additional genes—*rmbA, misL, fidL,* and *mart*—which show similarity to genes encoded by *E. coli* K12, however the genetic organization is different (Collazo and Galan 1996; Blanc-Potard et al. 1999). These genes encode proteins that include putative cytoplasmic proteins and putative membrane proteins. The two-component system, PhoP/PhoQ, regulates this operon through the Mg^{+2} sensor, PhoQ, and the activator, PhoP (Garcia Vescovi et al. 1996; Soncini et al. 1996). SPI-3 is required for survival in macrophages and virulence in the murine model of salmonellosis (Blanc-Potard and Groisman 1997).

Salmonella Pathogenicity Island-4 (SPI-4)

Salmonella pathogenicity island 4, SPI-4, was originally described as a 25 kb pathogenicity island that encoded 18 ORFs designated A–R (Wong et al. 1998). The recently completed annotation of SPI-4 has revealed, however, that SPI-4 actually encodes 6 ORFs (McClelland et al. 2001). SPI-4 gene expression is regulated by the transcriptional regulator, SirA, and SPI-4 is thought to play a role in invasion (Ahmer et al. 1999; Allen 2001). Allen (2001) showed that the genes encoded within SPI-4 played a role in the invasion of cultured epithelial cells. This may explain why deletion of SPI-1 did not eliminate the ability of *Salmonella* to invade epithelial cells (Murray and Lee 2000). SPI-4 may also be required for intramacrophage survival and virulence in mice infected intraperitoneally (Libby, unpublished).

Salmonella Pathogenicity Island-5 (SPI-5)

SPI-5 was first identified in *S.* Dublin at centisome 25 on the chromosome (Wood et al. 1998; Murray and Lee 2000). The genes encoded within SPI-5 include *pipA, pipB, pipC, pipD,* and *orfX*. A mutation in any one of these genes results in diminished fluid/chloride secretion and poor inflammatory responses in the bovine ileal loop model (Wood et al. 1998). Also encoded within SPI-5 is *sopB,* which codes for a protein required for fluid secretion and neutrophil recruitment, however the SopB protein is translocated by the SPI-1 *inv*/*spa* locus (Galyov et al. 1997; Hardt and Galan 1997; Fu and Galan 1998; Wood et al. 1998). Collectively, these data demonstrate that SPI-5 is required for enteric but not systemic salmonellosis. This is a clear example of the intricate and complicated mechanism of cross talk between genes located on different pathogenicity islands.

Salmonella Virulence Plasmids

Salmonella of serotypes Dublin, Pullorum, Gallinarum, Choleraesuis, Abortusovis, and some strains of Typhimurium and Enteritidis harbor virulence plasmids that encode genes required for the ability to cause systemic disease (Gulig 1990; Poppe et al. 1998). These serotypes are predominantly the host-adapted ones that cause severe systemic disease. It is interesting that *S.* Typhi lacks such a plasmid (Gulig 1990). Isolates of *S.* Typhimurium carry a 90 kb virulence plasmid with a conserved 8 kb region encoding the *spv* genes (Friedrich et al. 1993; Guiney et al. 1994). The *spv* locus encodes five genes (*spvR* and *spvABCD*) that affect bacterial growth during the systemic phase of infection inside macrophages (Gulig and Doyle 1993). SpvA is a negative regulator of the *spv* operon, while SpvD is most likely secreted (El-Gedaily et al. 1997). Expression of *spvABCD* relies on both the SpvR positive activator and RpoS (Guiney et al. 1995). SpvB encodes an ADP-ribosylating enzyme that targets actin and is essential for virulence in the murine model (Lesnick et al. 2001; Garcia-del

Portillo 2001). The *spv* genes are expressed by intracellular *Salmonella* and increase virulence by 10- to 10,000-fold, with the degree of enhancement varying with the serotype. It has been suggested that the Spv proteins might function by attracting macrophages to the infection sites, thereby providing a supply of permissive cells.

The virulence plasmid encodes additional proteins that are important for *Salmonella* pathogenesis. The plasmid-encoded fimbriae, *pef*, are involved in attachment to the intestinal epithelium (Baumler et al. 1996a). Rck, an outer membrane protein that is involved in intestinal epithelial cell invasion and resistance to host complement factors, is also encoded within the virulence plasmid, but is found only in *S.* Typhimurium (Cirillo et al. 1996).

Intracellular Survival

Ability to survive within the phagosomal compartment of macrophages is critical to the ability of *Salmonella* to establish in the intestine and in systemic organs. Survival within this compartment requires that *Salmonella* withstand both the oxygen-dependent and oxygen-independent killing mechanisms of professional phagocytes. The oxygen-independent mechanisms of killing by host phagocytes include reduced phagosomal pH, nutrient limitation, and antimicrobial peptides (Finlay and Falkow 1997). The PhoP/PhoQ regulon plays a major role in resistance to these factors. PhoP-activated genes (*pag*s) encode proteins that include cation transporters, outer membrane proteins, enzymes that alter lipid A structure, an acid phosphatase, and a two-component regulatory system (Ernst et al. 2001). These proteins are able to respond to the inhospitable environment in the SCV by, among other things, changing the composition of LPS and the outer membrane and reducing the charge of the bacterial surface. For example, PagP, a palmitoyl transferase, converts hexa-acyl lipid A to hepta-acyl lipid A. Other changes include alterations to the charged phosphates on lipid A, and synthesis of new outer-membrane proteins. These changes contribute to a modified outer membrane that is less permeable and more resistant to antimicrobial peptides.

The *Salmonella*-containing vacuole (SCV) is acidified to pH 4-5, which is needed for transcription of virulence genes of the SPI-2 system. Resistance to the oxygen-dependent killing mechanisms of professional phagocytes is the most important aspect of resistance of *Salmonella* (Vazquez-Torres and Fang 2001).

Oxidative killing by professional phagocytes is oxygen dependent and involves the production of toxic and highly reactive molecules that can damage microbial DNA, protein, and lipids (Demple and Halbrook 1983). As a result of the selective pressure exerted by this mechanism, many bacteria have evolved oxidative stress-resistance mechanisms that are essential for their survival within the phagosomal compartment. The ability of *Salmonella* to survive and replicate within host macrophages is an essential aspect of its ability to cause systemic infection (Fields et al. 1986, 1989**;** Finlay and Falkow 1989b, 1997).

Genes that are required for intramacrophage survival and virulence include *slyA, phoP,* SPI-2-encoded genes*, mig-14, recA, rpoE, rpoS,* and *pagC*, among many others (Fang et al. 1992; Buchmeier et al. 1995; De Groote et al. 1997; Henderson et al. 1999; Chamnongpol and Groisman 2000, 2002). Oxidative stress resistance in *E. coli* and *S.* Typhimurium involves more than 60 genes (Farr and Kogoma 1991), only some of which are required for intramacrophage survival and virulence. For example, a *Salmonella* strain harboring mutations in two catalase genes required for hydrogen peroxide resistance, *katE* and *katG*, is sensitive to *in vitro* derived oxidative stress, but remains fully virulent in the murine model of salmonellosis (Buchmeier et al. 1995). Of the genetic loci that are required for intramacrophage survival and virulence, only *slyA*, *sodC*, *rpoE*, *recA*, and *recBC* have clearly been demonstrated to be required for oxidative stress resistance *in vitro,* and they are also essential for intramacrophage survival and virulence in mice *in vivo* (Buchmeier et al. 1993, 1995, 1997; Battistoni et al. 2000; Kaneko et al. 2002; Testerman et al. 2002).

The respiratory burst of professional phagocytic cells, including neutrophils, involves the production of superoxide anion ($\bullet O_2^-$) from the univalent reduction of molecular oxygen through the action of NADPH oxidase (Babior 1984). Specifically, upon phagocytosis of *Salmonella*, the p67, p47, and p40 cytosolic components of NADPH oxidase translocate via the cytoskeleton for association with p22, gp91, and Rap1A membrane-bound subunits along with the cytosolic Rac proteins to the *Salmonella*-containing phagosome (Vazquez-Torres et al. 2000). As a result, superoxide anion is produced that can dismutate to form hydrogen peroxide (H_2O_2). Unlike superoxide, hydrogen peroxide is a membrane-permeable species that is capable of reacting with lipids, proteins, and DNA (Vazquez-Torres and

Fang 2001). Hydrogen peroxide can also react with Fe(II) in the Fenton reaction to produce the toxic hydroxyl radical (OH^-). Superoxide can react with nitric oxide (NO), which leads to the production of peroxynitrite ($ONOO^-$). Hydrogen peroxide can also react with NO by a mechanism that appears to be iron dependent (Pacelli et al. 1995).

The importance of the respiratory burst in resistance to *Salmonella* has been shown by the observation that *Salmonella* mutants defective in DNA repair genes, *recA* and *recBC*, are able to survive and replicate within murine macrophages lacking the ability to generate a respiratory burst, but are killed in burst-competent macrophages (Buchmeier et al. 1995). Mice that have a defined mutation in the gp91 subunit of the NADPH oxidase (*phox* knockouts) are susceptible to *Salmonella* infection, and macrophages from these mice are unable to control *Salmonella* replication (De Groote et al. 1997). By contrast, a *Salmonella* mutant lacking *sodA*, the Mn-cytoplasmic superoxide dismutase, remains virulent in mice and is able to replicate in macrophages.

Salmonella contains two periplasmic Cu, Zn-SODs, *sodCI* (encoded on a cryptic lambdalike bacteriophage) and *sodCII*, which are important for protection against exogenous oxidative damage. The *sodCII* gene is maximally expressed in stationary phase in an RpoS-dependent manner, while *sodCI* seems to be maximally expressed at the transition between exponential and stationary phase independent of RpoS (Fang et al. 1992; Lee et al. 1995; Fang et al. 1999; Gort et al. 1999). A *sodC* mutant was attenuated for virulence in mice and was unable to replicate in respiratory-competent macrophages, but restoration of virulence and macrophage growth was observed in gp91*phox*$^{-/-}$ mice (Tsolis et al. 1995; De Groote et al. 1997; Farrant et al. 1997).

Salmonella are also able to inhibit fusion of phagosomes with lysosomes (Vazquez-Torres and Fang 2001) by a SPI-2-mediated process. The mechanism by which this is achieved is not known, but SpiC appears to be responsible. Other SPI-2 functions that are related to intravacuole survival include the assembly of a meshwork of F-actin around the SCV, and induction of slow cell death.

Lipopolysaccharide (LPS)

The lipopolysaccharide of *Salmonella* is a major component of the outer membrane and an important toxin that interacts with the host immune system to induce inflammation and produce septic shock, fever, and death. LPS consists of three major components: lipid A, core, and the highly antigenic O polysaccharide. Lipid A is made up of fatty acid and phosphates coupled to a central glucosamine dimmer, and is responsible for the endotoxic effects of LPS, through overstimulation of the host cytokine response. Death of animals from systemic infection with *Salmonella* is attributed to the endotoxic effects of LPS, and mutants of *S.* Typhimurium that have a lipid A defect can grow in the liver and spleen of mice to as high as 10^9 without causing death, whereas death occurs at 1/100th to 1/1,000th this level with wild-type organisms (Garcia-del Portillo 2001).

Salmonella with incomplete LPS ("rough" mutants) are sensitive to complement-mediated killing and antimicrobial peptides, are killed more efficiently by host phagocytes, and are more sensitive to bile salts and detergents (Yethon et al. 2000). Rough LPS mutants are less able to cause lethal infection in mice. It is perhaps not surprising that most clinical isolates of *Salmonella* appear as "smooth" colonies having a complete LPS. *Salmonella* can modulate the structure of the O-antigen as a means of dampening host innate immune responses, an action that presumably enhances the organism's ability to persist and survive in the host (Ernst et al. 1999, 2001). *Salmonella* can also modify its LPS so that the organism is less susceptible to killing by host antimicrobial agents. These changes include formation of hepta-acylated lipid A as a result of addition of palmitate by the PagP protein, addition of phosphate and phosphoethanolamine to the core, and modification of phosphate groups of lipid A with ethanolamine and phosphoethanolamine.

The inflammatory response induced by LPS is mediated primarily by its interaction with macrophages (Freudenberg et al. 2001). LPS binds to CD14 and Toll-like receptor 4 (TLR4) on the surface of macrophages, and signals through TLR4. The *lps* gene, whose defective allele *lps*^{d} confers LPS resistance on mice, has been shown to encode TLR4. Because host resistance to infection depends on recognition of LPS and generation of a response to it, mice that carry the *lps*^{d} mutation are more susceptible to *S.* Typhimurium than are normal mice.

Fimbriae

In order to establish a successful infection, *Salmonella* must colonize the intestinal tract of the host. Genome sequence analysis has demonstrated that *Salmonella* serotypes contain a large number of fim-

brial gene sequences located in the chromosome and on the virulence plasmid. In *S.* Typhi, 14 putative fimbrial operons have been identified, suggesting that this large number of fimbrial sequences is important for virulence and that *S.* Typhi has evolved redundant mechanisms for adherence to the host. *In vitro* adherence assays have demonstrated that *Salmonella* may utilize different fimbriae for attachment to different cell types. Two fimbrial operons, *lpf* and *pef*, are used for adherence to Peyer's patches and villous intestine in a mouse model of infection. Loss of these two fimbrial operons diminishes the ability of *Salmonella* to associate with these areas (Baumler et al. 1996a, 1996b, 1996c, 1997a, 1997b). The role of fimbriae in the virulence of *Salmonella* in the murine model has been investigated. Inactivation of the *lpf, pef,* and *agf* fimbrial operons was required to observe a modest effect on virulence in mice (van der Velden et al. 1998). The roles of the fimbrial operons in the virulence of *Salmonella* infection in swine, cattle, or chickens have not been established, but the large number of fimbrial operons suggests that *Salmonella* may employ different adherence mechanisms when in various hosts.

Flagella

Most pathogenic *Salmonella* are biphasic and can produce two distinct antigenic types of flagellin. Switching between the two phases is accomplished by flipping of an invertible genetic element that permits transcription of one flagellin in one direction and the second flagellin when it is in the other direction. The variation between the phases appears to assist in immune evasion as antibody against flagellin of one phase selects for *Salmonella* that produce flagellin of the other phase.

The roles of flagella in virulence of *Salmonella* are not clear. Flagella play different roles in the models of septicemic (*S.* Typhimurium in mice) compared with enteric disease (*S.* Typhimurium in calves) (Schmitt et al. 2001). In the mouse model, neither flagella nor the flagella export system played a role in pathogenicity. In contrast, flagella contributed to intestinal invasiveness, PMN recruitment, and fluid secretion in infected calves. The authors speculate that the flagella TTSS may be able to secrete effector proteins that are secreted by the SPI-1 TTSS. Flagellin is known to strongly induce production of the potent PMN chemoattractant IL-8 by intestinal epithelial cells and likely plays a role in the inflammatory response in the intestine. The flagellar regulon is complex, involving more than 50 genes and 15 operons, but it is clear that there is coordinated regulation of virulence genes and flagella operons. The regulatory proteins RtsA and RtsB have been reported to coordinate induction of invasion genes and repression of motility in *S.* Typhimurium (Ellermeier and Slauch 2003). RtsA activates expression of hilA, hilD, hilC, and the invF operon, thereby increasing expression of the SPI-1 invasion genes, whereas RtsB represses flagellar gene expression.

PATHOGENESIS

Getting to the Intestinal Epithelial Cells

Salmonella commonly enter the host through ingestion, although inhalation may be an important route in swine. Following ingestion, the organism must survive exposure to the low pH of the stomach. Despite having a number of systems to resist acidity, only a small percentage of ingested *Salmonella* usually survive exposure to the low pH and move on into the small intestine. Because pH of the stomach fluctuates in relation to ingestion of milk and food, and exposure to stomach fluids varies with the vehicle with which the bacteria are associated, the effectiveness of gastric acidity as a barrier to infection is highly variable.

Salmonella can resist both inorganic and organic acids and can survive exposure to about pH 3. Two types of acid-tolerance systems have been described: one that is activated on exposure to an acidic environment during log phase growth and one that develops in stationary phase (Lee et al. 1995; Audia et al. 2001). The former is regulated by the ferric uptake regulator (Fur), is activated by exposure to pH 4.5, and is short lived. The latter is dependent on the alternate sigma factor RpoS and is sustained. In addition to Fur and Rpos, PhoP and OmpR are also pH-response regulators. Resistance to acids is achieved through production of a number of acid-shock proteins.

In the intestine, *Salmonella* encounter protective antibacterial factors and mechanisms such as bile salts, intestinal mucus, lysozyme, lactoferrin, peristalsis, and organic acids. In the large intestine, the abundant and complex established normal flora is a major obstacle to colonization. Factors that impair these host defenses will predispose to intestinal colonization and disease.

The role of the normal flora in protecting against establishment of *Salmonella* is illustrated by the effectiveness of adult cecal flora to protect young

chickens from infection with *Salmonella*. Another indication of the importance of the normal flora is the observation that oral antibiotics are a risk factor for development of salmonellosis in humans and animals. Experimental data also bear directly on this issue. Early studies demonstrated that germ-free mice were exquisitely susceptible to infection and developed lethal systemic infection following oral administration of only a few organisms of *S.* Enteritidis, whereas conventional mice required about a million organisms to achieve the same effect.

Invasion of Intestinal Epithelium

It is now clear that (1) *Salmonella* must be able to adhere to and invade the epithelial cell layer lining the intestine in order to cause enteritis and/or systemic disease (Tsolis et al. 1999), and (2) diarrhea and enteritis caused by *Salmonella* are due to products of a specialized secretion apparatus and effector proteins that are injected into the host cell (fig. 14.1; Galan 2001; Zhang et al. 2003).

In experimental infection of mice with *S.* Typhimurium (a model of typhoid fever), the primary sites of invasion are Peyer's patches, which contain specialized membranous epithelial cells (M cells) in the follicle-associated epithelium (FAE) that overlay aggregations of lymphoid cells (fig. 14.2). In this systemic infection, intestinal invasion occurs without damage to the intestinal epithelium. This contrasts with the enteric infection in calves, in which intestinal epithelial damage and enteritis occur without systemic infection (Zhang et al. 2003). The M cells sample antigenic material, including particulate material, from the lumen contents and present it to the immune system (Hohmann et al. 1978; Jones et al. 1994; Jones and Falkow 1996). Features of the FAE that may facilitate uptake of bacteria include low quantities of mucus, associated with the absence of goblet cells; low concentrations of SIgA, because of the absence of polymeric immunoglobulin receptors; a thinner glycocalyx; and an irregular brush border (Jepson and Clark 2001). *Salmonella* interaction with the M cells facilitates host colonization during macropinocytosis through a mechanism of cell membrane ruffling that results in the internalization of the bacteria into membrane-bound endocytic vacuoles (Takeuchi 1967; Finlay and Falkow 1990; Francis et al. 1992; Galan et al. 1992; Jones et al. 1993; McCormick et al. 1993). Although recognition of M cells by *Salmonella* is likely important in initiation of infection, almost nothing is known about the mechanisms that are involved. It is known that long polar fimbriae (Lpf) are required in *S.* Typhimurium.

Salmonella are capable of invading enterocytes as well as M cells. In fact, much of the intestinal invasion by *S.* Typhimurium in cattle occurs through enterocytes (Santos et al. 2001). Given that M cells occupy only about 0.01% of the intestine, invasion of enterocytes may be important. Dendritic cells (DC) have also been implicated in transfer of *Salmonella* across the intestinal epithelium (Wick 2001).

The framework of our understanding of the uptake of *Salmonella* into the intestine was laid by electron microscopic studies of *Salmonella* in the intestine of guinea pigs 35 years ago (Takeuchi 1967). Takeuchi observed that as *Salmonella* came in close proximity to enterocytes, the microvilli and the tight junctions underwent morphological changes and the bacteria entered the enterocytes through these altered areas. *Salmonella* were taken up into a membrane-bound vacuole that moved toward the base of the cell and the enterocyte morphology was restored. It is likely that the changes occurred in response to contact of the bacteria with the enterocytes, and it is now known that profound rearrangement of the actin cytoskeleton of the enterocyte occurs following contact with *Salmonella,* which stimulate host signal transduction pathways that result in the changes in the actin cytoskeleton (Galan 2001).

Details of the interaction of *Salmonella* with the intestine continue to be provided by current research. The bacteria are taken up by macropinocytosis, involving formation of membrane protrusions (called ruffles). Several effector proteins secreted by the SPI-1 TTSS play roles in the entry of *Salmonella* into the nonphagocytic epithelial cells. These effectors include SopE, SopE2, and SopB that activate Rho-family GTPases Cdc42 and Rac1, which promote rearrangements of the actin cytoskeleton; SipA and SipC, which bind actin and modulate its dynamics to facilitate rearrangement of the cytoskeleton; and SptP, which reverses the activation of Cdc42 and Rac1 caused by SopE, SopE2, and SopB. It is the action of SptP that restores the morphology of the epithelial cell.

Invasion of the M cells or enterocytes stimulates a strong inflammatory response characterized by an abundance of polymorphonuclear leukocytes (PMNs). Stimulation of a proinflammatory response is attributed to the action of SopE, SopE2, and SopB, which activate the Cdc42 and Rac1 GTPases leading

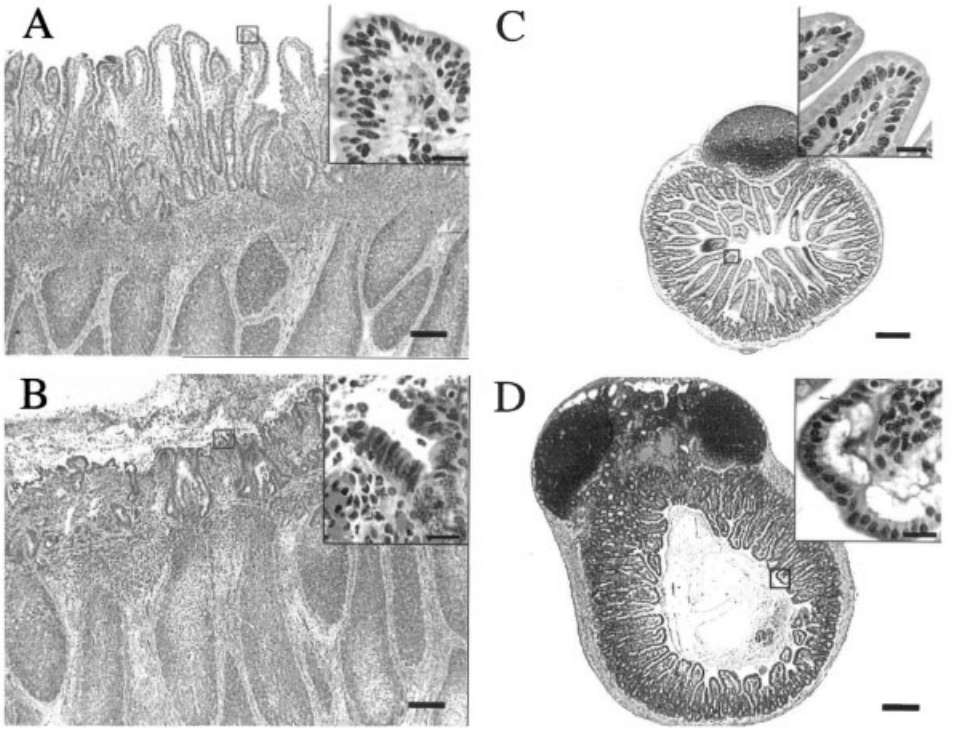


Figure 14.2. Small intestinal epithelial damage occurs in the calf (*A, B*) but not in the mouse (*C, D*) following infection with *Salmonella* Typhimurium. (*A*) shows the appearance of an uninfected Peyer's patch, whereas (*B*) shows blunted villi, loss of integrity of the epithelium, and an inflammatory exudate in the infected calf. (*C*) shows an uninfected murine Peyer's patch, and (*D*) shows that the intestinal epithelium remains intact in the infected mouse. (From Zhang et al. 2003).

to stimulation of MAP kinase pathways Erk, p38, and Jnk (Galan 2001). Production of IL-8 at the basolateral border and of pathogen-elicited epithelial chemoattractant at the apical border results in migration of PMNs from blood vessels into the lamina propria, and from this site to the lumen of the intestine. Disruption of the tight junctions occurs, thereby permitting access of bacteria and pathogen-associated molecular patterns such as LPS and flagella to Toll-like receptors (TLRs) on the basolateral border of enterocytes. This activity results in increased production of pro-inflammatory cytokines. Further exacerbation of the inflammation may occur as *Salmonella* kill macrophages in the lamina propria.

Invasion of M cells and enterocytes and the ensuing inflammation are not without adverse consequences. Cell death and sloughing of the FAE provide new opportunities for bacteria to invade the submucosal tissues. The mechanisms by which *Salmonella* damage these cells are not known, but the SPI-1 system appears to make a contribution. Macrophage lysis and the inflammatory response in the intestine have also been proposed to be potential contributors (Jepson and Clark 2001). PMNs are thought to cause damage as they move between cells and as they release reactive oxygen intermediates.

Transcytosis of M cells in Peyer's patches provides access to the underlying follicle dome of the lamina propria, where the organisms encounter host phagocytes. Some macrophages are killed rapidly by a caspase-1-mediated process, leading to release of IL-13 and IL-18. This process shares features of both apoptosis and necrosis. Most *Salmonella* have the ability to survive and replicate within these cells, leading to the extraintestinal colonization of other organs of the body, including the spleen and liver (Finlay 1994). The SEM images in figure 14.3 demonstrate *S.* Typhimurium associating with the M cells of the small intestine of a pig (Meyerholz et al.

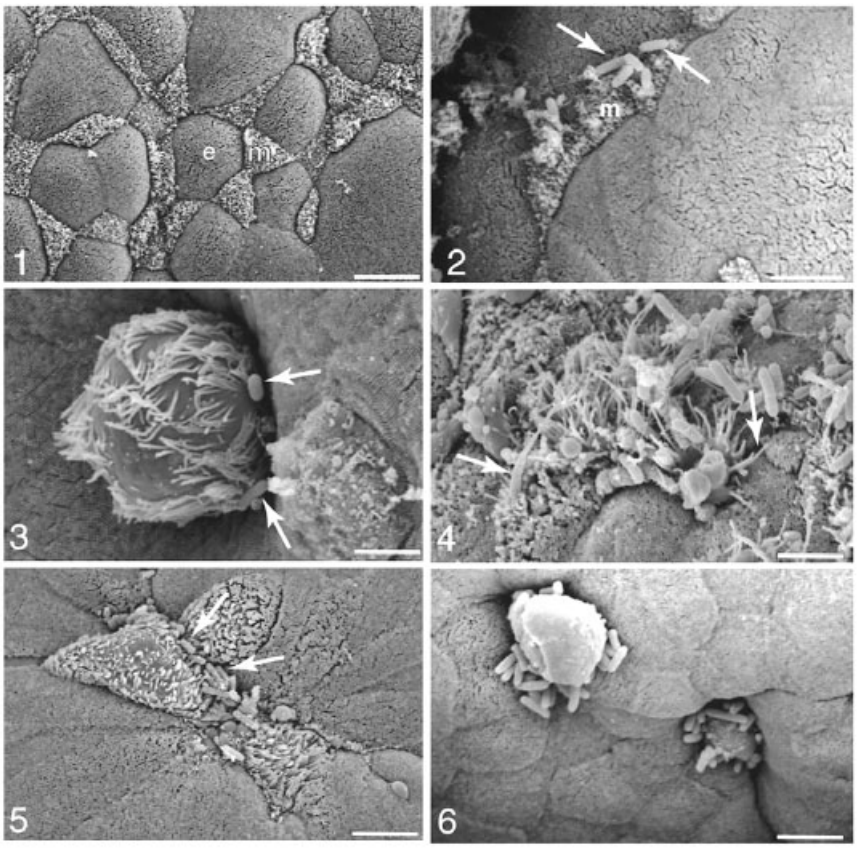


Figure 14.3. *Salmonella* Typhimurium interaction with M cells and follicle-associated epithelium of ligated ileal loops. (1) Scanning electron micrograph of the ileum demonstrating enterocytes (e) and M cells (m) inoculated with sterile media. (2) Ilial loop inoculated with *Salmonella* Typhimurium after 5 minutes. Arrows point to bacteria adhering to M cells. (3) Bacteria adhering to a cell extrusion. (4) Bacteria adhering to absorptive epithelium enterocytes 25 minutes following infection. (5) Bacteria adhering to sloughing cells. (6) Bacteria adhering to crevices formed by sloughing cells of the M cell.

2002; Meyerholz and Stabel 2003)**.** Late cell death, due to SPI-1-encoded genes and OmpR occurs in macrophages in which *Salmonella* have survived and replicated (Monack et al. 2001).

Diarrhea

Earlier investigations of the mechanisms of *Salmonella*-induced enteritis and diarrhea detected various cholera-like toxins, enterotoxins, and toxins of unknown composition, which were suggested to contribute to the enteritis and diarrhea seen in *Salmonella* infections in humans and animals. However, no toxin has been purified or demonstrated to induce the enteritis and diarrhea seen in natural infections. Furthermore, the completed genome sequences of *Salmonella enterica* serovars Typhimurium, Typhi, Dublin, and Choleraesuis have not revealed the identity of any toxinlike protein with homology to well-characterized toxins.

Salmonella-induced enteritis has long been described as secretory diarrhea with significant inflammation. Acute enteritis is characterized by an inflammatory infiltrate that is made up mostly of neutrophils (fig. 14.4; Zhang et al. 2003). To address the roles of selected virulence genes, Tsolis et al. (1999, 2000) tested the effects of *S.* Typhimurium mutants for their ability to induce diarrhea in calves. These researchers found that mutants defective in *spvR*, *sopB* (encoded by SPI-5), or *spiB* (encoded in SPI-2) were still able to cause diarrhea and deaths in calves, whereas

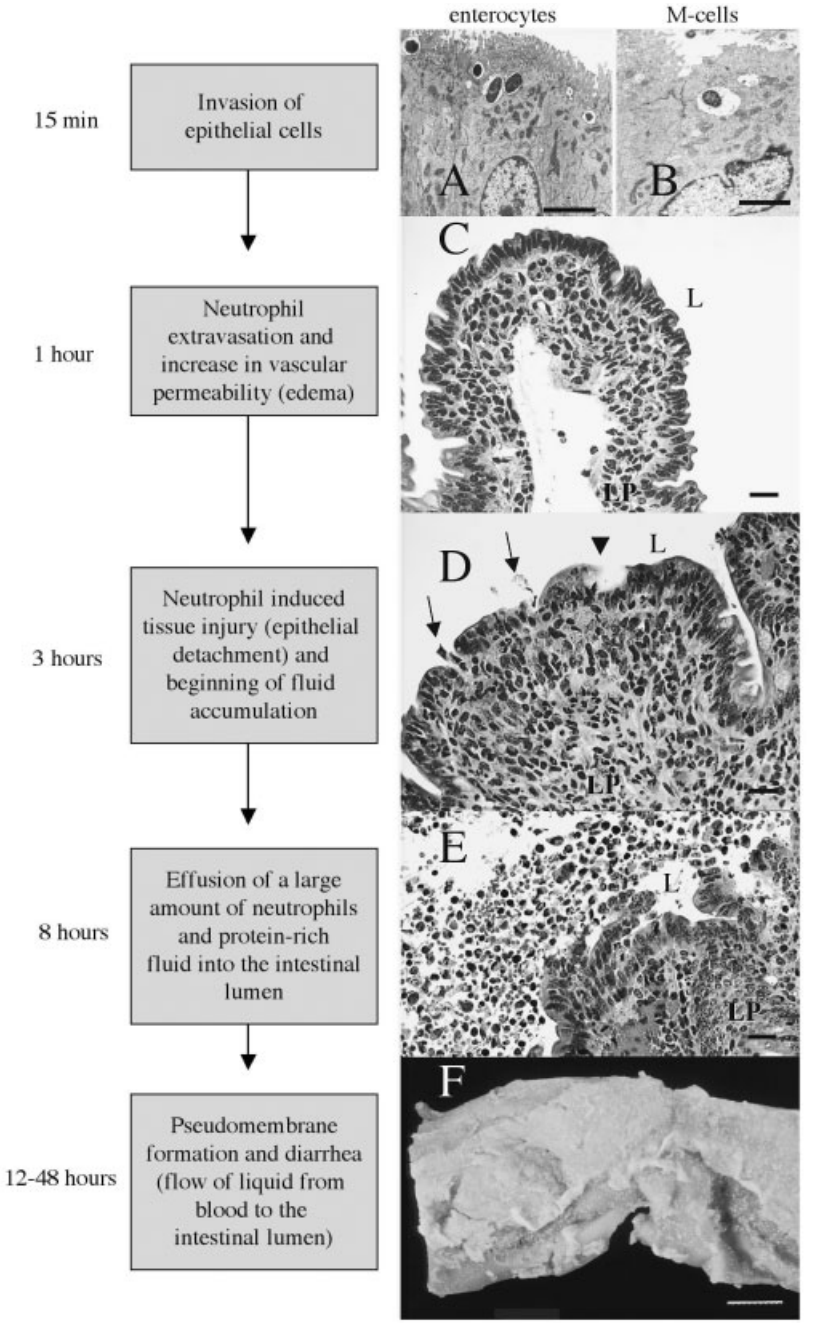


Figure 14.4. Steps in the development of inflammatory diarrhea in *Salmonella* Typhimurium-infected ligated ileal loops (*A–E*) in calves and in an orally infected calf (*F*). (*A, B*) 15 minutes postinfection: transmission electron micrographs show invasion of enterocytes (*A*) and an M cell (*B*) in a Peyer's patch. (*C*) 1 h postinfection: infiltration of the lamina propria with neutrophils. (*D*) 3 h postinfection: blunting of villi, loss of integrity of the epithelium, transmigration of neutrophils into the lumen, hemorrhage, and massive infiltration of the lamina propria with neutrophils. (*E*) 8 h postinfection: neutrophil-rich exudate. (F) 18–24 h postinfection: formation of a pseudomembrane over the damaged epithelium. (From Zhang et al. 2003)

mutants defective in SPI-1 function or in aromatic amino acid biosynthesis (*aroA*) showed significant reduction in ability to cause diarrhea. These studies emphasized the critical contribution of intestinal colonization, rather than systemic invasion, in the enteric disease in calves. In order to investigate the mechanism by which *Salmonella* Typhimurium causes enteritis, Zhang and colleagues (2003) investigated the effects of specific mutants in the effector and needle complex coding genes of SPI-1 in calves and in ligated ileal loops in calves. The severity of enteritis was quantitated by the amount of fluid accumulation in the loops as well as the level of inflammation determined by histochemistry. The investigators showed that in contrast to the wild-type organism, a *sipA sopABDE2* mutant failed to induce fluid accumulation, PMN infiltration, or expression of CXC chemokines in the ligated intestine.

The following all appear to contribute to development of diarrhea (Jones 2000):

1. There is increased secretion of fluid by epithelial cells. The SPI-1 effector proteins SopB and SopE have been identified as affecting inositol phosphate metabolism, and accumulation of fluid in *Salmonella*-infected intestine. SopB has inositol phosphatase activity and SopE has been suggested to activate an endogenous inositol phosphate phosphatase (Galan 2001).
2. The inflammatory response leads to production of prostaglandins, leukotrienes, histamine, and reactive oxygen intermediates. Prostaglandin E2 causes hypersecretion of Cl-, with water following by osmotic drag.
3. Protein-rich fluid is lost from the intestinal tissue. This includes fluid lost through blood vessels damaged by the effects of LPS.
4. Loss of epithelial cells represents a loss of absorptive intestinal surface.

Systemic Invasion

Salmonella that survive the battle in the intestine are taken to the regional lymph nodes where they are taken up by macrophages, whose actions frequently limit the infection to the intestine. Failure to contain the infection at these sites results in passage to the thoracic duct into the circulation, from which the bacteria are removed by cells of the reticuloendothelial system, notably in the liver and spleen. *Salmonella* are found in CD18+ phagocytic cells (macrophages and PMNs) in these sites. The major sites of proliferation appear to be in macrophages in the lymph nodes, liver, and spleen.

S. Typhimurium infection in mice models septicemic disease, whereas *S.* Typhimurium infection in calves models enteric disease. These animal models have contributed much to our understanding of pathogenesis (Santos et al. 2001). It is evident from studies with these model systems that the virulence factors that are important in the enteric infection are different from those required for systemic disease. Thus, the TTSS encoded by SPI-1, and its effector molecules, are necessary for the enteric phase of infection in both models of disease. However, the TTSS and effector molecules associated with SPI-2, the *spv* operon, and core LPS biosynthesis genes, which are needed for growth at systemic sites in the mouse, are not important in the calf (Zhang et al. 2003). The importance of SPI-2 to virulence in mice is shown by the observation that there is a million-fold increase in the LD_{50} for a SPI-2-deficient mutant compared with the wild-type organism.

IMMUNITY

Innate immune responses are important in controlling the early phases of infection with *Salmonella*. PMNs, macrophages, and DCs are all involved in the response at the sites of invasion in the intestine. PMNs are attracted to these sites and ingest and kill *Salmonella*. Macrophages are invaded by *Salmonella*, which can multiply in these cells and use them as a means of dissemination. Dendritic cells, which link innate and adaptive immune responses, may also be important in immunity to *Salmonella* (Wick 2003). DCS and splenic subsets of DCs can process *Salmonella* and present peptides from *Salmonella* to $CD4^+$ and $CD8^+$ T cells. *Salmonella*-infected DCs and macrophages in which apoptosis has been induced by the bacteria can not present antigens directly, but bystander DCs can ingest apoptotic material from affected macrophages and present the peptides from *Salmonella* antigens on MHC-I and MHC-II.

Shortly after oral exposure to *Salmonella*, there may be a period when nonimmune protection is afforded by competitive exclusion. This period may be followed by a time when there is nonspecific resistance mediated by the effects of macrophage activation, activities of TNF-α, IL-12, IFN-γ, and NK cells (Maestroni and Ménager 2003). Finally, the specific immune response provides long-term protection.

Oral infection with live attenuated *Salmonella* results in development of cell-mediated immunity

(CMI), systemic antibodies, and mucosal antibodies. CMI is considered to be of enormous significance for protection of the host against *Salmonella*, as the organism's ability to cause disease depends on its survival and multiplication in macrophages in susceptible hosts. Protection is conferred by transfer of sensitized T cells but not by transfer of macrophages, B cells, or hyperimmune serum. Calves that are naturally or experimentally infected with *Salmonella* develop delayed-type hypersensitivity (DTH), detected by increased skin thickness at sites of intradermal injection of *Salmonella* antigens, such as homologous LPS, whole organisms, or ribosome preparations. The reaction sites show massive infiltration of mononuclear cells. However, the accuracy of DTH as measured by increase in skin thickness is somewhat compromised by Arthus reaction and other hypersensitivity reactions that occur in response to antigen preparations, possibly as a result of their LPS content (Barrow and Wallis 2000). The degree of cell-mediated immunity appears to reflect protection against challenge in calves vaccinated by systemic routes. However, in calves vaccinated orally with a live vaccine, protection may be achieved although the DTH reaction is negative. It is likely that specific SIgA, induced by this route of exposure, may provide protection.

Antibodies also play a role in immunity to salmonellosis, and the infrequent occurrence of infection in neonatal calves may be due to the presence of antibodies in colostrum and milk. Secretory IgA is considered to be particularly important, as these antibodies may bind to the bacteria and prevent their uptake by M cells and epithelial cells in the intestine. Serum antibody levels do not appear to correlate with protection in vaccinated animals or animals that have recovered from disease.

Live, orally administered vaccines are considered to be superior because they induce CMI and both systemic and mucosal antibodies. In addition to stimulation of CMI, live vaccines present a repertoire of antigens similar to that to which the host is exposed in a low-level infection with the pathogen. Cross-protection has been reported in a number of studies. For example, calves vaccinated with an *aroA* mutant of *S.* Dublin showed protection not only against the homologous serotype but also against *S.* Typhimurium. Also an *S.* Choleraesuis vaccine that was effective in pigs also protected vaccinated calves from infection with *S.* Dublin. However, the interval between vaccination and challenge was often quite short. By contrast, evidence that even infection-related protection was serotype specific was provided by studies that demonstrated an absence of cross-protection between *S.* Dublin and *S.* Typhimurium in calves immunized with live attenuated mutants and challenged by the ligated ileal loop method 3 weeks later (Barrow and Wallis 2000).

Several live attenuated vaccines have been developed, based on mutations in a number of genes, including *aroA*, *galE, phoP*, *htrA, cya*, and *crp.* Optimally, for reasons of safety, a vaccine strain should contain at least two defined mutations, each of which is attenuating. Live attenuated vaccines have been effective against the host-adapted serotypes but have been less effective against the unadapted serotypes (Barrow and Wallis 2000). In part, this may be due to the fact that in the former, protection is aimed primarily at septicemic disease, whereas in the latter, protection is aimed at carriage of the bacteria and a relatively benign enteric infection. Live attenuated vaccines that are effective against the host-adapted serotype *S.* Gallinarum, which causes invasive septicemic disease, have been in use for decades. Attenuation of *S.* Gallinarum has been achieved by selection for rough mutants, virulence plasmid-cured derivatives, and *aroA* mutants. These derivatives have varying degrees of immunogenicity and residual virulence—the two having an inverse relationship. A variety of attenuated mutants of non-host-adapted serovars *S.* Enteritidis and *S.* Typhimurium have been tested in poultry, but the effectiveness of these vaccines has been highly variable.

Administration of several live attenuated vaccines appears to induce a period of hyporesponsiveness for a few weeks postvaccination (Eisenstein 2001). Eisenstein has indicated that the period of immunosuppression that occurs following administration of live *Salmonella* vaccines is attributable to production of nitric oxide (NO) by macrophages that have ingested *Salmonella*. The series of events is suggested to be as follows. Production of IL-12 by the macrophages causes NK cells to release IFN-γ, which up-regulates iNOS in macrophages and leads to production of NO. High levels of NO result in killing of the bacteria but also damage bystander lymphocytes, thereby causing immunosuppression against exogenous antigens.

Killed vaccines have not been consistently effective, but there are several studies that suggest that when the bacteria are grown under suitable conditions, killed organisms may be effective in stimulating serotype-specific protective immunity (Barrow and Wallis 2000; Eisenstein 2001). These vaccines

pose less risk compared with live vaccines, but they are often reactogenic.

ANTIBIOTIC RESISTANCE AND VIRULENCE

In recent years, *Salmonella* isolates obtained from humans and from food-producing animals have exhibited resistance to an increasing variety of antimicrobials. A common serotype that is also often multiresistant is Typhimurium. Recent studies have shown that, in some cases, over 90% of isolates obtained from food-producing animals are multiresistant (Edrington et al. 2001; Farrington et al. 2001). Among isolates obtained from humans, multiresistance is not as common, but recent data show that 26% of all isolates, and 50% of serotype Typhimurium, are now resistant to at least one antimicrobial (NARMS 2000). A common resistance among animal isolates is to tetracyclines, an antibiotic class used routinely in animal agriculture as a growth promoter. Resistance to several other antibiotics has been commonly found, including sulfamethoxazole, streptomycin, and ampicillin (FDA/USDA/CDC 2001). Also of concern is decreasing susceptibility to two antimicrobials important in the treatment of human disease, ciprofloxacin and ceftriaxone. Although resistance to these two remains low (less than 2% of isolates from humans), it has increased significantly during the last several years.

Among both animal and human isolates, resistance to ampicillin, chloramphenicol, tetracycline, streptomycin, and sulfonamide is common and is most often associated with phage type DT104, which has been responsible for numerous disease outbreaks (Poppe et al. 1998). This phage type encodes its resistance factors as a chromosomal element, with physical linkage of all five resistance genes (Briggs and Fratamico 1999). Three of the resistance elements (ampicillin, streptomycin, and sulfonamide) are encoded on integrons, genetic elements capable of acquiring and exchanging resistance factors. A second common resistance pattern is to ampicillin, kanamycin, tetracycline, streptomycin, and sulfonamide, frequently associated with the phage type DT193. Unlike DT104, this phage type carries its resistance factors on one or more conjugative plasmids, and so can transfer its resistance factors to other *Salmonella* strains or bacterial species (Gebreyes et al. 2000; Gebreyes and Altier 2002).

To date, there has been no evidence that drug resistance and virulence are linked in *Salmonella*. However, increasing prevalence of multidrug resistance in *Salmonella* will likely increase the frequency with which host susceptibility is enhanced by antibiotic therapy.

CONCLUDING REMARKS

Salmonellosis is particularly complex, as pathogenesis varies with strain, phage type, serotype, host, dose, route, immunity, environmental stresses, and host genetics. *Salmonella* virulence mechanisms have been studied at the biochemical, genetic, cellular, and molecular levels, mostly using animal model systems that have been designed to replicate human *S.* Typhi infection. In recent years, however, there have been excellent studies in animals that have shed light on enteric disease and variations in response of animals to host-adapted and nonadapted serotypes. There is a vast literature regarding how *Salmonella* causes disease, invades host intestinal epithelial cells, survives host phagocytes, and causes enteritis. This research has benefited greatly from genome sequencing projects in the United States and Europe. The Sanger Center (UK) and National Center for Biological Information (NCBI-USA) maintain user-friendly sites for investigators interested in obtaining sequence information regarding these bacteria. The genomes of two clinically important serovars, Typhimurium and Typhi, have been completed. The genomic sequences for serotypes Dublin, Enteritidis, and Choleraesuis are currently being completed. With the genome information available, several centers have produced DNA microarrays to examine global changes in gene expression as the result of various environmental or host challenges. Studies with these methodologies have begun to highlight the enormous variability within *Salmonella* and the spectacular complexity of its interactions with a variety of animal hosts.

REFERENCES

Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis, and F. Heffron. 1999. Salmonella SirA is a global regulator of genes mediating enteropathogenesis. Mol. Microbiol. 31:971–982.

Allen, C. A. 2001. Analysis of *Salmonella enterica* serovar Typhimurium virulence determinants. Ph.D. Thesis. North Carolina State University, Raleigh, NC.

Allen, C. A., P. J. Fedorka-Cray, A. Vazquez-Torres, M. Suyemoto, C. Altier, L. R. Ryder, F. C. Fang, and S. J. Libby. 2001. *In vitro* and *in vivo* assessment of *Salmonella enterica* serovar Typhimurium DT104 virulence. Infect. Immun. 69:4673–4677.

Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer. 2000b. Characterization of two novel regulatory genes affecting Salmonella invasion gene expression. Mol. Microbiol. 35:635–646.

Altier, C., M. Suyemoto, and S. D. Lawhon. 2000a. Regulation of *Salmonella enterica* serovar typhimurium invasion genes by *csrA*. Infect. Immun. 68:6790–6797.

Audia, J. P., C. C. Webb, and J. W. Foster. 2001. Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. Int. J. Med. Microbiol. 291:97–106.

Babior, B. M. 1984. The respiratory burst of phagocytes. J. Clin. Invest. 73:599–601.

Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee. 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. 22:703–714.

Bakshi, C. S., V. P. Singh, M. W. Wood, P. W. Jones, T. S. Wallis, and E. E. Gallyov. 2000. Identification of SopE2, a Salmonella secreted protein which is highly homologous to SopE and involved in bacterial invasion of epithelial cells. J. Bacteriol. 182:2341–2344.

Barrow, P. A., and T. S. Wallis. 2000. Vaccination against *Salmonella* infections in food animals: rationale, theoretical basis and practical application. In Salmonella in domestic animals. C. Wray and A. Wray (eds.). Wallingford, Oxon, UK: CABI Publishing, pp. 323–339.

Battistoni, A., F. Pacello, S. Folcarelli, M. Ajello, G. Donnarumma, R. Greco, M. G. Ammendolia, D. Touati, G. Rotilio, and P. Valenti. 2000. Increased expression of periplasmic Cu, Zn superoxide dismutase enhances survival of *Escherichia coli* invasive strains within nonphagocytic cells. Infect. Immun. 68:30–37.

Baumler, A. J., A. J. Gilde, R. M. Tsolis, A. W. van der Velden, B. M. Ahmer, and F. Heffron. 1997a. Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operons during evolution of Salmonella serotypes. J. Bacteriol. 179:317–322.

Baumler, A. J., R. M. Tsolis, and F. Heffron. 1996a. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. Infect. Immun. 64:1862–1865.

———. 1996b. The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. Proc. Natl. Acad. Sci. U S A 93:279–283.

———. 1997b. Fimbrial adhesins of *Salmonella typhimurium*. Role in bacterial interactions with epithelial cells. Adv. Exp. Med. Biol. 412:149–158.

Baumler, A. J., R. M. Tsolis, F. A. Bowe, J. G. Kusters, S. Hoffmann, and F. Heffron. 1996c. The *pef* fimbrial operon of *Salmonella typhimurium* mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse. Infect. Immun. 64:61–68.

Baumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams. 1998. Evolution of host adaptation in *Salmonella enterica*. Infect. Immun. 66:4579–4587.

Behlau, I., and S. I. Miller 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. 175:4475–4484.

Blanc-Potard, A. B., and E. A. Groisman 1997. The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. EMBO J. 161:5376–5385.

Blanc-Potard, A. B., F. Solomon, J. Kayser and E. A. Groisman. 1999. The SPI-3 pathogenicity island of *Salmonella enterica*. J. Bacteriol. 181:998–1004.

Blum, G., M. Ott, A. Lischewski, A. Ritter, H. Imrich, H. Tschape, and J. Hacker. 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect. Immun. 62:606–614.

Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. Salmonella nomenclature. J. Clin. Microbiol. 38:2465–2467.

Briggs, C. E., and P. M. Fratamico. 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. Antimicrob. Agents. Chemother. 43:846–849.

Buchmeier, N., S. Bossie, C. Y. Chen, F. C. Fang, D. G. Guiney, and S. J. Libby. 1997. SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. Infect. Immun. 65:3725–3730.

Buchmeier, N. A., C. J. Lipps, M. Y. So, and F. Heffron. 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. Mol. Microbiol. 7:933–936.

Buchmeier, N. A., S. J. Libby, Y. Xu, P. C. Loewen, J. Switala, D. G. Guiney, and F. C. Fang. 1995. DNA repair is more important than catalase for *Salmonella* virulence in mice. J. Clin. Invest. 95: 1047–53.

Chamnongpol, S., and E. A. Groisman. 2000. Acetyl phosphate-dependent activation of a mutant PhoP response regulator that functions independently of its cognate sensor kinase. J. Mol. Biol. 300:291–305.

———. 2002. Mg2+ homeostasis and avoidance of metal toxicity. Mol. Microbiol. 44:561–571.

Chan, K., S. Baker, C. C. Kim, C. S. Detweiler, G. Dougan, and S. Falkow. 2003. Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar typhimurium DNA microarray. J. Bacteriol. 185:553–563.

Chengappa, M. M., J. Staats, R. D. Oberst, N. H. Gabbert, and S. McVey. 1993. Prevalence of *Salmonella* in raw meat used in diets of racing greyhounds. J. Vet. Diagn. Invest. 53:372–377.

Cirillo, D. M., E. J. Heffernan, L. Wu, J. Harwood, J. Fierer, and D.G. Guiney. 1996. Identification of a domain in Rck, a product of the *Salmonella typhimurium* virulence plasmid, required for both serum resistance and cell invasion. Infect. Immun. 64:2019–2023.

Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow. 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. 30:175–188.

Collazo, C. M., and J. E. Galan. 1996. Requirement for exported proteins in secretion through the invasion-associated type III system of *Salmonella typhimurium*. Infect. Immun. 649: 3524–31.

———. 1997a. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. Mol. Microbiol. 24:747–756.

———. 1997b. The invasion-associated type-III protein secretion system in Salmonella—a review. Gene 1921:51–59.

Collier-Hyams, L. S., H. Zeng, J. Sun, A. D. Tomlinson, Z. Q. Bao, H. Chen, J. L. Madara, K. Orth, and A. S. Neish. 2002. Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. J. Immunol. 169:2846–2850.

De Groote, M. A., U. A. Ochsner, M. U. Shiloh, C. Nathan, J. M. McCord, M. C. Dinauer, S. J. Libby, A. Vazquez-Torres, Y. Xu, and F. C. Fang. 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. Proc. Natl. Acad. Sci. U S A 94:13997–14001.

Deiwick, J., T. Nikolaus, J. E. Shea, C. Gleeson, D. W. Holden, and M. Hensel**.** 1998. Mutations in Salmonella pathogenicity island 2 SPI2 genes affecting transcription of SPI1 genes and resistance to antimicrobial agents. J. Bacteriol. 180:4775–4780.

Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel. 1999. Environmental regulation of Salmonella pathogenicity island 2 gene expression. Mol. Microbiol. 31:1759–1773.

Demple, B., and J. Halbrook 1983. Inducible repair of oxidative DNA damage in Escherichia coli. Nature 304:466–468.

Edrington, T. S., R. B. Harvey, L. A. Farrington, and D. J. Nisbet. 2001. Evaluation of subtherapeutic use of the antibiotics apramycin and carbadox on the prevalence of antimicrobial-resistant Salmonella infection in swine. J. Food Prot. 64:2067–2070.

Eichelberg, K., and J. E. Galan. 1999. Differential regulation of *Salmonella typhimurium* type III secreted proteins by pathogenicity island 1 SPI-1-encoded transcriptional activators InvF and hilA. Infect. Immun. 67:4099–4105.

Eisenstein, T. K. 2001. Implications of *Salmonella*-induced nitric oxide (NO) for host defense and vaccines: NO, an antimicrobial, antitumour, immunosuppressive and immunoregulatory molecule. Microbes Infect. 3:1223–1231.

El-Gedaily, A., G. Paesold, and M. Krause. 1997. Expression profile and subcellular location of the plasmid-encoded virulence Spv proteins in wild-type *Salmonella dublin*. Infect. Immun. 65: 3406–3411.

Ellermeier, C. D., and J. M. Slauch. 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 185:5096–5108.

Ernst, R. K., T. Guina, and S. I. Miller. 1999. How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. J. Infect. Dis. 179 Suppl. 2:S326–330.

———. 2001. *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect. 3:314–315:1327–34.

Fang, F. C., M. A. DeGroote, J. W. Foster, A. J. Baumler, U. Ochsner, T. Testerman, S. Bearson, J. C. Giard, Y. Xu, G. Campbell, and T. Laessig**.** 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc. Natl. Acad. Sci. U S A 96:7502–7507.

Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney. 1992. The alternative sigma factor katF rpoS regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. U S A 89:11978–11982.

Farr, S. B., and T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55:561–585.

Farrant, J. L., A. Sansone, J. R. Canvin, M. J. Pallen, P. R. Langford, T. S. Wallis, G. Dougan, and J. S. Kroll. 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. Mol. Microbiol. 25: 785–796.

Farrington, L. A., R. B. Harvey, S. A. Buckley, R. E. Droleskey, D. J. Nisbet, and P. D. Inskip. 2001. Prevalence of antimicrobial resistance in Salmonellae isolated from market-age swine. J. Food Prot. 64:1496–1502.

FDA/USDA/CDC. 2001. FDA/USDA/CDC National Antimicrobial Resistance Monitoring System—enteric bacteria veterinary isolates final report. http//:www.arru.saa.ars.usda.gov/narms/narms/htm.

Feng, X., R. Oropeza, and L. J. Kenny. 2003. Dual regulation by phospho-OmpR of *ssrA/B* gene expression in Salmonella pathogenicity island 2. Mol. Microbiol. 48:1131–1143.

Fields, P. I., E. A. Groisman, and F. Heffron. 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science 243:1059–1062.

Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron. 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. U S A 83:5189–5193.

Finlay, B. B. 1994. Molecular and cellular mechanisms of Salmonella pathogenesis. Curr. Top. Microbiol. Immunol. 192:163–185.

Finlay, B. B., and S. Falkow. 1989a. Common themes in microbial pathogenicity. Microbiol. Rev. 53:210–230.

———. 1989b. Salmonella as an intracellular parasite. Mol. Microbiol. 31:1833–1841.

———. 1990. Salmonella interactions with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. 162:1096–1106.

———. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61: 136–169.

Francis, C. L., M. N. Starnbach, and S. Falkow. 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. Mol. Microbiol. 62:3077–3087.

Freudenberg, M. A., T. Merlin, M. Gumenscheimer, C. Kalis, R. Landmann, and C. Galanos. 2001. Role of lipopolysaccharide susceptibility in the innate immune response to *Salmonella typhimurium* infection: LPS, a primary target for recognition of Gram-negative bacteria. Microbes Infect. 3:1213–1222.

Friedman, C. R., C. Torigian, P. J. Shillam, R. E. Hoffman, D. Heltzel, J. L. Beebe, G. Malcolm, W. E. DeWitt, L. Hutwagner, and P. M. Griffin. 1998. An outbreak of salmonellosis among children attending a reptile exhibit at a zoo. J. Pediatr. 132:802–807.

Friedrich, M. J., N. E. Kinsey, J. Vila, and R. J. Kadner. 1993. Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. Mol. Microbiol. 8: 543–58.

Fu, Y., and J. E. Galan. 1998. The *Salmonella typhimurium* tyrosine phosphatase SptP is translocated into host cells and disrupts the actin cytoskeleton. Mol. Microbiol. 27:359–368.

Galan, J. E. 1996. Molecular genetic bases of Salmonella entry into host cells. Mol. Microbiol. 20:263–271.

———. 2001. *Salmonella* interactions with host cells: type III secretion at work. Annu. Rev. Cell. Dev. Biol. 17:53–86.

Galan, J. E., and R. Curtiss, III. 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. 58:1879–1885.

Galan, J. E., J. Pace, and M. J. Hayman. 1992. Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhimurium*. Nature 357:588–589.

Galyov, E. E., M. W. Wood, R. Rosqvist, P. B. Mullan, P. R. Watson, S. Hedges, and T. S. Wallis. 1997. A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. Mol. Microbiol. 25: 903–912.

Garcia-del Portillo, F. G. 2001. *Salmonella* intracellular proliferation: where, when, and how? Microbes Infect. 3:1305–1311.

Garcia Vescovi, E., F. C. Soncini, and E. A. Groisman. 1996. Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. Cell 84:165–174.

Gebreyes, W. A., and C. Altier 2002. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolates from swine. J. Clin. Microbiol. 40:2813–2822.

Gebreyes, W. A., P. R. Davies, W.E. Morrow, J.A. Funk, and C. Altier. 2000. Antimicrobial resistance of Salmonella isolates from swine. J. Clin. Microbiol. 38:4633–4636.

Gort, A. S., D. M. Ferber, and J. A. Imlay. 1999. The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. Mol. Microbiol. 32:179–191.

Groisman, E. A., and H. Ochman 1997. How *Salmonella* became a pathogen. Trends Microbiol. 5:343–349.

Guiney, D. G., F. C. Fang, M. Krause, and S. Libby. 1994. Plasmid-mediated virulence genes in non-typhoid Salmonella serovars. FEMS Microbiol. Lett. 124:1–9.

Guiney, D. G., F. C. Fang, M. Krause, S. Libby, N. A. Buchmeier, and J. Fierer. 1995. Biology and clinical significance of virulence plasmids in *Salmonella* serovars. Clin. Infect. Dis. 21 Suppl 2: S146–51.

Gulig, P. A. 1990. Virulence plasmids of *Salmonella typhimurium* and other salmonellae. Microb. Pathog. 8:3–11.

Gulig, P. A., and T. J. Doyle. 1993. The *Salmonella typhimurium* virulence plasmid increases the growth rate of salmonellae in mice. Infect. Immun. 612: 504–11.

Hansen-Wester, I., and M. Hensel 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes Infect. 3:549–559.

Hardt, W. D., and J. E. Galan 1997. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. Proc. Natl. Acad. Sci. USA 94:9887–9892.

Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galan. 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell 93:815–826.

Henderson, I. R., J. Czeczulin, C. Eslava, F. Noriega, and J. P. Nataro. 1999. Characterization of Pic, a secreted protease of *Shigella flexneri* and enteroaggregative *Escherichia coli*. Infect. Immun. 67:5587–5596.

Hensel, M., J. E. Shea, A. J. Baumler, C. Gleeson, F. Blattner, and D. W. Holden. 1997. Analysis of the boundaries of Salmonella pathogenicity island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. J. Bacteriol. 1794:1105–1111.

Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science 269:400–403.

Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden. 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. 301:163–74.

Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky. 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. U S A 96:2396–2401.

Hohmann, A. W., G. Schmidt, and D. Rowley. 1978. Intestinal colonization and virulence of *Salmonella* in mice. Infect. Immun. 22:763–770.

Hou, Y. M. 1999. Transfer RNAs and pathogenicity islands. Trends Biochem. Sci. 248:295–298.

Jepson, M. A., and M. A. Clark. 2001. The role of M cells in *Salmonella* infection. Microbes Infect. 3:1183–1190.

Jones, B. D., and S. Falkow. 1996. Salmonellosis: host immune responses and bacterial virulence determinants. Annu. Rev. Immunol. 14:533–561.

Jones, B. D., H. F. Paterson, A. Hall, and S. Falkow. 1993. *Salmonella typhimurium* induces membrane ruffling by a growth factor-receptor-independent mechanism. Proc. Natl. Acad. Sci. USA 90:10390–10394.

Jones, B. D., N. Ghori, and S. Falkow. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. 180:15–23.

Jones, S. L. 2000. *Salmonella*: a troublesome intracellular pathogen. Proc. 18th ACVIM, Seattle, WA, pp. 206–208.

Kaneko, A., M. Mita, K. Sekiya, H. Matsui, K. Kawahara, and H. Danbara. 2002. Association of a regulatory gene, *slyA* with a mouse virulence of *Salmonella* serovar Choleraesuis. Microbiol. Immunol. 46:109–113.

Kauffmann, F. 1966. The bacteriology of *Enterobacteriaceae*. Copenhagen, Denmark: Munksgaard.

Kingsley, R. A., and A. J. Baumler 2002. Pathogenicity islands and host adaptation of Salmonella serovars. Curr Top Microbiol. Immunol. 2641:67–87.

Knodler, L. A., and B. B. Finlay. 2001. *Salmonella* and apoptosis; to live or let die? Microbes Infect. 3:1321–1326.

Lee, A. K., C. S. Detweiler, and S. Falkow. 2000. OmpR regulates the two-component system SsrA-ssrB in *Salmonella* pathogenicity island 2. J. Bacteriol. 182:771–781.

Lee, C. A., B. D. Jones, and S. Falkow. 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. U S A 89:1847–1851.

Lee, I. S., J. Lin, H. K. Hall, B. Bearson, and J. W. Foster. 1995. The stationary-phase sigma factor sigma S RpoS is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol. Microbiol. 17:155–67.

Le Minor, L., and M. Y. Popoff. 1987. Request for an opinion. Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*. Int. J. Syst. Bacteriol. 37:465–468.

Lesnick, M. L., N. E. Reiner, J. Ferier, and D. G. Guiney. 2001. The *Salmonella* spvB virulence gene

encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. Mol. Microbiol. 39:1464–1470.

Liu, G. R., A. Rahn, W. Q. Liu, K. E. Sanderson, R. N. Johnston, and S. L. Liu. 2002. The evolving genome of *Salmonella enterica* serovar Pullorum. J. Bacteriol. 184:2626–2633.

Lucas, R. L., and C. A. Lee 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 183:2733–2745.

Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee. 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar typhimurium. J. Bacteriol. 182: 1872–1882.

Maestroni, P., and N. Ménager. 2003. Development of acquired immunity to *Salmonella*. J. Med. Microbiol. 52: 453–459.

McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413:852–856.

McCormick, B. A., S. P. Colgan, C. Delp-Archer, S. I. Miller, and J. L. Madara. 1993. *Salmonella typhimurium* attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. J. Cell Biol. 123:895–907.

McDonough, P. L., D. Fogelman, S. J. Shin, M. A. Brunner, and D. H. Lein. 1999. *Salmonella enterica* serotype Dublin infection: an emerging infectious disease for the northeastern United States. J. Clin. Microbiol. 37: 2418–2427.

Meyerholz, D. K., and T. J. Stabel 2003. Comparison of early ileal invasion by *Salmonella enterica* Serovars Choleraesuis and Typhimurium. Vet. Pathol. 40:371–375.

Meyerholz, D. K., T. J. Stabel, M. R. Ackermann, S. A. Carlson, B. D. Jones, and J. Pohlenz. 2002. Early epithelial invasion by *Salmonella enterica* serovar Typhimurium DT104 in the swine ileum. Vet. Pathol. 39:712–720.

Mills, D. M., V. Bajaj, and C. A. Lee. 1995. A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. Mol. Microbiol. 15:749–759.

Monack, D. M., W. W. Navarre, and S. Falkow. 2001. Salmonella-induced macrophage death: the role of caspase-1 in death and inflammation. Microbes Infect. 3:1201–1212.

Murray, M. J. 1996. Salmonellosis in horses. J. Am. Vet. Med. Assoc. 209:558–560.

Murray, R. A., and C. A. Lee. 2000. Invasion genes are not required for *Salmonella enterica* serovar typhimurium to breach the intestinal epithelium: evidence that salmonella pathogenicity island 1 has alternative functions during infection. Infect. Immun. 68:5050–5055.

NARMS (National Antimicrobial Resistance Monitoring System). 2000. Annual report. http://www.cdc.gov/narms/annual/2000/annual_pdf.

Nielsen, B., L. Alban, H. Stege, L. L. Sorensen, V. Mogelmose, J. Bagger, J. Dahl, and D. L. Baggesen. 2001. A new *Salmonella* surveillance and control programme in Danish pig herds and slaughterhouses. Berl. Munch. Tierarztl. Wochenschr. 114:323–326.

Norris, F. A., M. P. Wilson, T. S. Wallis, E. E. Galyov, and P. W. Majerus. 1998. SopB, a protein required for virulence of *Salmonella dublin*, is an inositol phosphate phosphatase. Proc. Natl. Acad. Sci. U S A 95:14057–14059.

Ochman, H., and A. C. Wilson 1987. Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. J. Mol. Evol. 261–2:74–86.

Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman. 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. U S A 9315:7800–7804.

Pacelli, R., D. A. Wink, J. A. Cook, M. C. Krishna, W. DeGraff, N. Friedman, M. Tsokos, A. Samuni, and J. B. Mitchell. 1995. Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. J. Exp. Med. 1825:1469–1479.

Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller. 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. Mol. Microbiol. 17:169–181.

Pelludat, C., S. Mirold, and W. D. Hardt. 2003. The SopEPhi phage integrates into the *ssrA* gene of *Salmonella enterica* serovar Typhimurium A36 and is closely related to the Fels-2 prophage. J. Bacteriol. 185:5182–5191.

Popoff, M. Y., and L. Le Minor. 1997. Antigenic formulas of the *Salmonella* serovars, 7th revision. Paris, France: World Health Organization Collaborating Centre for Reference and Research on *Salmonella*, Pasteur Institute.

Popoff, M. Y., J. Bockemuhl, and F. W. Brenner. 2000. Supplement 1998 no. 42 to the Kauffmann-White scheme. Res. Microbiol. 151:63–65.

Poppe, C., N. Smart, R. Khakhria, W. Johnson, J. Spika, and J. Prescott. 1998. *Salmonella typhimurium* DT104: a virulent and drug-resistant pathogen. Can Vet. J. 399:559–65.

Prager, R., W. Rabsch, W. Streckel, W. Voight, E. Tietze, and H. Tschape. 2003. Molecular properties of *Salmonella enterica* serotype Paratyphi B distinguish between its systemic and its enteric pathovars. J. Clin. Microbiol. 41:4270–4278.

Rabsch, W., H. Tschape, and A. J. Baumler. 2001. Non-typhoidal salmonellosis: emerging problems. Microbes Infect. 3:237–247.

Rabsch, W., H. L. Andrews, R. A. Kingsley, R. Prager, H. Tschape, L. G. Adams, and A. J. Baumler. 2002. *Salmonella enterica* serotype Typhimurium and its host-adapted variants. Infect. Immun. 70:2249–2255.

Rantala, M., and E. Nurmi 1973. Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. Br. Poult. Sci. 146:627–630.

Reeves, M. W., G. M. Evins, A. A. Heiba, B. D. Plikaytis, and J. J. Farmer III. 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. J. Clin. Microbiol. 27:313–320.

Santos, R. L., S. Zhang, R. M. Tsolis, R. A. Kingsley, L. G. Adams, and A. J. Baumler. 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. Microbes Infect. 3:1335–1344.

Schechter, L. M., S. M. Damrauer, and C. A. Lee. 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the *hilA* promoter. Mol. Microbiol. 32:629–642.

Schmitt, C. K., J. S. Ikeda, S. C. Darnell, P. R. Watson, J. Bispham, T. S. Wallis, D. L. Weinstein, E. S. Metcalf, and A. D. O'Brien. 2001. Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. Infect. Immun. 69:5619–5625.

Shea, J. E., C. R. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden. 1999. Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infect. Immun. 67:213–219.

Smith, R. L., M. T. Kaczmarek, L. M. Kucharski, and M. E. Maguire. 1998. Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* and *mgtCB* during invasion of epithelial and macrophage cells. Microbiology 144:1835–1843.

Soncini, F. C., E. Garcia Vescovi, F. Solomon, and E. A. Groisman. 1996. Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. J. Bacteriol. 178:5092–5099.

Spier, S. J. 1993. Salmonellosis. Vet. Clin. North Am. Equine Pract. 9:385–397.

Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt. 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol. Microbiol. 36: 1206–1211.

Takeuchi, A. 1967. Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Pathol. 50:109–136.

Testerman, T. L., A. Vazquez-Torres, Y. Xu, J. Jones-Carson, S. J. Libby, and F. C. Fang. 2002. The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. Mol. Microbiol. 43:771–782.

Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. Stevens, S. Maloy, J. Parkhill, G. Dougan, and A. J. Baumler. 2001. Serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infect. Immun. 69:2890–2894.

Tsolis, R. M., A. J. Baumler, and F. Heffron. 1995. Role of *Salmonella typhimurium* Mn-superoxide dismutase SodA in protection against early killing by J774 macrophages. Infect. Immun. 635:1739–1744.

Tsolis, R. M., L. G. Adams, M. J. Hantman, C. A. Scherer, T. Kimbrough, R. A. Kingsley, T. A. Ficht, S. I. Miller, and A. J. Baumler. 2000. SspA is required for lethal *Salmonella enterica* serovar Typhimurium infections in calves but is not essential for diarrhea. Infect. Immun. 68:3158–3163.

Tsolis, R. M., L. G. Adams, T. A. Ficht, and A. J. Baumler. 1999. Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. Infect. Immun. 67:4879–4885.

Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman. 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. EMBO J. 181:3924–3933.

Valdivia, R. H., and S. Falkow. 1997. Probing bacterial gene expression within host cells. Trends Microbiol. 5:360–363.

van der Velden, A. W., A. J. Baumler, R. M. Tsolis, and F. Heffron. 1998. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. Infect. Immun. 66:2803–2808.

Vazquez-Torres, A., and F. C. Fang. 2001. *Salmonella* evasion of the NADPH phagocyte oxidase. Microbes Infect. 3:1313–1320.

Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang. 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science 2875458: 1655–1658.

Watson, P. R., S. M. Paulin, P. W. Jones, and T. S. Wallis. 2000. Interaction of *Salmonella* serotypes with porcine macrophages in vitro does not correlate with virulence. Microbiology 146:1639–1649.

Wick, M. J. 2003. The role of dendritic cells in the immune response to *Salmonella*. Immunol. Lett 85:99–102.

Wong, K. K., M. McClelland, L. C. Stillwell, E. C. Sisk, S. J. Thurston, and J. D. Saffer. 1998. Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a *Salmonella* pathogenicity island located at 92 minutes on the chromosome map of *Salmonella enterica* serovar typhimurium LT2. Infect. Immun. 66:3365–3371.

Wood, M. W., M. A. Jones, P. R. Watson, S. Hedges, T. S. Wallis, and E. E. Galyov. 1998. Identification of a pathogenicity island required for Salmonella enteropathogenicity. Mol. Microbiol. 29:883–891.

Yethon, J. A., J. S. Gunn, R. K. Ernst, S. I. Miller, L. Laroche, D. Malo, and C. Whitfield. 2000. *Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence *in vivo*. Infect. Immun. 68:4485–4491.

Zhang, S., R. A. Kingsley, R. L. Santos, H. Andrews-Polymenis, M. Raffatellu, J. Figueiredo, J. Nunes, R. M. Tsolis, L. G. Adams, and A. J. Baumler. 2003. Molecular pathogenesis of *Salmonella enterica* serotype typhimurium-induced diarrhea. Infect Immun. 71:1–12.

Zhou, D., M. S. Mooseker, and J. E. Galan. 1999. Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. Science 283:2092–2095.

15
E. coli Shigella

T. Adam and C. L. Gyles

Members of the former "genus" Shigella comprising the four "species" *S. dysenteriae, S. flexneri, S. sonnei,* and *S. boydii* are now recognized as a group of *E. coli* clones that cause bacillary dysentery in humans and primates (Pupo et al. 1997, 2000; Lan and Reeves 2002). Phylogenetically, several lineages of *E. coli* Shigella clones have independently acquired the pINV plasmid conferring most of the pathogenetic traits of these bacteria. The three earliest derivatives have each given rise to variants of LPS composition, thus forming three clusters of *E. coli* Shigella serogroups. Clusters 1 and 2 arose 50,000–270,000 years ago and cluster 3 arose 35,000–170,000 years ago (Pupo et al. 2000). Five more recently emerged clones are serologically homogenous bringing the total number of currently recognized *E. coli* Shigella serogroups to 46 (fig. 15.1). Thus, Shigella seems to have diverged from nonpathogenic *E. coli* quite recently, correlating with the appearance of humans in the Paleolithic (Pupo et al. 2000).

Following a suggestion of Pupo et al. (2000), former Shigella species are referred to as *E. coli* Dysenteriae, *E. coli* Flexneri, *E. coli* Sonnei, or *E. coli* Boydii, respectively, while Shigella (not in italics) is meant to comprise all *E. coli* Shigella clones. Shigella are nonmotile, lactose and lysine decarboxylase negative bacteria. While the organism is quite fragile and requires rapid specimen transport for bacteriological diagnostics, Shigella clones are relatively acid resistant, facilitating passage through the stomach (Gorden and Small 1993). They are well adapted to a limited range of hosts. Thus, humans can be infected with infectious doses of as little as ten to one hundred bacteria (DuPont et al. 1989). *E. coli* Dysenteriae 1, the classical Shigella type strain described in 1887 and initially designated "Bacillus

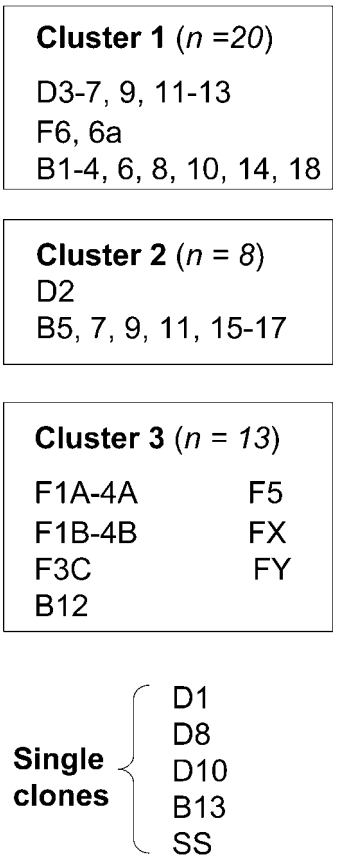


Figure 15.1. Phylogenetic classification of *E. coli* Shigella clones. *E. coli* Shigella clones have evolved eight times from nonpathogenic *E. coli.* Three early Shigella clones have given rise to three clusters of various serogroups while five more recently evolved clones are serologically homogeneous. D = dysenteriae, F = Flexneri, B = Boydii, SS = Sonnei. (Pupo et al. 2000)

dysentericus" (Yabuuchi 2002), seems to be the most virulent Shigella clone and can cause large epidemics.

DISEASE

Bacillary dysentery is endemic worldwide with an estimated 163 million human infections in developing countries and 1.5 million cases in industrialized countries (Kotloff et al. 1999). The disease is most prevalent in children from 0 to 4 years of age. In developing countries, 1.1 million humans are estimated to die from shigellosis each year, 60% being children younger than 5 years. There are approximately 3,000 human deaths annually in industrialized countries. Transmission is via the fecal-oral route. Due to the low infectious dose, transmission may occur not only by direct contact but also via contaminated food or drinking water. Shigellosis is also described as a sexually transmitted disease in homosexual men (Wolbert 1983). The incubation period is related to the number of ingested bacteria and varies from 1 to 4 days. After a short prodromal period with fever, headache, and fatigue, invasive intestinal disease is typically characterized by nausea, cramps, and frequent passage of low volumes of stool that contain blood, mucus, and pus, though initial discharges may resemble watery diarrhea. This clinical picture corresponds to recto-sigmoidal invasion of the pathogen followed by retrograde spread of the organisms to the colon. The proximal extent of ulcerative colitis is associated with severity of infection (Speelman et al. 1984). The clinical course may include intestinal complications: rectal prolapse, toxic megacolon, or colonic perforation. Extraintestinal complications include dehydration (may be lethal in children and the elderly), septicemia (*E. coli* Dysenteriae 1), neurologic manifestations (convulsions, seizures, encephalopathy), hemolytic uremic syndrome (HUS), and reactive arthritis (ReA) with or without urethritis and conjunctivitis (Reiter's syndrome). HUS only occurs after infections with *E. coli* Dysenteriae 1 and is associated with the ability of this strain to synthesize Shiga toxin. ReA preferentially occurs in individuals of the HLA-B27 histocompatibility group.

SHIGELLOSIS IN ANIMALS

Infection of animals with Shigella is of threefold relevance.

1. Shigella is one of the important bacterial infections of captive monkeys, in which it is frequently responsible for outbreaks of acute disease. Old world primates are most commonly affected but new world primates and apes may also be infected. Young animals appear to be more susceptible than adults. *E. coli* Dysenteriae is rarely implicated and *E. coli* Flexneri is most commonly associated with infection. While excretion of *E. coli* Shigella ranges from 0–70% among symptomatic and carrier monkeys in captivity (Banish et al. 1993; Vore et al. 2001), epidemiological data on free-living animals is scarce. Increasing prevalence of Shigella excretion in free-ranging gorillas habituated to humans indicates that anthropozoonotic transmission of the pathogen may be important in these cases (Nizeyi et al. 2001). Stresses, including the stress of altered environment, may precipitate disease in carrier animals. Rapid spread of infection in nonhuman primate colonies is facilitated by defecation on the cage floor and contact of food with the contaminated floor. Clinical signs include diarrhea (bloody or watery), rectal prolapse, edema of the face and neck, inappetence, and depression. Reactive arthritis has been reported as a sequel to shigellosis in primate colonies (Urvater et al. 2000). Severe gingivitis has been reported to be caused by Shigella infection and may occur with or without shedding of the organisms in the feces. Control measures are based on quarantine of newly acquired primates, thorough cleaning and sterilization of cages, prompt waste disposal, control of insects, and prevention of crowding. There are reports of successful use of a combination of antibiotic therapy and management changes to eliminate shigellosis and possibly the carrier state as well in a colony (Wolfensohn 1998). Shigella is not implicated in disease in other animal species. However, the organism may be recovered for limited periods from dogs in highly endemic areas.
2. Monkeys, at least in captivity, may carry and excrete Shigella for months (Banish et al. 1993) and thus serve as a Shigella reservoir for human infections. Only minimal contact is required for transmission to occur, as the infectious dose for humans is very low.
3. There is a great variety of animal models for human shigellosis. While experimental infection of monkeys with *E. coli* Shigella mimics clinical and histopathological aspects of

human disease (Ogawa et al. 1966; Takasaka et al. 1969; Karnell et al. 1991), infectious doses required to induce animal disease are in the range of 10^{10} bacteria compared to 10^2 in human infections. Nonmonkey animal models are used for studies of particular aspects of human disease. Thus, invasive properties of virulent strains can be studied using an experimental conjunctivitis model in guinea pigs (Sereny 1957) or an ileal-loop model in rabbits (Arm et al. 1965; Rabbani et al. 1995). Inflammatory responses to Shigella can be studied in a pulmonary model in mice (Way et al. 1999). Shigella-induced apoptosis has been studied in the ileal-loop model in rabbits or using knockout mice (Sansonetti et al. 2000). Mouse models are also used to study neurological complications of shigellosis (Nofech-Mozes et al. 2000).

VIRULENCE FACTORS OF *E. COLI* SHIGELLA

Pathogenic *E. coli* Shigella clones differ from nonpathogenic *E. coli* strains in several respects that may have contributed to access to, spread between, and replication within enterocytes as a novel ecological niche for *E. coli*. Most important, Shigella clones have picked up pathogenicity factors via horizontal gene transfer as parts of mobile genetic elements. Thus, Shigella genomes were found to contain large numbers of phage-specific or insertion (IS) sequences. Many of these elements are still mobile, but some have lost mobility. Acquired virulence genes may be part of the 213 kbp virulence plasmid (pINV) or of chromosomal loci including pathogenicity islands (PAI). In addition to acquisition of virulence genes, recent data indicate that loss of genes regularly present in nonpathogenic *E. coli* may be important for expression of full virulence by Shigella (Maurelli et al. 1998). It is assumed that in a primary step, some *E. coli* clones acquired genes of the invasion plasmid necessary for infection of and replication within enterocytes. Further adaptation to the novel niche was accompanied by changes to the chromosome including acquisition of pathogenicity islands and loss of genes that could impede Shigella's novel intracellular lifestyle. Since Shigella clones do not have a single evolutionary origin, phenotypic properties that are common to all or most Shigella clones (e.g., inability to ferment lactose, decarboxylate lysine, or move by means of flagella) are the result of convergent evolution. In general, genes for invasion of and spread within the colonic epithelial cell layer are located on the large virulence plasmid while some regulatory elements as well as an enterotoxin, serine proteases, colicin immunity factors, an iron acquisition system, or proteins for modification of lipopolysaccharide (LPS) are encoded by the chromosome. Important virulence factors of Shigella are summarized in table 15.1.

GENOME

The genomes of the two *E. coli* Flexneri 2a strains that have been sequenced comprise 4.6×10^6 bp with an average G + C content of 51%. The genome is interspersed with multiple mobile elements including about 300 IS elements on the chromosome and about 90 IS elements on the virulence plasmid pINV, making the Shigella genome the most IS-rich among Enterobacteriaceae (Buchrieser et al. 2000; Jin et al. 2002; Wei et al. 2003). Most virulence factors are part of PAIs situated on the chromosome or on pINV. Seven gene islands are associated with genes encoding tRNAs: *selC*, *leuX*, *aspV*, *asnT*, *argW*, *pheV*, and *glyU,* four of them include integrases (Wei et al. 2003). In addition to the PAIs described below, eight other islands are associated with iron uptake and utilization or code for putative adhesins (Wei et al. 2003). The *E. coli* Shigella genome is characterized not only by the acquisition of PAIs but also by the presence of a large number of pseudogenes. Loss of gene functions is thought to be associated with adoption of an intracellular lifestyle in a limited range of hosts, similar to *S. typhi* (Jin et al. 2002).

PATHOGENICITY ISLANDS (PAIS)

PAIs are typically stretches of 10–200 kb of DNA that differ in G + C content from core sequences of the host microorganism, code for virulence factors and mobility genes, are flanked by direct repeats, and are often associated with tRNA genes (Hacker and Kaper 2000). While most PAIs are integrated into the chromosome, PAIs may also be situated on plasmids (Hacker and Kaper 2000). PAIs associated with tRNA genes can often be deleted from the genome leaving a truncated tRNA gene. Therefore, tRNA genes with identical copies in the genome (e.g., pheV/pheU), with wobble capacity or nonessential tRNAs (e.g., *selC*) serve as integration sites for PAIs.

Shi-1 PAI (*she* PAI)

This PAI comprises 46603 bp, is located at the 3'-terminus of *pheV* tRNA, contains direct repeats at the boundaries of the PAI and shows similarities

Table 15.1. Virulence Factors of *E. coli* Shigella

Factor or gene	Location of gene(s)					Possible function; comment
	Chromosome			Plasmid		
	Shi-1	Shi-2	Other	pINV	pHS-2	
IpaA				x		Invasin; impedes actin polymerization beneath entering bacterium
IpaB				x		Translocator of TTSS, invasin, apoptosis, regulation of TTSS
IpaC				x		Translocator of TTSS, invasin, induction of macropinocytosis
IpaD				x		Invasin, regulation of TTSS
IpgB1				x		Regulation of TTSS
IpgD				x		Invasin, PIP_2-phosphatase
mxi operon				x		Structure and function of TTSS
spa operon				x		Structure and function of TTSS
IpgC				x		Chaperone
IpgE				x		Chaperone
Spa15				x		Chaperone
IpaH9.8				x		?; transported to host cell nucleus, member of *ipalt* gene family, LRR motif
IcsB				x		Lysis of vacuole; cell-to-cell spread
VirB				x		Regulator of *1pa-mxi-spa* operons and of *virA*
virA				x		*cis*-acting effect on *icsA* promoter
VirF				x		Positive regulator of *virB* and *icsA*
SepA				x		Tissue invasion; autotransporter, IgA1 protease family
senA/ospD3				x		Encodes enterotoxin ShET2
MsbB2				x		Lipid A synthesis; inflammatory response to LPS
MsbB1			x			Lipid A synthesis; inflammatory response to LPS
Wzz_{pHS-2}					x	Long O side chains of LPS; partial masking of icsA; intracellular movement
Set1A	x					Subunit of enterotoxin ShET1; in F2a
Set1B	x					Subunit of enterotoxin ShET1; in F2a
SigA	x					IgA protease homolog; autotransporter
Imm/ShiD		x				Immunity to colicin V
StxB			x			Subunit of Shiga toxin; in D1
Tgt			x			tRNA modification; translation of VirF
MiaA			x			tRNA modification; translation of VirF
Fis			x			Positive regulator of *virF*
IHF			x			Positive regulator of *virF*
H-NS			x			Negative regulator of *virF*, *virB, ipa-mxi-spa* operons. *IcsA*
IspA			x			Intercellular spread
VpsC			x			Intercellular spread
VacJ			x			Intercellular spread

with the locus of enterocyte effacement (LEE) PAI of enterohemorrhagic E. coli (EHEC). This PAI is an unstable genetic element that deletes at a frequency of 10^{-5}–10^{-6} (Rajakumar et al. 1997b).

Apart from phage-associated and mobile genetic elements that played a role in transfer and composition of the PAI, this island encodes the following virulence genes: (1)*sigA*, which encodes a 139.6 kDa *S*higella IgA-like protease homolog that belongs to a family of autotransporter proteases; (2) *pic*, which encodes a 146 kDa (*p*rotein involved in *i*ntestinal *c*olonization) autotransporter protease with mucinase, serum resistance (PMSF-inhibitable intrinsic protease activity required), and hemagglutination activities (Henderson et al. 1999); (3) *set1A* and *set1B,* which encode the 55 kDa *Sh*igella *ent*erotoxin ShET1 consisting of SetA (20 kDa) and SetB (7 kDa); and (4) *sap* (*s*imilarity to *a*utotransported surface *p*rotein), which encodes a paralog of a protein that mediates autoaggregation in *E. coli*.

SHI-2 PAI (*selC* PAI)

This recently described PAI is located at a tRNA gene that has been shown to be a frequent integration site of phages, the *selC* locus (Moss et al. 1999; Vokes et al. 1999). This PAI comprises the aerobactin operon and shows some similarity to the pColV-K30 plasmid. IucA-D form the siderophore, IutA is the bacterial receptor for the iron-siderophore complex, and Imm (in *E. coli* Flexneri 2a) or ShiD (in *E. coli* Flexneri 5) confers immunity to colicin V. In addition, this PAI encodes genes of unknown function (*shiA-G)*.

SRL PAI, Shigella Resistance Locus

The locus encodes resistance to streptomycin, ampicillin, chloramphenicol, and tetracycline(Rajakumar et al. 1997a). The SRL resistance locus is situated on a deletable element that is part of the larger PAI (66 kb). The PAI is integrated into the 3'-end of tRNA gene *serX* or its paralog *serW* (Turner et al. 2003) and is again part of a larger structure, MRDE (*m*ultiple-antibiotic-*r*esistance *d*eletable *e*lement). It is interesting that nested deletions of all three genetic structures have been observed (Turner et al. 2001). Deletion of the 16.7 kb SRL locus involves IS*1* elements, the deletion of the 66 kb PAI is integrase-mediated, while deletion of the 99 kb MRDE involves flanking IS*91* elements. The SRL PAI contains components of plasmid-, transposon-, and integron-encoded resistance determinants (Rajakumar et al. 1997a; Luck et al. 2001) and has been found in cluster 1 and cluster 3 Shigella as well as in some strains of *E. coli* Sonnei (Turner et al. 2003). In addition, the SRL PAI also incorporates components of an iron transport system (Luck et al. 2001). The two completely sequenced *E. coli* Flexneri 2a strains did not contain the SRL PAI.

Shi-O PAI, Serotype-specifying Locus

It has long been known that *E. coli* Flexneri serogroups 1–5 can be interconverted by infection with different bacteriophages (Petrovskaya and Bondarenko 1995). The serogroups differ by variations of the basic O-antigen tetrasaccharide unit represented by serogroup Y. Three phage-encoded genes are required for conversion of serogroups, *gtrA*, *gtrB,* and $gtr_{(type)}$. While the first two genes are highly conserved among serotype-converting phages, the type-specific *gtr(type)* gene confers serogroup specificity. The functions of these proteins are not completely understood. GtrA could be a flippase for transport of the UndPP-O-unit precursor across the inner membrane (Guan et al. 1999). *GtrB* encodes a bactoprenol glucosyltransferase and *gtr(type)* encodes a site-sensitive glucosyltransferase. The genes conferring serogroup specificity to *E. coli* Flexneri 1a are part of a pathogenicity island resembling a bacteriophage that has lost its ability to excise from the chromosome. This 10.6 kb PAI is situated between the *dsdC* (D-serine dehydratase) and the *thrW* tRNA genes; the G + C content of this PAI is 40% compared to 51% of the total Shigella genome indicating acquisition of this PAI by horizontal transfer.

O Antigen Locus

Lipopolysaccharide (LPS) consists of three modules: lipid A, core sugars, and the O side chain. Lipid A consists of a glucosamine disaccharide with one N- and one O-linked hydroxy fatty acyl chain on each glucosamine. The hydroxy fatty acids of one glucosamine carry additional fatty acids as acyl-oxy-acyl substituents on the hydroxyl groups. The gene product that catalyzes acyl-oxy-acylation of the fatty acid at the 3'-position has recently been associated with the capacity of lipid A to induce TNF-alpha-mediated responses during Shigella infection (d'Hauteville et al. 2002). Lipid A and the core sugars are synthesized in one continuous process at the cytoplasmic side of the inner membrane, then translocated (flipped) through the membrane to the periplasmic side. Similarly, repetitive units of O antigens are synthesized on an isoprenoid carrier in the inner membrane, flipped, polymerized to form complete

O chains, and subsequently transferred to the core sugars of lipid A/core to make LPS, which is then transported to and constitutes the major component of the outer leaflet of the outer membrane. In *E. coli*, lipid A and the inner core are conserved, while the outer core and the O antigen are variable.

It is interesting that genes for the synthesis of lipid A and the inner core are scattered within the *E. coli* genome, while genes for the synthesis of the more variable parts seem to be clustered on gene islands (Reeves and Wang 2002). LPS is an important virulence factor of Shigella involved in invasion, induction of inflammation, and tissue destruction (Hong and Payne 1997). In addition, the highly variable O antigen has been used for serotyping. Shigella strains express 1 of 33 distinct O antigens (there exist 187 *E. coli* O antigens including Shigella). Phage-encoded modifications of these antigens and the observation that *E. coli* Dysenteriae 2 and *E. coli* Boydii 15 possess identical O antigens, explain the number of 46 currently recognized serotypes (Wang et al. 2001). The chromosomal locus comprising the genes for O-antigen synthesis is situated between the *galF* and the *gnd* genes and encodes proteins for synthesis of sugar precursors, transfer of sugars to build the O unit, and genes for specific assembly or processing steps (e.g., flippase, O antigen polymerase) (Wang et al. 2001). LPS is a major target of the immune system, and variations of these highly exposed molecules may therefore bring a selective advantage over other O antigens. The high variability seen in this locus is probably the result of intra- or interspecies horizontal gene transfer.

PLASMIDS

pINV

Acquisition of the large virulence plasmid pINV is thought to have been the primary step of divergence of Shigella clones from nonpathogenic *E. coli*. Essential virulence traits like invasion of, motility in, and spread between epithelial cells, or induction of apoptosis in macrophages are encoded by the large virulence plasmid. Access to the cytoplasm of eukaryotic cells fundamentally changed the lifestyle of and selection pressure on Shigella leading to an accumulation of pseudogenes whose products were required no more in the new ecological niche. On the other hand, new traits optimizing intracellular life were incorporated into the genome. Thus, pINV probably contains the oldest Shigella-specific DNA. However, pINV shows the highest percentage of mobile elements of all known plasmids. Indeed, complete sequencing of the virulence plasmids from *E. coli* Flexneri 5 and 2a strains showed that the 213 kbp plasmid is a mosaic of blocks of DNA from different origins as indicated by varied G + C contents (Buchrieser et al. 2000; Venkatesan et al. 2001; Jin et al. 2002; Wei et al. 2003). In addition, one-third of pINV consists of IS elements. This finding suggests that the pINV plasmid seen in today's Shigella is the result of intense—and probably still ongoing—horizontal DNA transfer, and also explains variability among virulence plasmids from closely related Shigella clones.

Phylogenetic analysis revealed that the large virulence plasmid of Shigella belongs to either of two groups, A or B. Cluster 1 Shigella have pINV A, cluster 3 isolates harbor pINV B, and cluster 2 strains and single clones that have not diverged into different serogroups contain either pINV A or B plasmids indicating two ancestral donors for pINV (Lan et al. 2001). These results match with the previous finding that Shigella virulence plasmids belong to either of two compatibility groups. The exception is *E. coli* Dysenteriae 1 whose virulence plasmid seems to be a mixed type. Since this plasmid shares compatibility with group A plasmids, it is probable that pINV of *E. coli* Dysenteriae 1 evolved from a group A plasmid later incorporating sequences from a distantly related plasmid of group B (Lan et al. 2001).

Type III Secretion of Invasins

It has long been known that a 31 kb pINV fragment is required for the expression of the invasive phenotype of Shigella (Maurelli et al. 1985; Sasakawa et al. 1988). This region carries 34 genes coding for *i*nvasion *p*lasmid *a*ntigens (IpaA-D), IpgD, a type III secretion system (TTSS) (Mxi/Spa), the chaperone proteins IpgC (for IpaB, C), IpgE (for IpgD), Spa15 (for IpaA, IpgB1, and OspC3), for IcsB, a protein involved in the lysis of the phagocytic vacuole after transcellular migration, and for VirB, a regulator of transcription of *ipa-mxi-spa* genes (Adler et al. 1989). Ipa proteins are the major protein antigens for the serological immune response seen during convalescence. IpaB, C, and D are essential for bacterial invasion of epithelial cells (Menard et al. 1993). IpaB and C are the putative translocators of the TTSS forming a pore for subsequent injection of effectors secreted by the type III system. They also play roles as effectors—IpaC for Shigella-induced cytoskeletal rearrangements (Tran Van Nhieu et al. 1999) and IpaB for apoptosis in

macrophages (Zychlinsky et al. 1994; Hilbi et al. 1998).

In addition to IpaB and C, effectors of the TTSS comprise IpaA, IpaD, IpgB1, and IpgD. IpaA is a protein that impedes actin polymerization underneath the entering bacterium (Tran Van Nhieu et al. 1997), IpaD is a secreted protein that may bind to alpha5beta1-integrin receptors on host cells (Watarai et al. 1996) and regulate the TTSS together with IpaB (Menard et al. 1993, 1994). IpgB1 is a secreted protein of unknown function, and IpgD is a phosphatidylinositol 4,5-biphosphatase-specific 4-phosphatase that modulates the composition of phospholipids at the entry site of the host cell (Niebuhr et al. 2002).

Regulation of Synthesis of Invasion-associated Proteins

Optimal expression of the invasins occurs at 37°C, moderate levels of osmolarity, and at pH 7.4, conditions that are found in the large intestine of the primate host. Expression of invasion-associated proteins is the result of a regulatory cascade including proteins encoded by the chromosome and pINV (fig. 15.2). After a temperature shift to 37°C, transcription of the pINV-encoded regulator gene *virF* is activated by the chromosomally encoded DNA-binding proteins Fis and IHF (Porter and Dorman 1997; Falconi et al. 2001); efficient translation of VirF also requires two nucleoside modifications of tRNA, catalyzed by products of the *tgt* or *miaA* genes, respectively (Durand et al. 2000). VirF activates transcription of *virB* and *icsA*. IcsA is the major bacterial effector for intracellular motility by localized polymerization of actin at one pole of the bacterium. Expression of IcsA is posttranscriptionally regulated by VirK, another pINV-encoded protein (Nakata et al. 1992).

VirB, a DNA-binding protein, is the principal positive regulator of the three operons in the entry region of pINV, the *ipa*, *mxi,* and *spa* operons (Beloin et al. 2002). In addition, VirB induces transcription of *virA*, a pINV gene coding for a factor that is not implicated in epithelial cell invasion, but affects intracellular spread, probably by a *cis*-acting effect on the *icsA* promoter (Uchiya et al. 1995; Demers et al. 1998). Of note, it seems that *virA* and some *ipaH* genes are activated only after induction of type III secretion indicating a coregulatory role of the TTSS for the transcription of some genes that are not required for invasion of host cells (Demers et al. 1998). Temperature-dependent expression of the relevant genes or operons for the invasive phenotype of Shigella is counterregulated by the nucleoid structuring protein H-NS (VirR). This DNA-binding protein is a global regulator and represses *virF*, *virB*, *icsA*, *virA* genes and the *ipa*, *mxi,* and *spa* operons directly at temperatures below 32°C (Beloin and Dorman 2003).

In addition to the Ipa proteins and IpgD, the type III apparatus is able to secrete the following proteins: IpaH9.8, IpaH7.8, IpaH4.5, OspC1, VirA, MxiC, Spa32, OspB, OspF, IpgB1, OspD1, OspP, OspE1, MxiL (Buchrieser et al. 2000). IpaH proteins belong to a superfamily of LRR-proteins containing *l*eucine-*r*ich *r*epeats, a motif associated with protein-protein interaction that occurs in proteins of pathogenic bacteria (e.g., invasins InlA and InlB of *L. monocytogenes*) but also in mammalian receptors like TLRs (receptors in the cytoplasmic membrane) or NODs (receptors in the cytoplasm). IpaH9.8, a member of a protein family with five genes on pINV and four genes on the chromosome, is synthesized after induction of the TTSS and secreted in a second wave after the invasins, probably together with VirA (Demers et al. 1998; Toyotome et al. 2001). Interestingly, IpaH9.8 seems to be transported into the nucleus of the eukaryotic host cell making use of a microtubuli-dependent mechanism (Toyotome et al. 2001). IpaH7.8, another secreted member of the IpaH family, may play a role during lysis of the phagocytic vacuole (Fernandez-Prada et al. 2000).

The Type III Secretion System (TTSS)

TTSSs form a canal that transiently connects the cytoplasm with the exterior of the cell thus bypassing the periplasm. TTSSs are composed of the structure proteins of the canal, translocators that are first secreted upon activation of the system and often form a pore in the host-cell membrane, and effectors that are subsequently injected into the host cell via the pore formed by the translocators. The translocators may or may not have effector functions. The structure resembles a needle extending ~45–60 nm out of the bacterial surface that is integrated into the basal structure spanning the cell wall. The basal structure is composed of a double ring in the outer membrane and a bulblike structure that extends into the cytoplasm. The needle is essentially composed of MxiH (Tamano et al. 2000; Blocker et al. 2001) and may be capped by MxiI (Blocker et al. 2001). Needle length is controlled by Spa32 (Tamano et al. 2002) and by the expression level of MxiH (Tamano et al. 2000).

MxiD, G, and J are the major components of the basal structure that anchors the needle in the cell

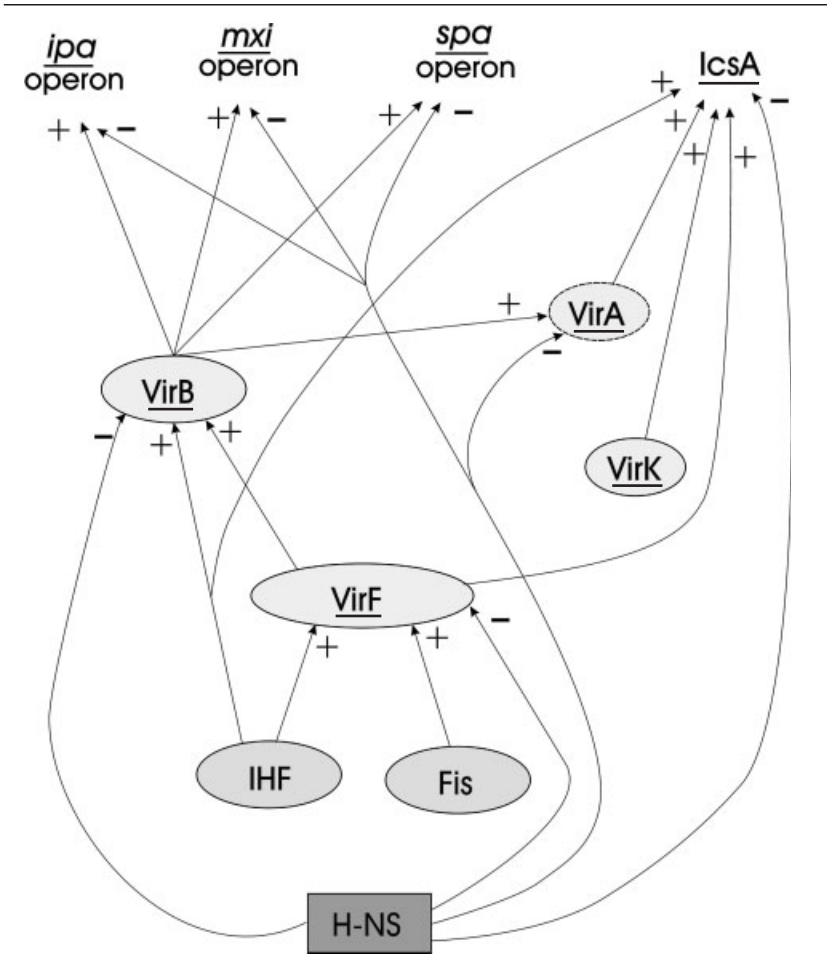


Figure 15.2. Regulation of proteins encoded by the *ipa*, *mxi*, or *spa* operons and regulation of IcsA. *Ipa* operon codes for IcsB, IpgA-C, IpaB, C, D, A, and VirB; *mxi* operon encodes IpgD-F, MxiH-K, N, L, M, E, D, C, A; *spa* operon codes for Spa47, 13, 32, 24, 9, 29, 40. pINV-encoded proteins or operons located on pINV are underlined; proteins encoded by the chromosome are in plain text. Ovals indicate positive regulators, H-NS is a global negative regulator. VirA is a regulator of IcsA and a potential effector.

wall. MxiD—possibly in association with MxiM—forms the ring structure associated with the outer membrane, while MxiG and J fix the bulb in the inner membrane (Blocker et al. 2001; Schuch and Maurelli 2001a). Spa33 is required for transport of invasins from the inner to the outer membrane (Schuch and Maurelli 2001b). Spa47 is a putative ATPase essential for the buildup of the needle and for secretion via the TTS system (Tamano et al. 2000). In *E. coli* Shigella, the TTSS is induced upon contact with host cells (Menard et al. 1994; Watarai et al. 1995) or after exposure to bile salts (Pope et al. 1995).

Other Proteins Encoded by pINV: IcsP/SopA, SepA, SenA/OspD3, MsbB2

IcsP/SopA is a protease cleaving IcsA, which induces polarized actin polymerization for intracellular movement (Shere et al. 1997). SepA is an autotransporter and member of the IgA1-protease family with a potential role in tissue invasion in the ileal loop model (Benjelloun et al. 1995). *SenA/ospD3* encodes the

enterotoxin ShET2. MsbB2, together with the chromosomally encoded MsbB1 protein, is required for complete acyl-oxy-acylation of the myristate at the 3' position of the lipid A component of LPS. This acylation is important for the capacity of Shigella LPS to induce production of TNFalpha in monocytes and destruction of the mucosa (d'Hauteville et al. 2002).

NR1: Conjugative Resistance Plasmid

NR1 is the archetype of a number of resistance plasmids found in Shigella (Womble and Rownd 1988). The plasmid confers resistance to mercury, sulfonamides, streptomycin, chloramphenicol, and tetracycline and is similar to the SRL pathogenicity island (see above). It is thought that the chromosomal SRL PAI arose following integration of a NR1-like plasmid (Rajakumar et al. 1997a).

pHS-2: Modification of LPS (Stieglitz et al. 1989)

The pHS-2 plasmid occurs in most cluster 3 Shigella (Adam et al. 2003) and essentially encodes Wzz_{pHS-2} (Cld_{pHS-2}), a chain length determinator of the O-side chain of LPS (Stevenson et al. 1995). Wzz proteins influence the number of oligosaccharide units (repeat units, RU) that are polymerized to make the O-side chain. *E. coli* Shigella clones harbor a chromosomal *wzz* gene, which is generally situated outside of the gene cluster for LPS synthesis (Morona et al. 1995; Wang et al. 2001). The activity of the chromosomal *wzz* gene results in a modal distribution of O-side chain lengths with a maximum at ~15 subunits (Morona et al. 1995). Shigella strains with an additional wzz_{pHS-2} show a bimodal distribution of O-side chain lengths with peaks at ~15 and >90 RU (Stevenson et al. 1995; Hong and Payne 1997).

The long O-side chains regulated by Wzz_{pHS-2} seem to partially mask IcsA, an inducer of actin polymerization, which is essential for intracellular movement. Thus, IcsA is accessible only at the old pole where high concentrations of this molecule are found (Morona and Van Den Bosch 2003). It is interesting that the pHS-2 plasmid occurs preferentially in Shigella serogroups that have been associated with reactive arthritis in HLA-B27-positive populations (Adam et al. 2003), a postinfectious complication of shigellosis that may lead to ankylosing spondylitis in humans.

PATHOGENESIS OF SHIGELLOSIS

Infection of Epithelial Cells

Oral uptake of the pathogen is followed by passage of the stomach, a critical step facilitated by the relative acid resistance of the microorganism. After intraluminal transport with intestinal content, the bacterium enters the rectosigmoidal mucosa via infection of microvillar or membraneous (M) cells. M cells are part of the follicle-associated epithelium (FAE) that covers lymphoid follicles of the mucosa-associated lymphoid tissue (MALT). They are closely associated with macrophages, lymphocytes, and dendritic cells and are made for endocytic transport of antigens from the lumen to the MALT. They express mucinlike surface molecules distinct from neighboring enterocytes (Lelouard et al. 1999; Lelouard et al. 2001).

From M cells, Shigella is handed over to macrophages that are killed by Shigella-induced apoptosis (Zychlinsky et al. 1992). Together with the bacterium, large quantities of pro-inflammatory cytokines IL-1β and IL-18 are released from the macrophage (Chen et al. 1996) attracting polymorphonuclear cells (PMN) to the entry site, while the pathogen gains access to the basolateral pole of enterocytes. Since Shigella is unable to penetrate into polarized epithelial cells via the apical pole (Mounier et al. 1992), bypass of the apical pole of enterocytes via infection of M cells and macrophages is a prerequisite for the pathogen to infect the epithelial cell layer of the rectosigmoid.

Initial inflammation is increased by secretion of pro-inflammatory IL-8 by infected epithelial cells (Sansonetti et al. 1999) and by transcytosis of Shigella LPS from the apical to the basolateral pole of enterocytes thus attracting PMN to Shigella-infected epithelia (Beatty and Sansonetti 1997; Beatty et al. 1999). Efflux of PMN further destabilizes the epithelial cell layer and may open up tight junctions between enterocytes thus providing direct access of intralumenal Shigella to the permissive basolateral pole of enterocytes. Therefore, initial inflammation promotes bacterial invasion of the intestinal mucosa (Sansonetti et al. 1995). The hyaluronic acid receptor CD44 (Skoudy et al. 2000; Lafont et al. 2002) and the fibrinogen receptor alpha5beta1 (Watarai et al. 1996) have been associated with initial steps of bacterial invasion of epithelial cells. CD44 is associated with the cytoskeleton via ERM (ezrin-radixin-moesin) proteins. One of these proteins (ezrin) has been attributed a functional role for bacterial entry (Skoudy et al. 1999). CD44 has also been reported to accumulate during bacterial invasion in microdomains of the cytoplasmic membrane enriched in cholesterol and sphingolipids, called rafts (Lafont et al. 2002). The rafts concept implies temporary formation of membrane

islands of particular composition in lipids and proteins, thus facilitating interaction between components enriched in these structures (Simons and Toomre 2000). While rafts seem to be important for bacterial internalization, a coherent concept for the role of these structures has not yet been elaborated.

Upon contact with the cell, Shigella secretes a number of proteins via its TTSS, a needlelike structure that is composed of pINV-encoded proteins of the *mxi*/*spa* operons (Blocker et al. 1999, 2001). The TTSS forms a 2–3 nm canal from the bacterial cytoplasm to the exterior (Blocker et al. 2001). Secretion via TTSSs is fast and efficient because the system tunnels the bacterial envelope. Ipa proteins are the principal antigens that elicit a serological immune response in convalescent individuals. In the bacterial cytoplasm, IpaB and IpaC are each bound to the chaperone protein IpgC. Upon activation of the TTSS by contact with host cells (Menard et al. 1994; Watarai et al. 1995) or by exposure to bile salts (Pope et al. 1995), IpaB and IpaC are secreted as part of a protein complex while IpgC remains in the bacterial cytoplasm. IpaB and C then form a 25 Å pore in the host membrane, which serves as a port of entry for other invasins including IpaA and IpgD.

The C-terminus of IpaC is a major effector for actin nucleation and polymerization leading to the formation of cellular protrusions in the vicinity of the entering bacterium that are transformed to lamellipodia (Adam et al. 1995; Tran Van Nhieu et al. 1999) some of which finally engulf and internalize the bacterium (Adam et al. 1995). Initial induction of actin polymerization by IpaC and formation of membrane protrusions is facilitated by temporal detachment of F-actin from the membrane by the action of IpgD (Niebuhr et al. 2002), a phosphatidyl-inositol,4,5 biphosphate ($PI4,5P_2$)-specific phosphatase (fig. 15.3). The subsequent cytoskeletal rearrangements require transient activation of Rho-like small G proteins of the host including CDC42, Rac, and Rho (Adam et al. 1996; Watarai et al. 1997; Mounier et al. 1999). Thus, inhibition of Rho-like small G proteins abolishes bacterial invasion.

Efficient internalization of the bacterium requires inhibition of the formation of cellular projections underneath the bacterium in order to prevent repulsion of the microorganism. This is accomplished by the activity of the TTSS-secreted protein IpaA (fig. 15.3). This protein binds to the cellular actin-binding protein vinculin, thus promoting binding of vinculin to and depolymerization of actin filaments (F-actin) (Tran Van Nhieu et al. 1997). The highly organized cytoskeletal structures that resemble macropinocytosis and promote bacterial internalization (fig. 15.4) are the result of recruitment as well as temporal and spatial regulation of the activities of a great number of actin-binding proteins, adaptors, and regulators. Activities represented by a variety of proteins of the invasion complex include nucleation, polymerization, cross-linking, severing, and binding to the membrane of actin.

Rho proteins are key regulators of Shigella-induced cytoskeletal rearrangements. While CDC42 is important for the basic processes of actin nucleation and polymerization and Rac is implicated in the formation of membrane ruffles, Rho recruits Src and ezrin into the entry complex thus linking cytoskeletal rearrangements to signaling cascades of the cell and fine-tuning cytoskeletal remodeling. Src tyrosine phosphorylates cortactin and paxillin (Watarai et al. 1997) during bacterial entry. Cortactin is implicated in the spatial organization of membrane-associated actin polymerization via the Arp2/3 complex. Actin-cross-linking activity of cortactin is reduced by Src-mediated phosphorylation. Interestingly, Src-mediated phosphorylation can be inhibited by interferon-alpha leading to reduced invasion efficiency (Dumenil et al. 1998). Paxillin is an adaptor protein with binding sites for the focal adhesion kinase (FAK), a tyrosine kinase and substrate for Src, and vinculin, thus connecting tyrosine phosphorylation cascades to cytoskeletal remodeling. In addition, paxillin binds to a protein complex containing PAK. Since PAK is an upstream regulator of CDC42 and Rac, this activity may link tyrosine phosphorylation cascades to CDC42/Rac-dependent signaling. FAK is a substrate of Src and forms a link to ERK2-signaling via the adaptor protein Grb2 (Schlaepfer et al. 1994) or via phosphorylation of p130Cas and downstream activation of Nck (Schlaepfer et al. 1997). However, the precise signaling mechanisms that regulate cytoskeletal rearrangements are poorly understood.

In a second step, the cellular projections are stabilized and transformed to a membranous structure resembling macropinocytosis, a basic mechanism of epithelial cells to internalize larger quantities of fluid (fig. 15.4). For this process, molecules that bind actin to the membrane and actin-cross-linking proteins are important. It is interesting that Rho isoforms are differentially recruited during bacterial invasion. RhoA accumulates early in the membrane next to the bacterium and is attached to the phagosome containing the microorganism. In contrast, RhoC accumulates in the tips of cellular projections. While IpaA prevents formation of cellular projec-

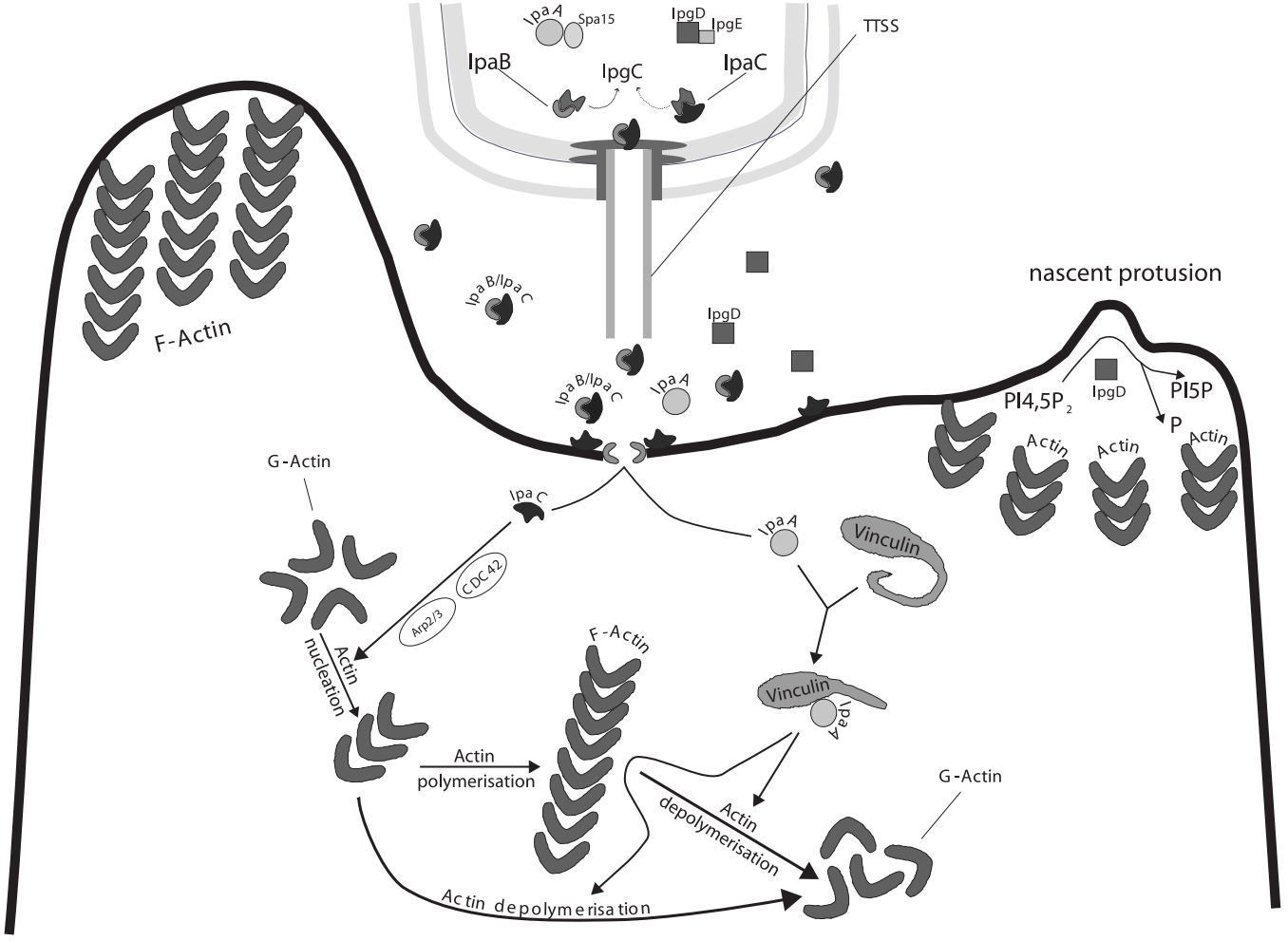
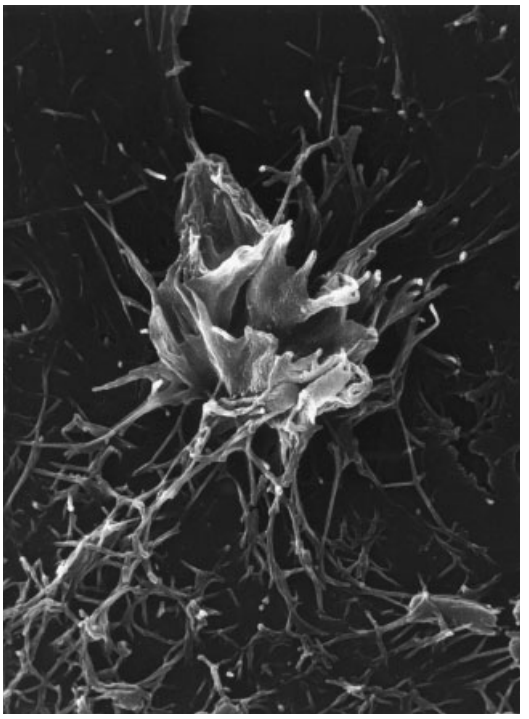
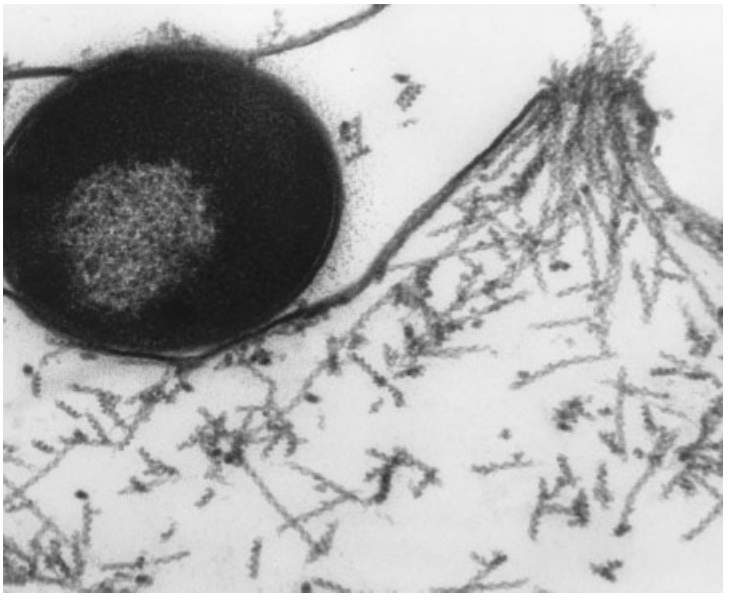


Figure 15.3. Secretion of invasins via the TTSS and cytoskeletal rearrangements during early stages of epithelial cells invasion. IpaC is the major bacterial inducer of actin nucleation and polymerization leading to cellular protrusions aside of the entering bacterium, which later engulf and internalize the microorganism. Note localized inhibition of actin polymerization beneath the bacterium by IpaA and IpgD-facilitated membrane extensions in nascent protrusions.



A



B

Figure 15.4. Shigella invasion of epithelial cells. (*A*) Macropinocytotic membrane folding; scanning-EM. (*B*) Cytoarchitecture of Shigella-induced entry structure. Cellular protrusions are sustained by actin filaments with the positive ("barbed") ends directed toward the cytoplasmic membrane; S1-myosin labeling of F-actin; transmission-EM. Bar: 1 μm. (From Adam et al. 1994 with permission)

tions underneath the entering microorganism, a class IX myosin antagonizes Rho isoforms that accumulate in the cellular protrusions (Graf et al. 2000) due to a C-terminal Rho-GAP module rendering these G proteins into the inactive, GDP-bound state. Finally, membrane protrusions fuse above the bacterium thus internalizing the microorganism.

Lysis of the Phagosome and Intracellular Movement

Once inside the cell, Shigella rapidly lyses the phagosome. This IpaB- and IpaC-dependent process (High et al. 1992) is followed by passive intracellular movement. Two forms of movement have been

described: locomotion along stress fibers or organellelike movement (OLM) in centripetal direction toward the nucleus in fibroblasts (Vasselon et al. 1991), and IcsA-dependent free movement through polarized actin polymerization in epithelial cells. IcsA (VirG) is a 120 kDa secreted protein encoded by the virulence plasmid (pINV). Its GC content is 41% and thus varies considerably from that of the invasins (Ipa, 34%) indicating that *icsA* and *ipa* genes may have different origins. The transport of IcsA through the inner membrane is *sec* dependent while transport through the outer membrane is autocatalyzed. In the outer membrane, the protein is cleaved by the pINV-encoded protease IcsP (SopA) releasing a 95 kDa protein to the exterior that is anchored at the old pole (after cell division) of the bacterium.

IcsA interacts specifically with N-terminal domains of the Wiskott-Aldrich-Syndrome Protein N-WASP, but not WASP, to promote Arp2/3-mediated actin nucleation and polymerization. Since hematopoietic cells do not express N-WASP, Shigella is not motile in macrophages showing that presence of N-WASP is a prerequisite for IcsA-mediated motility of Shigella in host cells (Suzuki et al. 2002). In addition, factors that keep the concentration of G-actin "fuel" high (cofilin, capping protein), cap G-actin, and promote actin polymerization (profilin) or stabilize the F-actin tail structure (actin cross-linking proteins or vinculin) are also important for IcsA-mediated intracellular movement. The velocity of Shigella propulsion by this mechanism is quite high (6–60 μm/min). *IcsA* mutants that are able to penetrate into but unable to move within and spread between epithelial cells are not virulent (Sansonetti et al. 1991), showing the importance of intracellular movement for efficient infection of the epithelial cell layer in shigellosis.

Cell-to-Cell Spread

Upon contact of the bacterium with the inner leaflet of the cytoplasmic membrane, a fingerlike cellular protrusion is formed reaching into the neighboring cell. Internalization and subsequent lysis of the membranes that surround the bacteria result in transition of Shigella from cell to cell without leaving the cytoplasm of host cells. Cell-to-cell transition is not a merely mechanical process due to bacterial propulsion, as formation of the protrusions requires expression of cadherins, normally involved in homophilic cell-cell interactions (Sansonetti et al. 1994). Furthermore, myosin seems to be involved in cell-to-cell spread since inhibitors of the myosin light chain kinase (MLCK) dramatically decrease intercellular spread indicating a role for nonmuscle myosin II in this process (Rathman et al. 2000).

In addition to IcsA, the pInv-encoded bacterial factors required for intercellular spread comprise IcsB (Allaoui et al. 1992) as well as IpaB, IpaC, and their chaperone IpgC, probably needed for lysis of the double membrane vacuole that surrounds Shigella (Page et al. 1999). Chromosomal Shigella genes involved in intercellular spread are *ispA, vacJ,* and *vpsC* whose precise function for transcellular migration has not yet been defined (Suzuki et al. 1994; Mac Siomoin et al. 1996, Hong et al. 1998). By these mechanisms, Shigella is able to rapidly infect the colon in proximal direction starting from the rectosigmoidal entry site. Enterocytes of the host are the privileged target cells of Shigella infection, since it is in these cells that the pathogen multiplies in the safe and rich haven of host-cell cytoplasm. In contrast to macrophages, Shigella does not induce major damage to or a stress response in these cells thus preserving host-cell resources for growth.

Clearance of the Infection

In parallel, continuing efflux of PMN attracted by pro-inflammatory cytokines released from apoptotic macrophages and infected epithelial cells demarcates the infected zone thus limiting further spread of the infection at the expense of destruction of colonic mucosa. PMN efficiently phagocytose the pathogen, and *E. coli* Shigella is not able to evade PMN phagosomes. This is in contrast to macrophages or epithelial cells and may be due to degradation of bacterial virulence factors by neutrophil elastase (Weinrauch et al. 2002). Finally, the necrotic epithelial cell layer is shed into the lumen together with the bacteria that are excreted to start a novel cycle of infection in a new host.

IMMUNITY

A number of first-line defense mechanisms plays a role in exclusion of Shigella from the intestinal mucosa. Lactoferrin, a glycoprotein present in human mucosal secretions including milk, induces proteolytic degradation of the invasin IpaB (Gomez et al. 2003). On the other hand, Shigella is able to bind to and extract iron from lactoferrin (Lawlor et al. 1987; Tigyi et al. 1992). Another mechanism of innate immunity is secretion of antibacterial peptides onto mucosal surfaces. While some antibacterial peptides secreted to the intestine (alpha-defensins) are increased during Shigella infection,

others (LL-37, beta-defensin-1) are down-regulated by the bacterium (Islam et al. 2001).

A further barrier to Shigella infection is the mucus layer that covers intestinal epithelia and contains large amounts of mucin glycoproteins. Intestinal mucins may prevent Shigella from access to epithelial cells. On the other hand, mucins may serve as receptor for adherence of the pathogen thus promoting infection. Mucins seem to be important for the host range of Shigella as well as for the site of invasion (rectosigmoid) within the intestine since Shigella adheres to colonic mucus of humans, but does not bind to colonic mucins from the rat or to human mucins from the small intestine (Rajkumar et al. 1998). On the other hand, mucins can be cleaved by Pic, a virulence-associated bacterial protease encoded by the chromosome (Henderson et al. 1999).

Shigellosis can also be prevented by Shigella-specific secretory IgA (SIgA) as part of the adaptive immune system. These antibodies are secreted onto mucosal surfaces as covalently coupled dimers or oligomers in conjunction with secretory component, a factor that provides correct localization and orientation of the antibodies in mucus (Phalipon et al. 2002). Secreted IgA antibodies are mostly directed toward Shigella LPS and may provide transient, serotype-specific protection against Shigella infection in animal models (Phalipon et al. 1994; Phalipon et al. 1995). Clinical symptoms of Shigellosis are essentially the result of inflammatory reactions of the host in an attempt to clear the infection, and the secretion of proinflammatory cytokines is important for a successful immune response. On the other hand, initial inflammation is exploited by Shigella for facilitated access to and multiplication within the enterocytes of the colon. Thus, early responses of the innate immune system after bacterial invasion of the rectosigmoidal mucosa are pivotal for the course and the outcome of shigellosis.

A key factor for the orientation of initial immune responses to shigellosis is induction of apoptosis in macrophages. Induction of apoptosis is via a cascade of proteases including IL-1β converting enzyme, which in turn activates by cleavage IL-1β and IL-18, two pro-inflammatory factors that are released from the dying cell. IpaB directly binds to IL-1β converting enzyme. Recent data indicate that Shigella is also able to activate tripeptidyl peptidase II, a protease upstream of IL-1β-converting enzyme, to promote apoptosis (Hilbi et al. 2000). In addition, Shigella has also been shown to release lipoproteins that activate Toll-like receptor (TLR)-2 in monocytes and may induce apoptosis in TLR-2-expressing cells (Aliprantis et al. 1999). In contrast to macrophages, monocytes undergo a delayed form of apoptosis only after having killed the pathogen. In this form of apoptosis, release of pro-inflammatory cytokines is suppressed (Hathaway et al. 2002); however, the precise mechanism of this process has not yet been elucidated.

Finally, Shigella also induces apoptosis in dendritic cells, another type of antigen-presenting cell, thus further impeding generation of an adaptive immune response (Edgeworth et al. 2002). While IL-1β and IL-8 are key molecules for early inflammation, IL-18, another pro-inflammatory cytokine of the IL-1 family, is required for clearing the infection (Sansonetti et al. 2000) indicating a protective role for IL-18-mediated immune mechanisms. Attraction to the site of invasion and activation of PMN and monocytes are crucial steps for early inflammation that promotes bacterial infection as well as for the resolution of the infection. Chemoattractants are primarily released from apoptotic antigen-presenting cells and from epithelial cells after internalization of Shigella LPS or the complete pathogen. It seems that epithelial cells are the major source of IL-8 production. This requires intracellular sensing of pathogen-associated molecular patterns (PAMPs).

TLR-expressing cells are able to sense bacterial components like lipoprotein, LPS, flagella, at the plasma membrane and trigger responses that may lead to apoptosis or to the transcription of pro-inflammatory genes via NF-kB, ERK, or AP signaling to the host nucleus (fig. 15.5). In addition, signaling pathways that involve release of IL-12 thus triggering a type 1 response can be initiated by TLRs-mediated sensing of extracellular PAMPs. However, the intracellular lifestyle of Shigella requires intracellular monitoring of bacteria that are intruded into the cytoplasm. Indeed, in epithelial cells, NOD1 may act as an intracellular sensor of bacterial components that are released from bacterial cell walls possibly after lysosomal degradation (fig. 15.5). NOD1 recognizes a muropeptide specific for gram-negative bacteria (Girardin et al. 2003) and is a key component for Shigella-triggered, NF-kB-mediated induction in epithelial cells of the synthesis of pro-inflammatory proteins including IL-8 (Philpott et al. 2000), a major chemoattractant for PMNs. Although Shigella induces necrosis in PMN contributing to tissue damage in early infection by release of granular enzymes, PMN are able and

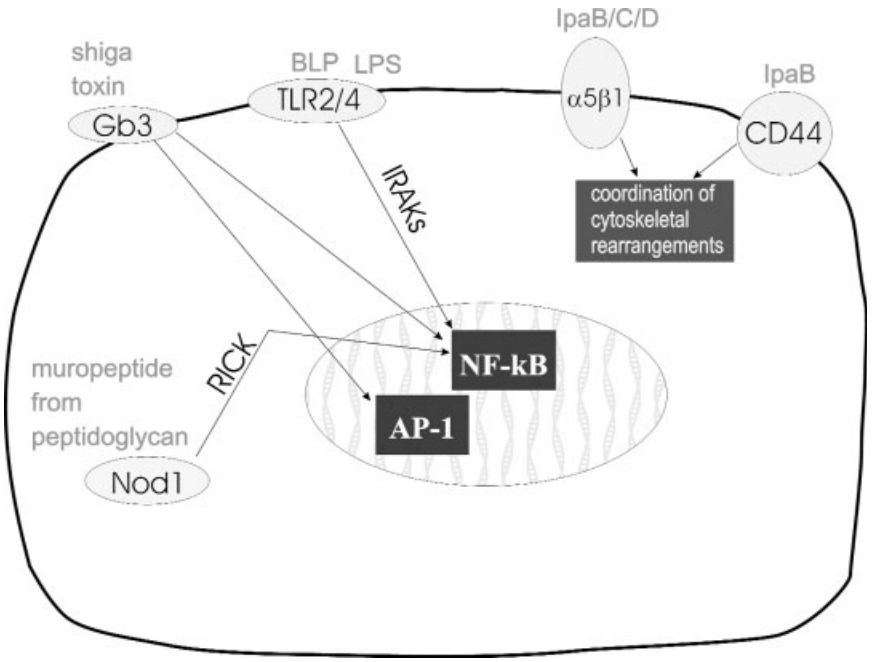


Figure 15.5. Shigella components that interact with eukaryotic receptors and induction of pro-inflammatory transcription factors NF-kB and AP-1. Bacterial factors: LPS (lipopolysaccharide), BLP (bacterial lipoprotein), Ipa (Invasion plasmid antigen). Receptors: TLR (*T*oll-*l*ike *r*eceptor), membranous sensor of pathogen-associated molecular patterns (PAMPs), α5β1 (integrin), Gb3 (glycolipid receptor globotriaosylceramide), CD44 (hyaluronic acid receptor), Nod1 (cytoplasmic sensor of PAMPs), IRAK (*I*L-1 *r*eceptor-*a*ssociated *k*inase), RICK (*R*IP-like *i*nteracting *C*LARP *k*inase).

required to efficiently contain Shigella infection (Mandic et al. 1997; Zhang et al. 2001).

Although eliciting an adaptive immune response is impeded by Shigella-mediated induction of apoptosis in antigen-presenting cells, anti-LPS antibody responses and to a lesser extent anti-Ipa antibodies are regularly triggered (Li et al. 1994). However, specificity of protective antibodies is controversial. While some investigators found a correlation of anti-LPS-antibodies with protection (Cohen et al. 1997), others did not (Kotloff et al. 1996). Anti-Ipa antibodies may also be important for postinfectious resistance to Shigellosis (Oberhelman et al. 1991). In addition, recent data also give a hint to relevant cell-mediated immunity. Thus, several studies have provided evidence for an important role for IFN-gamma in resistance to shigellosis (Samandari et al. 2000; Raqib et al. 2002). This type 1 response can be elicited with small infectious doses in the range of 10^2 bacteria (Samandari et al. 2000). With increasing inoculum size, antibody responses in addition to IFN-gamma can be induced, while type 2 responses are negligible (Samandari et al. 2000). Later, immunmodulatory IL-10 is produced that down-regulates type 1 responses, NK activity, and pro-inflammatory IL-1 release, while B lymphocytes may be stimulated. Thus, type 1 responses seem to play a role in immunity to shigellosis.

Taken together, early steps of the innate immune response to Shigella are crucial for the induction of efficient antibacterial activity in the host. While initial pro-inflammatory apoptosis is accompanied by release of large amounts of activated IL-1β and IL-18, signaling via membrane-bound TLR and the intracellular sensor NOD1 lead to increased transcription of pro-inflammatory genes including IL-8 in epithelial cells. Efflux of PMN and a type 1 immune response may be important for clearance of the infection. It seems that serotype-specific secretory anti-LPS antibodies and perhaps anti-Ipa anti-

bodies and the type 1 immune response are the only relevant reactions of the adaptive immune system.

TOXINS

SHIGA TOXIN

E. coli Dysenteriae 1 is the only *E. coli* Shigella serotype that regularly expresses Shiga toxin (see also chapter 16). Infection with Shiga toxin-producing bacteria has been associated with the hemolytic uremic syndrome (HUS), characterized by thrombocytopenia, microangiopathic hemolytic anemia, and renal failure (Koster et al. 1984; Khin Maung et al. 1987). Shiga toxin belongs to the AB family of toxins and is composed of 1 A and 5 B subunits, StxA and StxB. The genes encoding the subunits of the toxin are part of an operon located on the chromosome and associated with phage-borne sequences (Unkmeir and Schmidt 2000). However, *E. coli* Dysenteriae 1 has lost most of the late genes of the prophage, probably by recombination with insertion sequences (IS). The 1A:5B subunit stoichiometry of the holotoxin is maintained by a more efficient ribosome binding site of the *stxB* transcript and by an additional promoter of the *stxB* gene. Enzymatic activity is exerted by the A subunit, while B subunits bind to globotriaosylceramide (Gb_3), a glycolipid receptor (Waddell et al. 1988). The toxin is transported from the cytoplasmic membrane to the Golgi and the endoplasmic reticulum (ER). Subunit A is cleaved into subunits A1 and A2 by proteolysis and reduction of an internal disulfide bond. After release in the cytosol, the A1 fragment, a RNA N-glycosidase, inhibits protein synthesis by removal of adenine from adenosine in position 4324 of 28S rRNA thus preventing elongation factor-1-dependent binding of aminoacyl-tRNA to the ribosome (Endo et al. 1988; Saxena et al. 1989). Recently, oral administration of an *E. coli* mutant expressing a modified LPS that mimics the Shiga toxin receptor Gb_3 was shown to protect mice from Shiga-toxin-mediated pathogenicity (Paton et al. 2000). This finding paves the way for future "probiotic" protection of animals or humans from the deleterious effects of Shiga toxin.

ENTEROTOXINS

Two enterotoxins have been described in *E. coli* Shigella, ShET1 and ShET2.

ShET1

ShET1 is encoded by two chromosomal genes, *setA* and *setB*, which are oppositely oriented and embodied within *pic*, a gene encoding an autotransporter situated within a pathogenicity island (Behrens et al. 2002). The toxin occurs in a 1A:5B configuration (Fasano et al. 1997), is prevalent almost exclusively in *E. coli* Flexneri 2a strains (Yavzori et al. 2002), induces fluid accumulation in the rabbit ileal loop, and may account for initial watery diarrhea that can occur in early stages of *E. coli* Flexneri 2a infections (Fasano et al. 1997).

ShET2

ShET2 is encoded by the *sen* gene on the virulence plasmid pINV and occurs in virtually all *E. coli* Shigella isolates and in enteroinvasive *E. coli* (EIEC) (Nataro et al. 1995; Yavzori et al. 2002). While the complete pINV sequence of an *E. coli* Flexneri 5 strain revealed that *senA/ospD3* belongs to a gene family of at least two genes with 38% sequence identity, it is unknown whether both genes are expressed in the same conditions (Buchrieser et al. 2000). The toxin increases transepithelial conductance in an *in vitro* model (Nataro et al. 1995); however, the relevance of the toxin for clinical disease is unknown.

CONCLUDING REMARKS

Though some of the major pathogenic processes that cause shigellosis are understood, present knowledge of the molecular processes is essentially based on cell culture or animal models that only partially mimic disease in primates. Thus, in addition to further elucidation of molecular mechanisms of host-parasite interaction, some basic questions remain to be answered:

- Which factors determine the host range of *E. coli* Shigella?
- Is there a nonprimate reservoir for Shigella?
- What is the precise epidemiological role of acquired immunity to Shigella?
- Which factors cause epidemics with *E. coli* Dysenteriae 1?
- What is the particular role of *E. coli* Shigella during evolution of primates: Did Shigella play a relevant role for primate evolution as a selective counterpart or did the bacteria only adapt to evolutionary changes of its hosts?

REFERENCES

Adam, T., M. Arpin, M. C. Prévost, P. Gounon, and P. J. Sansonetti. 1995. Cytoskeletal rearrangements and the functional role of T-plastin during entry of *Shigella flexneri* into HeLa cells. J. Cell Biol. 129:367–381.

———. 1994. Die Invasion von Epithelzellen durch *Shigella flexneri*. In Ökosystem Darm VI. W. F. Caspary, M. Kist, and M. Zeitz (eds.). Berlin: Springer, pp. 231–238.

Adam, T., M. Giry, P. Boquet, and P. Sansonetti. 1996. Rho-dependent membrane folding causes Shigella entry into epithelial cells. EMBO J. 15:3315–3321.

Adam, T., R. Siewerdt, I. Offermann, J. Lang, H. Tschape, J. Sieper, and B. Graf. 2003. Prevalence and molecular diversity of pHS-2 plasmids, marker for arthritogenicity, among clinical *Escherichia coli* Shigella isolates. Microbes Infect. 5:579–92.

Adler, B., C. Sasakawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa. 1989. A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol. Microbiol. 3:627–635.

Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285:736–739.

Allaoui, A., J. Mounier, M. C. Prevost, P. J. Sansonetti, and C. Parsot. 1992. icsB: a *Shigella flexneri* virulence gene necessary for the lysis of protrusions during intercellular spread. Mol. Microbiol. 6:1605–1616.

Arm, H. G., T. M. Floyd, J. E. Faber, and J. R. Hayes. 1965. Use of ligated segments of rabbit small intestine in experimental shigellosis. J. Bacteriol. 89:803–809.

Banish, L. D., R. Sims, D. Sack, R. J. Montali, L. Phillips, and M. Bush. 1993. Prevalence of shigellosis and other enteric pathogens in a zoologic collection of primates. J. Am. Vet. Med. Assoc. 203:126–132.

Beatty, W. L., and P. J. Sansonetti. 1997. Role of lipopolysaccharide in signaling to subepithelial polymorphonuclear leukocytes. Infect. Immun. 65:4395–4404.

Beatty, W. L., S. Meresse, P. Gounon, J. Davoust, J. Mounier, P. J. Sansonetti, and J. P. Gorvel. 1999. Trafficking of Shigella lipopolysaccharide in polarized intestinal epithelial cells. J. Cell Biol. 145:689–698.

Behrens, M., J. Sheikh, and J. P. Nataro. 2002. Regulation of the overlapping *pic*/*set* locus in *Shigella flexneri* and enteroaggregative *Escherichia coli*. Infect. Immun. 70:2915–2925.

Beloin, C., and C. J. Dorman. 2003. An extended role for the nucleoid structuring protein H-NS in the virulence gene regulatory cascade of *Shigella flexneri*. Mol. Microbiol. 47:825–38.

Beloin, C., S. McKenna, and C. J. Dorman. 2002. Molecular dissection of VirB, a key regulator of the virulence cascade of *Shigella flexneri*. J. Biol. Chem. 277:15333–15344.

Benjelloun, T. Z., P. J. Sansonetti, and C. Parsot. 1995. SepA, the major extracellular protein of *Shigella flexneri*: autonomous secretion and involvement in tissue invasion. Mol. Microbiol. 17:123–135.

Blocker, A., N. Jouihri, E. Larquet, P. Gounon, F. Ebel, C. Parsot, P. Sansonetti, and A. Allaoui. 2001. Structure and composition of the *Shigella flexneri* "needle complex", a part of its type III secreton. Mol. Microbiol. 39:652–63.

Blocker, A., P. Gounon, E. Larquet, K. Niebuhr, V. Cabiaux, C. Parsot, and P. Sansonetti. 1999. The tripartite type III secreton of *Shigella flexneri* inserts IpaB and IpaC into host membranes. J. Cell Biol. 147:683–693.

Buchrieser, C., P. Glaser, C. Rusniok, H. Nedjari, H. D'Hauteville, F. Kunst, P. Sansonetti, and C. Parsot. 2000. The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. Mol. Microbiol. 38:760–71.

Chen, Y., M. R. Smith, K. Thirumalai, and A. Zychlinsky. 1996. A bacterial invasin induces macrophage apoptosis by binding directly to ICE. EMBO J. 15:3853–3860.

Cohen, D., S. Ashkenazi, M. S. Green, M. Gdalevich, G. Robin, R. Slepon, M. Yavzori, N. Orr, C. Block, I. Ashkenazi, J. Shemer, D. N. Taylor, T. L. Hale, J. C. Sadoff, D. Pavliakova, R. Schneerson, and J. B. Robbins. 1997. Double-blind vaccine-controlled randomised efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. Lancet 349:155–159.

Demers, B., P. J. Sansonetti, and C. Parsot. 1998. Induction of type III secretion in *Shigella flexneri* is associated with differential control of transcription of genes encoding secreted proteins. EMBO J. 17:2894–2903.

d'Hauteville, H., S. Khan, D. J. Maskell, A. Kussak, A. Weintraub, J. Mathison, R. J. Ulevitch, N. Wuscher, C. Parsot, and P. J. Sansonetti. 2002. Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. J. Immunol. 168:5240–5251.

Dumenil, G., J. C. Olivo, S. Pellegrini, M. Fellous, P. J. Sansonetti, and G. T. Nhieu. 1998. Interferon alpha inhibits a Src-mediated pathway necessary for Shigella-induced cytoskeletal rearrangements in epithelial cells. J. Cell Biol. 143:1003–1012.

DuPont, H. L., M. M. Levine, R. B. Hornick, and S. B. Formal. 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. 159:1126–1128.

Durand, J. M., B. Dagberg, B. E. Uhlin, and G. R. Bjork. 2000. Transfer RNA modification, temperature and DNA superhelicity have a common target in the regulatory network of the virulence of *Shigella flexneri*: the expression of the *virF* gene. Mol. Microbiol. 35:924–935.

Edgeworth, J. D., J. Spencer, A. Phalipon, G. E. Griffin, and P. J. Sansonetti. 2002. Cytotoxicity and interleukin-1beta processing following *Shigella flexneri* infection of human monocyte-derived dendritic cells. Eur. J. Immunol. 32:1464–1471.

Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi. 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur. J. Biochem. 171:45–50.

Falconi, M., G. Prosseda, M. Giangrossi, E. Beghetto, and B. Colonna. 2001. Involvement of FIS in the H-NS-mediated regulation of *virF* gene of Shigella and enteroinvasive *Escherichia coli*. Mol. Microbiol. 42:439–452.

Fasano, A., F. R. Noriega, F. M. Liao, W. Wang, and M. M. Levine. 1997. Effect of shigella enterotoxin 1 (ShET1) on rabbit intestine in vitro and in vivo. Gut 40:505–511.

Fernandez-Prada, C. M., D. L. Hoover, B. D. Tall, A. B. Hartman, J. Kopelowitz, and M. M. Venkatesan. 2000. *Shigella flexneri* IpaH (7.8) facilitates escape of virulent bacteria from the endocytic vacuoles of mouse and human macrophages. Infect. Immun. 68:3608–3619.

Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem. 278:8869–8872.

Gomez, H. F., T. J. Ochoa, L. G. Carlin, and T. G. Cleary. 2003. Human lactoferrin impairs virulence of *Shigella flexneri*. J. Infect. Dis. 187:87–95.

Gorden, J., and P. L. Small. 1993. Acid resistance in enteric bacteria. Infect. Immun. 61:364–367.

Graf, B., M. Bahler, P. Hilpela, C. Bowe, and T. Adam. 2000. Functional role for the class IX myosin myr5 in epithelial cell infection by *Shigella flexneri*. Cell. Microbiol. 2:601–616.

Guan, S., D. A. Bastin, and N. K. Verma. 1999. Functional analysis of the O antigen glucosylation gene cluster of *Shigella flexneri* bacteriophage SfX. Microbiology 145 (Pt 5):1263–1273.

Hacker, J., and J. B. Kaper. 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. 54:641–679.

Hathaway, L. J., G. E. Griffin, P. J. Sansonetti, and J. D. Edgeworth. 2002. Human monocytes kill *Shigella flexneri* but then die by apoptosis associated with suppression of proinflammatory cytokine production. Infect. Immun. 70:3833–3842.

Henderson, I. R., J. Czeczulin, C. Eslava, F. Noriega, and J. P. Nataro. 1999. Characterization of Pic, a secreted protease of *Shigella flexneri* and enteroaggregative *Escherichia coli*. Infect. Immun. 67:5587–5596.

High, N., J. Mounier, M. C. Prevost, and P. J. Sansonetti. 1992. IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole. EMBO J. 11:1991–1999.

Hilbi, H., J. E. Moss, D. Hersh, Y. Chen, J. Arondel, S. Banerjee, R. A. Flavell, J. Yuan, P. J. Sansonetti, and A. Zychlinsky. 1998. Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. J. Biol. Chem. 273:32895–32900.

Hilbi, H., R. J. Puro, and A. Zychlinsky. 2000. Tripeptidyl peptidase II promotes maturation of caspase-1 in *Shigella flexneri*-induced macrophage apoptosis. Infect. Immun. 68:5502–5508.

Hong, M., and S. M. Payne. 1997. Effect of mutations in *Shigella flexneri* chromosomal and plasmid-encoded lipopolysaccharide genes on invasion and serum resistance. Mol. Microbiol. 24:779–791.

Hong, M., Y. Gleason, E. E. Wyckoff, and S. M. Payne. 1998. Identification of two *Shigella flexneri* chromosomal loci involved in intercellular spreading. Infect. Immun. 66:4700–4710.

Islam, D., L. Bandholtz, J. Nilsson, H. Wigzell, B. Christensson, B. Agerberth, and G. Gudmundsson. 2001. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nature Med. 7:180–185.

Jin, Q., Z. Yuan, J. Xu, Y. Wang, Y. Shen, W. Lu, J. Wang, H. Liu, J. Yang, F. Yang, X. Zhang, J. Zhang, G. Yang, H. Wu, D. Qu, J. Dong, L. Sun, Y. Xue, A. Zhao, Y. Gao, J. Zhu, B. Kan, K. Ding, S. Chen, H. Cheng, Z. Yao, B. He, R. Chen, D. Ma, B. Qiang, Y. Wen, Y. Hou, and J. Yu. 2002. Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. Nucleic Acids Res. 30:4432–4441.

Karnell, A., F. P. Reinholt, S. Katakura, and A. A. Lindberg. 1991. *Shigella flexneri* infection: a histopathologic study of colonic biopsies in monkeys infected with virulent and attenuated bacterial strains. Apmis 99:787–796.

Khin Maung, U., K. Myo, A. Tin, A. Myo Min, A. Soe Soe, M. Thane Oke Kyaw, and N. Khin Than. 1987. Clinical features, including haemolytic-uraemic syndrome, in *Shigella dysenteriae* type 1 infection in children of Rangoon. J. Diarrhoeal Dis. Res. 5:175–177.

Koster, F. T., V. Boonpucknavig, S. Sujaho, R. H. Gilman, and M. M. Rahaman. 1984. Renal histopathology in the hemolytic-uremic syndrome following shigellosis. Clin. Nephrol. 21:126–133.

Kotloff, K. L., F. Noriega, G. A. Losonsky, M. B. Sztein, S. S. Wasserman, J. P. Nataro, and M. M. Levine. 1996. Safety, immunogenicity, and transmissibility in humans of CVD 1203, a live oral *Shigella flexneri* 2a vaccine candidate attenuated by deletions in *aroA* and *virG*. Infect. Immun. 64:4542–4548.

Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine. 1999. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. Bull. World Health Organ. 77:651–666.

Lafont, F., G. Tran Van Nhieu, K. Hanada, P. Sansonetti, and F. G. van der Goot. 2002. Initial steps of Shigella infection depend on the cholesterol/sphingolipid raft-mediated CD44-IpaB interaction. EMBO J. 21:4449–4457.

Lan, R., and P. R. Reeves. 2002. *Escherichia coli* in disguise: molecular origins of Shigella. Microbes Infect. 4:1125–1132.

Lan, R., B. Lumb, D. Ryan, and P. R. Reeves. 2001. Molecular evolution of large virulence plasmid in Shigella clones and enteroinvasive *Escherichia coli*. Infect. Immun. 69:6303–6309.

Lawlor, K. M., P. A. Daskaleros, R. E. Robinson, and S. M. Payne. 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. 55:594–599.

Lelouard, H., H. Reggio, C. Roy, A. Sahuquet, P. Mangeat, and P. Montcourrier. 2001. Glycocalyx on rabbit intestinal M cells displays carbohydrate epitopes from Muc2. Infect. Immun. 69:1061–1071.

Lelouard, H., H. Reggio, P. Mangeat, M. Neutra, and P. Montcourrier. 1999. Mucin-related epitopes distinguish M cells and enterocytes in rabbit appendix and Peyer's patches. Infect. Immun. 67:357–367.

Li, A., C. R. Zhao, E. Ekwall, and A. A. Lindberg. 1994. Serum IgG antibody responses to Shigella invasion plasmid-coded antigens detected by immunoblot. Scand. J. Infect. Dis. 26:435–445.

Luck, S. N., S. A. Turner, K. Rajakumar, H. Sakellaris, and B. Adler. 2001. Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. Infect. Immun. 69:6012–6021.

Mac Siomoin, R. A., N. Nakata, T. Murai, M. Yoshikawa, H. Tsuji, and C. Sasakawa. 1996. Identification and characterization of *ispA*, a *Shigella flexneri* chromosomal gene essential for normal in vivo cell division and intracellular spreading. Mol. Microbiol. 19:599–609.

Mandic, M., I. J. Weiss, and A. Zychlinsky. 1997. *Shigella flexneri* is trapped in polymorphonuclear leukocyte vacuoles and efficiently killed. Infect. Immun. 65:110–115.

Maurelli, A. T., B. Baudry, H. d'Hauteville, T. L. Hale, and P. J. Sansonetti. 1985. Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. 49:164–171.

Maurelli, A. T., R. E. Fernandez, C. A. Bloch, C. K. Rode, and A. Fasano. 1998. "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive *Escherichia coli*. Proc. Natl. Acad. Sci. U S A 95:3943–3948.

Menard, R., P. J. Sansonetti, and C. Parsot. 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. 175:5899–5906.

Menard, R., P. Sansonetti, and C. Parsot. 1994. The secretion of the Shigella flexneri Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. EMBO J. 13:5293–5302.

Morona, R., and L. Van Den Bosch. 2003. Lipopolysaccharide O antigen chains mask IcsA (VirG) in *Shigella flexneri*. FEMS Microbiol. Lett. 221:173–180.

Morona, R., L. Van-den-Bosch, and P. A. Manning. 1995. Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. J. Bacteriol. 177:1059–1068.

Moss, J. E., T. J. Cardozo, A. Zychlinsky, and E. A. Groisman. 1999. The *selC*-associated SHI-2 pathogenicity island of *Shigella flexneri*. Mol. Microbiol. 33:74–83.

Mounier, J., T. Vasselon, R. Hellio, M. Lesourd, and P. J. Sansonetti. 1992. *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. Infect. Immun. 60:237–248.

Mounier, J., V. Laurent, A. Hall, P. Fort, M. F. Carlier, P. J. Sansonetti, and C. Egile. 1999. Rho family GTPases control entry of *Shigella flexneri* into epithelial cells but not intracellular motility. J. Cell. Sci. 112:2069–2080.

Nakata, N., C. Sasakawa, N. Okada, T. Tobe, I. Fukuda, T. Suzuki, K. Komatsu, and M. Yoshikawa. 1992. Identification and characterization of *virK*, a virulence- associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. Mol. Microbiol. 6:2387–2395.

Nataro, J. P., J. Seriwatana, A. Fasano, D. R. Maneval, L. D. Guers, F. Noriega, F. Dubovsky, M.

M. Levine, and J. G. Morris, Jr. 1995. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and Shigella strains. Infect. Immun. 63:4721–8.

Niebuhr, K., S. Giuriato, T. Pedron, D. J. Philpott, F. Gaits, J. Sable, M. P. Sheetz, C. Parsot, P. J. Sansonetti, and B. Payrastre. 2002. Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the *S. flexneri* effector IpgD reorganizes host cell morphology. EMBO J. 21:5069–5078.

Nizeyi, J. B., R. B. Innocent, J. Erume, G. R. Kalema, M. R. Cranfield, and T. K. Graczyk. 2001. Campylobacteriosis, salmonellosis, and shigellosis in free-ranging human-habituated mountain gorillas of Uganda. J. Wildl. Dis. 37:239–244.

Nofech-Mozes, Y., Y. Yuhas, E. Kaminsky, A. Weizman, and S. Ashkenazi. 2000. Induction of mRNA for tumor necrosis factor-alpha and interleukin-1 beta in mice brain, spleen and liver in an animal model of Shigella-related seizures. Israel Med. Assoc. J. 2:86–90.

Oberhelman, R. A., D. J. Kopecko, E. Salazar-Lindo, E. Gotuzzo, J. M. Buysse, M. M. Venkatesan, A. Yi, C. Fernandez-Prada, M. Guzman, R. Leon-Barua, and et al. 1991. Prospective study of systemic and mucosal immune responses in dysenteric patients to specific Shigella invasion plasmid antigens and lipopolysaccharides. Infect. Immun. 59:2341–2350.

Ogawa, H., S. Honjo, M. Takasaka, T. Fujiwara, and K. Imaizumi. 1966. Shigellosis in cynomolgus monkeys (*Macaca irus*). IV. Bacteriological and histopathological observations on the earlier stage of experimental infection with *Shigella flexneri* 2 A. Jpn. J. Med. Sci. Biol. 19:22–32.

Page, A. L., H. Ohayon, P. J. Sansonetti, and C. Parsot. 1999. The secreted IpaB and IpaC invasins and their cytoplasmic chaperone IpgC are required for intercellular dissemination of *Shigella flexneri*. Cell. Microbiol. 1:183–193.

Paton, A. W., R. Morona, and J. C. Paton. 2000. A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. Nature Med. 6:265–270.

Petrovskaya, V. G., and V. M. Bondarenko. 1995. Antigenic variability of *Shigella flexneri* serovars 1-5. Mol. Gen. Mikrobiol. Virusol. JID—9315607:15–17.

Phalipon, A., A. Cardona, J. P. Kraehenbuhl, L. Edelman, P. J. Sansonetti, and B. Corthesy. 2002. Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. Immunity 17:107–115.

Phalipon, A., M. Kaufmann, P. Michetti, J. M. Cavaillon, M. Huerre, P. Sansonetti, and J. P. Kraehenbuhl. 1995. Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of *Shigella flexneri* lipopolysaccharide protects against murine experimental shigellosis. J. Exp. Med. 182:769–778.

Phalipon, A., P. Michetti, M. Kaufmann, J. M. Cavaillon, M. Huerre, J. P. Kraehenbuhl, and P. J. Sansonetti. 1994. Protection against invasion of the mouse pulmonary epithelium by a monoclonal IgA directed against *Shigella flexneri* lipopolysaccharide. Ann. N. Y. Acad. Sci. 730:356–358.

Philpott, D. J., S. Yamaoka, A. Israel, and P. J. Sansonetti. 2000. Invasive *Shigella flexneri* activates NF-kappa B through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells. J. Immunol. 165:903–914.

Pope, L. M., K. E. Reed, and S. M. Payne. 1995. Increased protein secretion and adherence to HeLa cells by Shigella spp. following growth in the presence of bile salts. Infect. Immun. 63:3642–3648.

Porter, M. E., and C. J. Dorman. 1997. Positive regulation of *Shigella flexneri* virulence genes by integration host factor. J. Bacteriol. 179:6537–6550.

Pupo, G. M., D. K. Karaolis, R. Lan, and P. R. Reeves. 1997. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. Infect. Immun. 65:2685–2692.

Pupo, G. M., R. Lan, and P. R. Reeves. 2000. Multiple independent origins of Shigella clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc. Natl. Acad. Sci. U S A 97:10567–10572.

Rabbani, G. H., M. J. Albert, H. Rahman, M. Islam, D. Mahalanabis, I. Kabir, K. Alam, and M. Ansaruzzaman. 1995. Development of an improved animal model of shigellosis in the adult rabbit by colonic infection with *Shigella flexneri* 2a. Infect. Immun. 63:4350–4357.

Rajakumar, K., C. Sasakawa, and B. Adler. 1997b. Use of a novel approach, termed island probing, identifies the *Shigella flexneri she* pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins. Infect. Immun. 65:4606–4614.

Rajakumar, K., D. Bulach, J. Davies, L. Ambrose, C. Sasakawa, and B. Adler. 1997a. Identification of a chromosomal *Shigella flexneri* multi-antibiotic resistance locus which shares sequence and organizational similarity with the resistance region of the plasmid NR1. Plasmid 37:159–168.

Rajkumar, R., H. Devaraj, and S. Niranjali. 1998. Binding of Shigella to rat and human intestinal mucin. Mol. Cell. Biochem. 178:261–268.

Raqib, R., F. Qadri, P. SarkEr, S. M. Mia, P. J. Sansonnetti, M. J. Albert, and J. Andersson. 2002. Delayed and reduced adaptive humoral immune responses in children with shigellosis compared with in adults. Scand. J. Immunol. 55:414–423.

Rathman, M., P. de Lanerolle, H. Ohayon, P. Gounon, and P. Sansonetti. 2000. Myosin light chain kinase plays an essential role in *S. flexneri* dissemination. J. Cell. Sci. 113 Pt 19:3375–3386.

Reeves, P. P., and L. Wang. 2002. Genomic organization of LPS-specific loci. Curr. Top. Microbiol. Immunol. 264:109–135.

Samandari, T., K. L. Kotloff, G. A. Losonsky, W. D. Picking, P. J. Sansonetti, M. M. Levine, and M. B. Sztein. 2000. Production of IFN-gamma and IL-10 to Shigella invasins by mononuclear cells from volunteers orally inoculated with a Shiga toxin-deleted *Shigella dysenteriae* type 1 strain. J. Immunol. 164:2221–2232.

Sansonetti, P. J., A. Phalipon, J. Arondel, K. Thirumalai, S. Banerjee, S. Akira, K. Takeda, and A. Zychlinsky. 2000. Caspase-1 activation of IL-1beta and IL-18 are essential for *Shigella flexneri*-induced inflammation. Immunity 12:581–590.

Sansonetti, P. J., J. Arondel, A. Fontaine, H. d'Hauteville, and M. L. Bernardini. 1991. OmpB (osmo-regulation) and *icsA* (cell-to-cell spread) mutants of *Shigella flexneri*: vaccine candidates and probes to study the pathogenesis of shigellosis. Vaccine 9:416–422.

Sansonetti, P. J., J. Arondel, J. M. Cavaillon, and M. Huerre. 1995. Role of interleukin-1 in the pathogenesis of experimental shigellosis. J. Clin. Invest. 96:884–892.

Sansonetti, P. J., J. Mounier, M. C. Prevost, and R. M. Mege. 1994. Cadherin expression is required for the spread of *Shigella flexneri* between epithelial cells. Cell 76:829–839.

Sansonetti, P. J., J. Arondel, M. Huerre, A. Harada, and K. Matsushima. 1999. Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. Infect. Immun. 67:1471–1480.

Sasakawa, C., K. Kamata, T. Sakai, S. Makino, M. Yamada, N. Okada, and M. Yoshikawa. 1988. Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. J. Bacteriol. 170:2480–2484.

Saxena, S. K., A. D. O'Brien, and E. J. Ackerman. 1989. Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28 S RNA when microinjected into Xenopus oocytes. J. Biol. Chem. 264:596–601.

Schlaepfer, D. D., M. A. Broome, and T. Hunter. 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. Mol. Cell. Biol. 17:1702–1713.

Schlaepfer, D. D., S. K. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 372:786–791.

Schuch, R., and A. T. Maurelli. 2001a. MxiM and MxiJ, base elements of the Mxi-Spa type III secretion system of Shigella, interact with and stabilize the MxiD secretin in the cell envelope. J. Bacteriol. 183:6991–6998.

———. 2001b. Spa33, a cell surface-associated subunit of the Mxi-Spa type III secretory pathway of *Shigella flexneri*, regulates Ipa protein traffic. Infect. Immun. 69:2180–2189.

Sereny, B. 1957. Experimental keratoconjunctivitis shigellosa. Acad. Sci. Hung. 4:367–376.

Shere, K. D., S. Sallustio, A. Manessis, T. G. D'Aversa, and M. B. Goldberg. 1997. Disruption of IcsP, the major Shigella protease that cleaves IcsA, accelerates actin-based motility. Mol. Microbiol. 25:451–462.

Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. Nat. Rev. Mol. Cell. Biol. 1:31–39.

Skoudy, A., G. T. Nhieu, N. Mantis, M. Arpin, J. Mounier, P. Gounon, and P. Sansonetti. 1999. A functional role for ezrin during *Shigella flexneri* entry into epithelial cells. J. Cell Sci. 112:2059–2068.

Skoudy, A., J. Mounier, A. Aruffo, H. Ohayon, P. Gounon, P. Sansonetti, and G. Tran Van Nhieu. 2000. CD44 binds to the Shigella IpaB protein and participates in bacterial invasion of epithelial cells. Cell. Microbiol. 2:19–33.

Speelman, P., I. Kabir, and M. Islam. 1984. Distribution and spread of colonic lesions in shigellosis: a colonoscopic study. J. Infect. Dis. 150:899–903.

Stevenson, G., A. Kessler, and P. R. Reeves. 1995. A plasmid-borne O-antigen chain length determinant and its relationship to other chain length determinants. FEMS Microbiol. Lett. 125:23–30.

Stieglitz, H., S. Fosmire, and P. Lipsky. 1989. Identification of a 2-Md plasmid from *Shigella flexneri* associated with reactive arthritis. Arthritis Rheum. 32:937–946.

Suzuki, T., H. Mimuro, S. Suetsugu, H. Miki, T. Takenawa, and C. Sasakawa. 2002. Neural Wiskott-Aldrich syndrome protein (N-WASP) is the specific

ligand for Shigella VirG among the WASP family and determines the host cell type allowing actin-based spreading. Cell. Microbiol. 4:223–233.

Suzuki, T., T. Murai, I. Fukuda, T. Tobe, M. Yoshikawa, and C. Sasakawa. 1994. Identification and characterization of a chromosomal virulence gene, *vacJ*, required for intercellular spreading of *Shigella flexneri*. Mol. Microbiol. 11:31–41.

Takasaka, M., S. Honjo, K. Imaizumi, H. Ogawa, and R. Nakaya. 1969. Experimental infection with *Shigella sonnei* in cynomolgus monkeys (*Macaca irus*). Jpn. J. Med. Sci. Biol. 22:389–393.

Tamano, K., E. Katayama, T. Toyotome, and C. Sasakawa. 2002. Shigella Spa32 is an essential secretory protein for functional type III secretion machinery and uniformity of its needle length. J. Bacteriol. 184:1244–1252.

Tamano, K., S. Aizawa, E. Katayama, T. Nonaka, O. S. Imajoh, A. Kuwae, S. Nagai, and C. Sasakawa. 2000. Supramolecular structure of the Shigella type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. EMBO J. 19:3876–3887.

Tigyi, Z., A. R. Kishore, J. A. Maeland, A. Forsgren, and A. S. Naidu. 1992. Lactoferrin-binding proteins in *Shigella flexneri*. Infect. Immun. 60:2619–2626.

Toyotome, T., T. Suzuki, A. Kuwae, T. Nonaka, H. Fukuda, S. Imajoh-Ohmi, T. Toyofuku, M. Hori, and C. Sasakawa. 2001. Shigella protein IpaH(9.8) is secreted from bacteria within mammalian cells and transported to the nucleus. J. Biol. Chem. 276:32071–32079.

Tran Van Nhieu, G., A. Ben-Ze'ev, and P. J. Sansonetti. 1997. Modulation of bacterial entry into epithelial cells by association between vinculin and the Shigella IpaA invasin. EMBO J. 16:2717–2729.

Tran Van Nhieu, G., E. Caron, A. Hall, and P. J. Sansonetti. 1999. IpaC induces actin polymerization and filopodia formation during Shigella entry into epithelial cells. EMBO J. 18:3249–3262.

Turner, S. A., S. N. Luck, H. Sakellaris, K. Rajakumar, and B. Adler. 2003. Molecular Epidemiology of the SRL Pathogenicity Island. Antimicrob. Agents Chemother. 47:727–734.

———. 2001. Nested deletions of the SRL pathogenicity island of *Shigella flexneri* 2a. J. Bacteriol. 183:5535–5543.

Uchiya, K., T. Tobe, K. Komatsu, T. Suzuki, M. Watarai, I. Fukuda, M. Yoshikawa, and C. Sasakawa. 1995. Identification of a novel virulence gene, *virA*, on the large plasmid of Shigella, involved in invasion and intercellular spreading. Mol. Microbiol. 17:241–250.

Unkmeir, A., and H. Schmidt. 2000. Structural analysis of phage-borne stx genes and their flanking sequences in shiga toxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 strains. Infect. Immun. 68:4856–4864.

Urvater, J. A., S. N. McAdam, J. H. Loehrke, T. M. Allen, J. L. Moran, T. J. Rowell, S. Rojo, J. A. Lopez de Castro, J. D. Taurog, and D. I. Watkins. 2000. A high incidence of Shigella-induced arthritis in a primate species: major histocompatibility complex class I molecules associated with resistance and susceptibility, and their relationship to HLA-B27. Immunogenetics. 51:314–325.

Vasselon, T., J. Mounier, M. C. Prevost, R. Hellio, and P. J. Sansonetti. 1991. Stress fiber-based movement of *Shigella flexneri* within cells. Infect. Immun. 59:1723–1732.

Venkatesan, M. M., M. B. Goldberg, D. J. Rose, E. J. Grotbeck, V. Burland, and F. R. Blattner. 2001. Complete DNA sequence and analysis of the large virulence plasmid of *Shigella flexneri*. Infect. Immun. 69:3271–3285.

Vokes, S. A., S. A. Reeves, A. G. Torres, and S. M. Payne. 1999. The aerobactin iron transport system genes in *Shigella flexneri* are present within a pathogenicity island. Mol. Microbiol. 33:63–73.

Vore, S. J., P. D. Peele, P. A. Barrow, J. F. Bradfield, and W. H. Pryor, Jr. 2001. A prevalence survey for zoonotic enteric bacteria in a research monkey colony with specific emphasis on the occurrence of enteric Yersinia. J. Med. Primatol. 30:20–25.

Waddell, T., S. Head, M. Petric, A. Cohen, and C. Lingwood. 1988. Globotriosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2. Biochem. Biophys. Res. Commun. 152:674–679.

Wang, L., W. Qu, and P. R. Reeves. 2001. Sequence analysis of four *Shigella boydii* O-antigen loci: implication for *Escherichia coli* and Shigella relationships. Infect. Immun. 69:6923–6930.

Watarai, M., S. Funato, and C. Sasakawa. 1996. Interaction of Ipa proteins of *Shigella flexneri* with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. J. Exp. Med. 183:991–999.

Watarai, M., T. Tobe, M. Yoshikawa, and C. Sasakawa. 1995. Contact of Shigella with host cells triggers release of Ipa invasins and is an essential function of invasiveness. EMBO J. 14:2461–2470.

Watarai, M., Y. Kamata, S. Kozaki, and C. Sasakawa. 1997. rho, a small GTP-binding protein, is essential for Shigella invasion of epithelial cells. J. Exp. Med. 185:281–292.

Way, S. S., A. C. Borczuk, and M. B. Goldberg. 1999. Adaptive immune response to *Shigella flexneri* 2a *cydC* in immunocompetent mice and mice lacking immunoglobulin A. Infect. Immun. 67:2001–2004.

Wei, J., M. B. Goldberg, V. Burland, M. M. Venkatesan, W. Deng, G. Fournier, G. F. Mayhew, G. Plunkett, 3rd, D. J. Rose, A. Darling, B. Mau, N. T. Perna, S. M. Payne, L. J. Runyen-Janecky, S. Zhou, D. C. Schwartz, and F. R. Blattner. 2003. Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect. Immun. 71:2775–2786.

Weinrauch, Y., D. Drujan, S. D. Shapiro, J. Weiss, and A. Zychlinsky. 2002. Neutrophil elastase targets virulence factors of enterobacteria. Nature 417:91–94.

Wolbert, J. 1983. Sexually transmitted diseases in homosexual men. Nurse Pract. 8:35–42.

Wolfensohn, S. 1998. Shigella infection in macaque colonies: case report of an eradication and control program. Lab. Anim. Sci. 48:330–333.

Womble, D. D., and R. H. Rownd. 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. 52:433–451.

Yabuuchi, E. 2002. *Bacillus dysentericus* (*sic*) 1897 was the first taxonomic rather than *Bacillus dysenteriae* 1898. Int. J. Syst. Evol. Microbiol. 52:1041.

Yavzori, M., D. Cohen, and N. Orr. 2002. Prevalence of the genes for shigella enterotoxins 1 and 2 among clinical isolates of shigella in Israel. Epidemiol. Infect. 128:533–535.

Zhang, Z., L. Jin, G. Champion, K. B. Seydel, and S. L. Stanley, Jr. 2001. Shigella infection in a SCID mouse-human intestinal xenograft model: role for neutrophils in containing bacterial dissemination in human intestine. Infect. Immun. 69:3240–3247.

Zychlinsky, A., B. Kenny, R. Menard, M. C. Prevost, I. B. Holland, and P. J. Sansonetti. 1994. IpaB mediates macrophage apoptosis induced by *Shigella flexneri*. Mol. Microbiol. 11:619–627.

Zychlinsky, A., M. C. Prevost, and P. J. Sansonetti. 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature 358:167–169.

16
Escherichia coli

C. L. Gyles and J. M. Fairbrother

Escherichia coli is a gram-negative, fermentative, rod-shaped bacterium that 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 organism in the intestinal tract of most animal species and is typically present at 10^7 to 10^9 organisms per gram in feces. *E. coli* is usually the dominant organism recovered on aerobic culture of feces, but pet birds appear to be an exception as *E. coli* are recovered from only a low percentage of healthy pet birds (Glunder 2002)

The sterile intestinal tracts of newborn animals quickly become contaminated with bacteria from the dam and 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 it increases progressively, with the maximum concentration in the large intestine. The vast majority of *E. coli* in the normal flora are nonpathogenic, but Shiga toxin-producing *E. coli* (STEC) in the normal flora of cattle and other ruminants may be highly pathogenic for humans. In most *E. coli* diseases, pathogenicity is associated with virulence genes encoded by plasmids, bacteriophages, or pathogenicity islands (PAI). These genes include the plasmid-encoded genes for enterotoxins and fimbriae or pili, the phage-encoded genes for Shiga toxins (Stx), and the PAI-encoded genes for the attaching and effacing (AE) lesion in enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) as well as the *pap*, *hly*, and *cnf*1 genes in uropathogenic *E. coli* (UPEC).

Differentiation of *E. coli* into various types is important for distinguishing pathogenic from nonpathogenic types and for conducting epidemiological investigations. Serotyping is a well-established and valuable method that was developed on the basis of differences in the O, K, and H antigens determined by the polysaccharide portion of lipopolysaccharide (LPS), capsular polysaccharide, and flagellar proteins, respectively (Ørskov and Ørskov 1992). The K antigens are no longer routinely determined and serotyping usually involves determination of O and H antigens. Presently there are 175 O antigens and 53 H antigens in the international typing scheme. F or fimbrial antigens are important 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 as the foundation of subspecies differentiation but it is expensive and is conducted by only a few laboratories.

Other procedures are often used to characterize isolates within a serotype. These methods include pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, amplified fragment length polymorphism (AFLP), ribotyping, and 16S rDNA sequencing. Biotyping on the basis of a selection of biochemical tests is also useful but has only moderate discriminatory power.

Detection of virulence factors that are unique to particular types of pathogenic *E. coli* is important for identification and characterization of pathogenic *E. coli.* 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 that characterize the method by which disease is caused. This system identifies broad classes of pathogenic *E. coli*, such as ETEC, EPEC, and EHEC. The term "pathotype" has also been used to refer to variants within these classes, based on differences in the complement of virulence genes (Ngeleka et al. 2002). For example, the latter system identifies ETEC variants based on the complement of fimbriae and enterotoxins among ETEC. We will use the term "virotype" to refer to these subtypes of pathotypes.

TYPES OF *E. COLI* IMPLICATED IN DISEASE

E. coli cause a wide variety of enteric and extraintestinal diseases in animals (tables 16.1 and 16.2). These diseases have been studied extensively in food-producing animals, but there is a paucity of information for 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. A schematic representation of the steps involved in the pathogenesis of infection due to the different classes of pathogenic *E. coli* in animals is shown in figure 16.1.

ENTEROTOXIGENIC *E. COLI* (ETEC)

ETEC is the most common cause of *E. coli* diarrhea in farm animals (Fairbrother et al. 2002). These strains produce one or more fimbrial adhesins and enterotoxins. The most frequently encountered fimbrial adhesins of ETEC in pigs are F4 (K88), F5 (K99), F6 (987P), F41, and F18. Isolates producing the F4 (K88) or F18 adhesin and certain isolates producing F6 (987P) are hemolytic. The most important fimbrial adhesins of ETEC in ruminants are F5 (K99), F41, and F17. Colonies of these isolates often tend to be mucoid and are rarely hemolytic. A newly described adhesin involved in diffuse adherence (AIDA) has also been found on certain ETEC isolates from pigs. Enterotoxins produced by ETEC may be heat stable (STa, STb, or enteroaggregative heat *E. coli* heat-stable enterotoxin 1 [EAST1]) or heat labile (LT). The most important virotypes are listed in tables 16.3–16.5.

Virulence Factors

Fimbriae

Fimbriae (or pili) are rodlike or fibrillar surface appendages on bacteria that mediate attachment to host tissues (Smyth et al. 1994). Each fimbrial unit consists of hundreds of copies of a major subunit that provides the structure and confers the antigenic specificity of the fimbriae. Fimbriae may also contain several copies of minor subunits, one of which is 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 (CFA or colonizing factor antigen). A system of F numbers was devised to designate fimbrial adhesions (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 in the F system. When both designations are used commonly in the literature, we will use a composite name in the following description.

F4 (K88). The F4 adhesin, initially thought to be a K antigen (K88), was subsequently found to be a fine fibrillar structure. F4 (K88) fimbriae are encoded by the *fae* locus on a plasmid that often also carries genes for raffinose fermentation. The O serogroups and virotypes most commonly associated with F4 (K88) fimbriae are listed in table 16.3. F4 (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 F4 (K88)-positive ETEC occurs in both neonatal and postweaning pigs and may be observed in finisher pigs. Adherence due to F4 (K88) is species specific, occurring mostly in pigs. There are three variants of F4 (K88), namely, ab, ac, and ad. The most commonly encountered variant is ac. Up to 50% of pigs lack the receptors for the F4 (K88) adhesin. The allele (S) for the receptor is dominant, and genetic resistance is inherited in a Mendelian fashion. Hence, three genotypes exist: ss (resistant), SS and Ss (sensitive). 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. A report by Francis et al. (1998) indicated that an intestinal brush border mucin-type glycoprotein may be a biologically more relevant receptor for F4 (K88) than the glycoprotein localized on the epithelial brush border, possibly explaining the often-observed lack of correlation of adherence of F4 (K88) variants to small intestinal brush borders *in vitro* with susceptibility of pigs.

F18 Fimbriae. F18 are long flexible filaments with a characteristic zigzag pattern (Nagy and Fekete 1999). They are encoded by the *fed* locus,

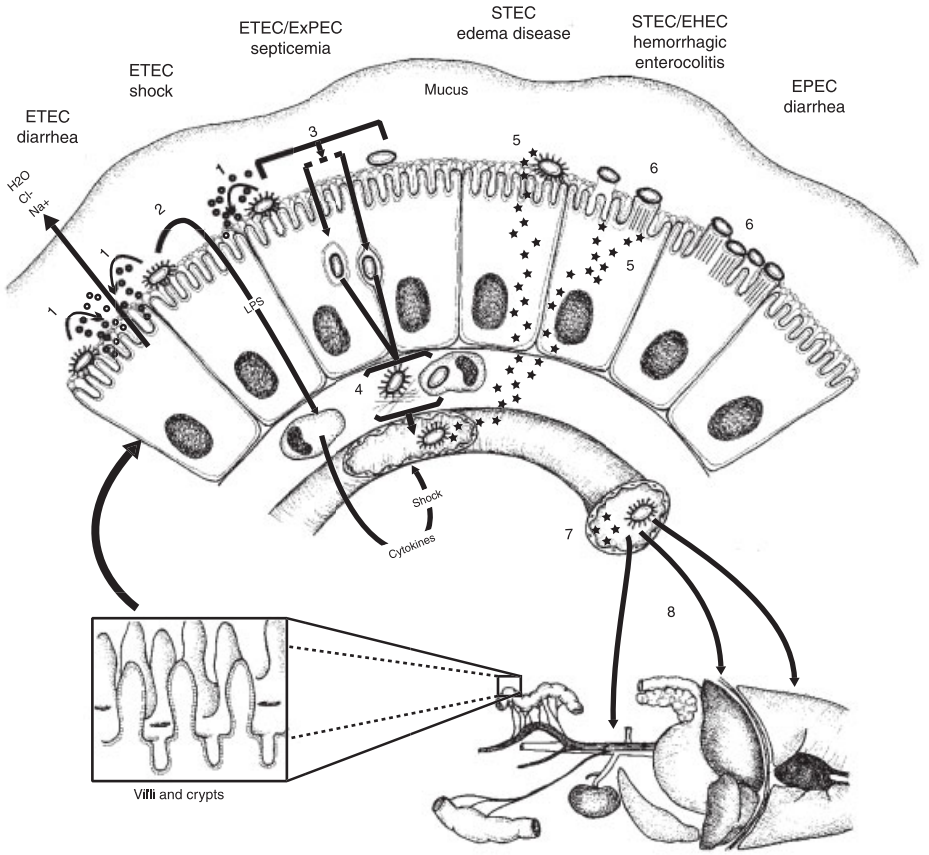


Figure 16.1. Schematic representation of the steps involved in the pathogenesis of infection due to the different classes of pathogenic *E. coli* in animals. (1) ETEC adhering to the small intestinal epithelium produce enterotoxins that stimulate the secretion of water and electrolytes into the intestinal lumen. (2) LPS released by ETEC may also pass through the epithelium and stimulate macrophages to produce mediators that act on the vascular endothelium to promote shock. (3) ETEC and ExPEC may pass through or between the epithelial cells to enter the bloodstream and (8) disseminate to extraintestinal organs. (4) These bacteria may adhere to extracellular matrix proteins and may also resist the bactericidal effects of complement and phagocytes. (5) STEC (F18-fimbriated edema disease *E. coli* in pigs and EHEC in dogs) produce Stx cytotoxins, which pass via the epithelial cells and enter the bloodstream (7) to act on the vascular endothelium at target sites. (6) STEC (EHEC in cattle and dogs) and EPEC attach intimately to epithelial cells and induce actin polymerization and pedestal formation beneath adherent bacteria. (Courtesy of Jacinthe Lachance and Eric Nadeau, The *Escherichia coli* Laboratory, Faculté de mæcine vétérinaire, Université de Montréal)

which is usually found on a plasmid. Two variants of F18, ab and ac, have been described, 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, 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. Genotypes susceptible and resistant to F18 adherence have been differentiated, and pigs with at least one copy of the dominant allele for receptor are susceptible to epithelial adherence *in vitro* and hence to intestinal colonization. The receptor gene has been localized on porcine chromosome 6, closely linked to the gene encoding halothane sensitivity.

F5 (K99) Fimbriae. F5 (K99) fimbriae were also initially thought to be capsular, but were subsequently

Table 16.1. Enteric Diseases of Animals Caused by *E. coli*

Animal species	Disease	Major Types of *E. coli*
Cattle	Neonatal diarrhea	STa$^+$, K99$^+$ 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$^+$ ETEC
		LT$^+$, STb$^+$ O149:K88ac ETEC
	Postweaning diarrhea	LT$^+$, 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 16.2. Extraintestinal Diseases of Animals Caused by *E. coli*

Host species	Type of disease	Comments
Cattle	Septicemia	Invasive *E. coli* that affect calves with hypogammaglobulinemia
	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	Urinary tract infection (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	
	Yolk sac infection	
	Swollen head syndrome	

found to be fibrillar appendages similar to F4 (K88) fimbriae. They are encoded by the *fan* locus, which is also found on a plasmid. F5 (K99) fimbriae are produced by ETEC isolates from pigs, cattle, and sheep (tables 16.3 and 16.4). F5 (K99) mediates attachment of ETEC mostly to the posterior small intestinal mucosa in younger but occasionally in older animals. Hence, diarrhea due to F5 (K99)-positive ETEC is observed mostly in neonatal animals but has recently been occasionally seen in pigs post weaning.

Table 16.3. Important Pathotypes, Virotypes, and Serogroups of *Escherichia 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:EAST-1: 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
Edema disease	STEC	Stx2e:F18ab:(AIDA), α-hemolysin	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 16.4. Important Pathotypes, Virotypes, and Serogroups of *Escherichia 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 16.5. Important Categories, Virulence Factors, and Serogroups of *Escherichia coli* Causing Disease in Poultry

Disease	Category	Virotypes	O Serogroups
Septicemia	APEC	Aerobactin, F1 (type 1), F11 (P fimbrial family), K1, Tsh	1,2,8,15,18,35,78,88, 109,115
Cellulitis	APEC	F1- and P-fimbriae, K1	2,25,71,78

F6 (987P) Fimbriae. F6 (987P) are large, rod-shaped fimbriae encoded by the *fas* locus found both on the chromosome and in plasmids. F6 (987P) mediates bacterial colonization mostly of the posterior small intestine in neonatal pigs. F6 (987P)-mediated intestinal colonization in older pigs is

rarely observed and is thought to be inhibited by preferential binding of bacteria to F6 (987P) receptors present in the mucus rather than to those on the intestinal epithelium (Dean-Nystrom and Samuel 1994). Hence, diarrhea due to F6 (987P)-positive ETEC is observed almost exclusively in neonatal pigs.

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 F5 (K99), although some strains 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 F5 (K99) is present or not.

F17 Fimbriae. F17 are rodlike fimbriae found mostly on bovine necrotoxigenic *E. coli* (NTEC) strains producing cytotoxic necrotizing factor (CNF) 1 or CNF2, being encoded by genes present 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 A. F17 has also been found on bovine ETEC strains, although its role in development of diarrhea due to these strains has not been fully elucidated.

AIDA. The adhesin involved in diffuse adherence (AIDA) is an autotransported, 100-kDa mature protein, which mediates bacterial attachment to intestinal epithelial cells (Benz and Schmidt 1992). AIDA was originally detected in *E coli* isolates from humans with diarrhea, and has been detected recently in *E. coli* strains from pigs with edema disease or postweaning diarrhea, particularly in strains of the virotypes Stx:F18 and F18 alone (Mainil et al. 2002). AIDA is encoded by genes present 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).

Enterotoxins

Two major classes of enterotoxin are produced by ETEC (Gyles 1994): heat-stable toxin (ST), which is resistant to treatment at 100°C for 15 minutes, and heat-labile toxin (LT), which is inactivated at 60°C for 15 minutes. 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, has been recently reported in ETEC isolates from pigs. The enterotoxins are plasmid encoded. LT enterotoxins are highly antigenic, whereas ST enterotoxins tend to be poorly antigenic. Enterotoxins 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 2000 Da (Gyles 1994). STa has been designated STaP (produced by bovine, porcine, and human ETEC) or STaH (produced by human ETEC), based on minor differences in the composition of the toxins. STa binds to a guanylyl cyclase intestinal epithelial receptor (De-Sauvage et al. 1991) and activates guanylate cyclase, which stimulates production of cyclic GMP. High levels of cyclic GMP in the cell inhibit the Na/Cl cotransport system and reduce the absorption of electrolytes and water from the intestine at villus tips (Dreyfus et al. 1984) and result in an elevated secretion of Cl^- and H_2O in crypt cells (Forte et al. 1992). The effects of STa are reversible. STa is active in infant mice and young pigs but is less active in older pigs, possibly due to a decreasing concentration of receptors with age (Cohen et al. 1988).

STb. STb (also called STII) is a 48-amino acid peptide of 5,000 Da, in its mature form, that is unrelated to STa in composition and biological activity (Dubreuil 1997). ETEC producing STb are mostly associated with pigs but have also been isolated sporadically from cases of diarrhea in humans, calves, and chickens. The intestinal epithelial cell receptor to which STb binds has not been fully elucidated. A 25 kDa protein in mouse intestinal cell membranes that binds STb has been identified (Hitotsubashi et al. 1994). More recently, sulfatide molecular species in the pig intestine have been shown to be recognized by STb (Beausoleil et al. 2002).

STb does not alter cGMP or cAMP levels in intestinal mucosal cells, thus differing in mechanism of action from STa and LT (Hitotsubashi et al. 1992). On the other hand, STb stimulates an increase in prostaglandin E_2 levels, probably inducing the duodenal and jejunal secretion of water and electrolytes by as yet unknown mechanisms. STb also stimulates the release of another secretagogue, 5-hydroxytryptamine, into intestinal fluid (Harville and Dreyfus 1995). STb may also act by opening a G-protein-linked, receptor-operated calcium channel in the plasma membrane (Dreyfus et al. 1993), leading to uptake of Ca^{2+} into cells and subsequent

stimulation of synthesis of prostaglandin E_2 and other secretagogues, and induction of diarrhea. STb is inactivated by trypsin, and, in the presence of trypsin-inactivator, is active in the intestines of mice, rats, and calves (Whipp 1990). The role of STb in the development of diarrhea has been questioned (Casey et al. 1998) although ETEC producing only STb can induce diarrhea (Fairbrother et al. 1989).

EAST1. EAST1 was first identified in enteroaggregative *E. coli* isolated from humans (Savarino et al. 1993). The *astA* gene that encodes EAST1 was subsequently reported in human ETEC and EPEC, and in ETEC from pigs and calves with diarrhea (Yamamoto and Nakazawa 1997). The *astA* gene is commonly found in F4 (K88)-positive ETEC strains from pigs with diarrhea and in F18:Stx2e strains from pigs with edema disease (Choi et al. 2001; table 16.1). EAST1 is a 38-amino-acid peptide of 4,100 Da that is different from STa and STb, although it shares 50% homology with the enterotoxic domain of STa (Savarino et al. 1993) and appears to interact with the STa receptor guanylate cyclase C to elicit an increase in cGMP. Hence, the mechanism of action of EAST1 is proposed to be identical to that of STa. However, the role of EAST1 in the development of diarrhea has yet to be defined (Ménard and Dubreuil 2002).

Heat-labile Enterotoxin (LT). Two subgroups of LT—LTI and LTII—have been described, only LTI being neutralized by anticholera toxin. LTI can be divided into LTh-I, produced by human ETEC, and LTp-I, produced by porcine ETEC, which have slight differences in composition. LTII consists of two antigenic variants, LTIIa and LTIIb, which are related to LTI in their A subunits but differ in their B subunits.

LTI is a high molecular weight toxin complex (approximately 88,000 daltons) that consists of five B subunits able to bind to GM1 ganglioside receptors on the intestinal epithelial cell surface and a biologically active A subunit (Gill et al. 1981). Other receptors to which LT binds are GD1b, asialo GM1, GM2, and a number of galactoproteins and galactose-containing glycolipids. The A subunit (30,000 daltons) consists of an A1 fragment (21,000 daltons) containing the active site and an A2 fragment, which links the A1 fragment 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 reported to account for the lower toxicity of LT compared with CT (Rodighiero et al. 1999). LTI is very similar to cholera toxin (CT), both structurally and functionally. A pathway highly homologous to the type II secretion pathway for secretion of CT was recently reported for secretion of LTI by ETEC (Tauschek et al. 2002).

After binding of the B subunits to their specific cell surface receptor, the A_1 fragment translocates into the cell where it activates the adenylate cyclase system, stimulating the production of cyclic AMP. The A_1 fragment possesses ADP-ribosyl transferase activity, by which it transfers an ADP-ribose moiety from NAD to the Gs-alpha regulator of adenyl cyclase. This action keeps the synthesis of cAMP by adenyl cyclase locked in the "on" position. ADP-ribosylation is enhanced by ADP-ribosylation factors (ARFs), which are 20 kDa regulatory GTPases that activate the LT A1 catalytic unit.

High levels of cAMP in the cell result in increased secretion and decreased absorption of Cl^- and Na^+ ions and water into the intestinal lumen. The effect of LT 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 has potent immunomodulatory effects, and derivatives of LT have been developed as adjuvants of both the mucosal and systemic immune systems. LTB alone has been shown to be an adjuvant by means of activation of cell signaling 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).

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, and pigs. The LTII enterotoxins share the A1:B5 structure, ADP-ribosyl transferase activity, and immunomodulatory properties of LT1 but differ in their binding specificities.

PATHOGENESIS

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. The ETEC then

proliferate rapidly to attain massive numbers to the order of 10^9/g in the midjejunum to the ileum. ETEC adhering closely to the intestinal epithelium produce enterotoxins that 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 (Qureshi et al. 1991). LPS stimulates the overproduction of mediators of inflammation including tumor necrosis factor-α, interleukin-1, and interleukin-6, which cause these symptoms (Whitfield et al. 1994). These mediators promote shock by affecting the vascular endothelium leading to increased vascular permeability. Modulation of the coagulation pathway may lead to fibrin deposition and clot formation. Aggregation and subsequent degranulation of neutrophils activated by these mediators may cause damage to the vascular endothelium.

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.

In the following sections, specific aspects of the pathogenesis of ETEC infections in different animal species will be discussed.

Pigs

Newborn piglets ingest ETEC found in their environment, especially from the mammary glands of the mother and the farrowing crate. These ETEC originate from the feces of piglets with ETEC diarrhea, asymptomatic carrier piglets, or sows (Fairbrother 1999). The O serogroups and virotypes of ETEC most commonly associated with diarrhea in newborn piglets are shown in table 16.3. 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.

In newborn pigs, the pH of the stomach and duodenum is less acidic and the production of digestive enzymes is low, 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. One or more animals in a group are affected. A less-watery diarrhea may also be observed in the first 1 to 2 weeks of age, or as early as 2 days postweaning, with low mortality and often with decreased weight gain. In piglets of this age group with diarrhea, a coinfection with other pathogens such as transmissible gastroenteritis virus, rotavirus, or coccidia, is often observed. F4 (K88)-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 some cutaneous cyanosis of the extremities, or less acutely with hyperthermia, diarrhea, and anorexia.

Postweaning diarrhea is fluid, yellowish or grey and most commonly starts at 3 to 5 days after weaning, lasting for up to a week and causing emaciation (Bertschinger 1999). Over several days, most of the pigs in a group may be affected and a mortality of up to 25% may be observed in the absence of antibiotic therapy. In farms where husbandry measures at weaning, such as addition of higher levels of protein of animal source, plasma, acidifying agents, and zinc oxide are being used, peaks of diarrhea and enteric colibacillosis complicated by shock may be observed often at 3 weeks after weaning, or even at 6 to 8 weeks after weaning, at the time when the pigs enter the growing barns (Fairbrother, unpublished data).

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 16.3.

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 (Dreau et al. 1994). The latter could result in changes such as a decrease in villous height, deepening of the crypts, and an increase in antisoya 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 2,400 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 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, as pigs weaned at 2 weeks of age or less were twice as likely to develop diarrhea as those weaned at 6–7 weeks of age (Svensmark et al. 1989).

On histopathology, layers of *E. coli* are observed adhering to the mucosa of the jejunum and ileum in newborn and postweaning pigs. Bacteria are usually located at a distance of approximately half to one bacterial width away from the microvilli, as observed on transmission electronmicroscopy (fig. 16.2). 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.

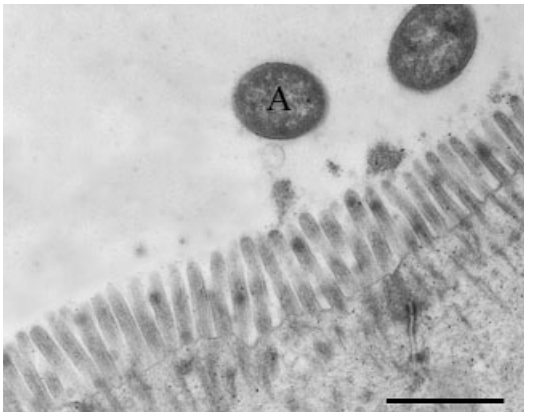


Figure 16.2. Attachment of ETEC to the ileal epithelial cells of a weaned pig, showing typical less-intimate bacterial adherence and no effect on epithelial cells. Bar = 1 μm. (Courtesy of Francis Girard, The *Escherichia coli* Laboratory, Faculté de médécine vétérinaire, Université de Montréal)

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 in color and occasionally containing flecks of blood. In acute cases, extensive loss of body water leads to a marked decrease in body weight within 6–8 hours 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 16.4. These ETEC often produce an acidic polysaccharide type of K antigen, such as K25, K28, K30, or K35, which may enhance intestinal colonization due to 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 epithelial attachment of ETEC is less clear. Nevertheless, F17-positive *E. coli* producing the cytotoxic necrotizing factor 2 (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.

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 (Beutin 1999). Most of the ETEC isolated from dogs with diarrhea are STa-positive, a small proportion of these also being STb-positive (Drolet et al. 1994; Hammermueller et al. 1995; Wasteson et al. 1988). No LT-positive ETEC have been reported in dogs with diarrhea. Canine ETEC belong to O serogroups, such as O42:H7, 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.

SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC)

Shiga toxin-producing *E. coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC), are implicated in diarrhea and dysentery in calves and lambs, cutaneous and renal glomerular vasculopathy (CRGV) in dogs, and edema disease (ED) in pigs. An understanding of Shiga toxins (Stxs), the development of the AE lesion, and potential STEC virulence factors are critical for understanding the diseases caused by STEC and are discussed below.

Virulence Factors

Shiga Toxins

Shiga toxins 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 major *E. coli* Stx toxins consist of Stx1, which is identical to Shiga toxin of *Shigella dysenteriae*, and Stx2, which is 56% homologous to Stx1. In addition, there are variants of Stx2 identified as Stx2c, Stx2d, Stx2e, and Stx2f. Stx2e is typically found as the only Stx in porcine edema disease strains of STEC, whereas Stx2f is found in *E. coli* isolated from healthy pigeons. Combinations of Stx1, Stx2, Stx2c, and Stx2d are found in STEC that are carried by healthy ruminants and implicated in diseases in humans. The *stx* genes are carried by temperate lambdoid bacteriophages, along with late genes of the phage genome, and appear to be regulated by a Q-like antiterminator (Schmidt 2001). Induction of phage by several agents, including certain 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, permitting the toxin molecule to be internalized by receptor-mediated endocytosis. Following retrograde transfer through the Golgi apparatus, the toxin becomes associated with the rough endoplasmic reticulum, 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. The Stx toxins also induce signaling pathways that result in apoptosis, as well as activation of nuclear factor κB (NF-κB), Src kinases, and activator protein-1(AP-1) (Heyderman et al. 2001).

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 (Acheson et al. 1996; 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 in animals.

The Attaching and Effacing (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; fig. 16.3). The LEE_{O157} is a 36.5-kb DNA fragment that consists of 41 genes, organized into 5 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 led to recognition of at least ten types of intimin in STEC and EPEC (Zhang et al. 2002). The gene *tir* encodes the translocated intimin receptor (Tir), a protein that 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. There is recent evidence that nucleolin, a host-cell protein, is also involved in the interaction of Tir and intimin (Sinclair and O'Brien 2002). 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 fimbriae and host epithelial cell is believed to be an early development in formation of the AE lesion. Long polar fimbriae in O157:H7 STEC (Lpf) (Torres et al. 2002) could play the role of making initial contact. Following the initial contact, the genes of the LEE are turned on and the TTSS allows transfer of *E. coli* secreted proteins (Esp) and transfer of Tir to proceed. In response to these proteins, 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. 1998; fig. 16.3). 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 leukocytes that migrate between epithelial cells into the intestinal lumen.

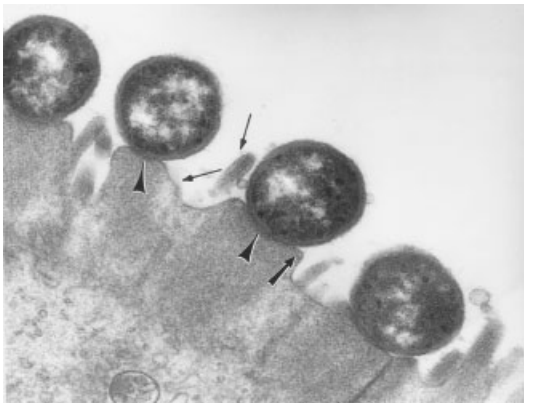


Figure 16.3. Attaching and effacing lesion in the colon of an experimentally infected calf. The intimate attachment of the bacteria, the accumulation of electron-dense material beneath the bacteria, and the effacement of microvilli are evident. (From Sandhu and Gyles 2002 with permission)

Adhesins with unidentified roles have been reported in LEE-positive and LEE-negative STEC. These include LPF (Doughty et al. 2002), a type IV fimbria (Srimanote et al. 2002), Iha of O157:H7 (Tarr et al. 2000), and plasmid-encoded Efa or ToxB (Nicholls et al. 2000; Tatsuno et al. 2001).

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 edema disease in pigs encode alpha hemolysin. Antibody to EHEC hemolysin 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. ED strains of STEC produce alpha hemolysin rather than Ehly.

EASTI. The O157:H7, O26:H11, O111, and O45 EHEC all carry the *astA* gene for the enteroaggregative heat-stable toxin I (EASTI). This enterotoxin could play a role in the diarrhea caused by these EHEC.

Acid Resistance. The O157:H7 EHEC (and certain other serotypes of STEC) are exceptionally acid

resistant *in vitro*, being able to survive exposure to pH 2.5 for over 3 hours, but there is considerable variation in acid resistance among strains of O157:H7 EHEC. At least three acid-resistance mechanisms have been identified in O157:H7 EHEC—a glutamate-dependent system, and acid-inducible arginine-dependent and oxidative systems (Audia et al. 2001). In addition, the O polysaccharide has been reported to contribute 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 a low infectious dose for humans. The O157:H7 EHEC 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*.

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 (fig. 16.1). STEC in the environment are ingested and pass through the stomach into the intestine, where they colonize and produce Shiga toxin. In LEE-positive STEC, formation of the AE lesion is a major feature of intestinal colonization. In LEE-negative STEC, binding of STEC to the epithelium occurs in a non-intimate 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. Other bacterial products, such as LPS, may contribute to pathology by induction of cytokines and up-regulation of receptors for Stx. Descriptions of pathogenesis of dysentery in sheep and lambs, CRGV in dogs, and edema disease in pigs will illustrate variations on this theme.

STEC in Calves and Lambs

A high percentage of cattle and sheep carry STEC without showing any signs of ill health. 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 be used to experimentally reproduce disease in calves (Dean-Nystrom et al. 1997). Recent studies indicate that O157 STEC are carried in cattle by persistent shedders, which are colonized over 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).

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 both by association with and experimental reproduction of disease (Moxley and Francis 1986; Wray et al. 1989; Dorn et al. 1993). Affected calves show AE lesions in the terminal ileum, colon, and rectum, with edema and 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.

Calves naturally or experimentally infected with STEC that induce enteric disease show no systemic signs. This has been attributed to lack of the Gb3 receptor in blood vessels of calves (Pruimboom-Brees et al. 2000; Hoey et al. 2002). There is also evidence that most O157 STEC from cattle secrete considerably lower amounts of proteins through the TTSS, suggesting that they may be less efficient colonizers compared with human O157 STEC (McNally et al. 2001).

Diarrhea has been associated with certain STEC, particularly of the O118 serogroup. The STEC that are implicated are typically *stx1* and *eae* positive.

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 diarrhea in this species. The hemolytic uremic syndrome (HUS) occurs occasionally in young dogs of several breeds. There is a prodrome of bloody diarrhea followed by thrombocytopenia, microangiopathic hemolytic anemia, and anuric acute renal failure (Carpenter et al. 1988; Holloway et al. 1993; Hertzke et al. 1995). HUS occurs in about 5% of the dogs that 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 cutaneous and renal glomerular vasculopathy (CRVG), has been reported in racing greyhounds fed poor-quality ground beef (Cowan et al. 1997; Fenwick and Cowan

1998). The O157:H7 EHEC 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.

Edema Disease in Pigs

Edema disease (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 nanograms per kg body weight) results in the characteristic signs, symptoms, and lesions of ED (MacLeod et al. 1991).

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 to 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 pili (Bertschinger and Gyles 1994). Pigs that lack the intestinal receptors for F18 pili 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), which may be required for synthesis of a glyconjugate receptor for the F18 pili. Resistance is present in only a low percentage of most breeds of pigs. Age-related expression of receptors for F18 pili (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 it 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 management factors. At the time of weaning, the intestinal epithelial cells of the pig undergo changes that lead to a period of temporary malabsorption. High-protein diets contribute to the presence in the intestine of an abundance of rich substrate for rapid proliferation of EDEC (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 the 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 bloodstream, 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.

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 and a low percentage of intact toxin passes through the intestinal epithelium (Acheson et al. 1996). 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 receptors 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.

Cases of 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, give 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 groups 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 that have been associated with ED or possess the *stx2e* genes include O groups 2, 9, 101,107, 120, 121, 125, 149, 154, and 157 (Gannon et al. 1988).

EDEC may or may not be enterotoxigenic. Those strains that are enterotoxigenic function as both ETEC and EDEC (Nagy and Fekete 1999).

ENTEROPATHOGENIC *E. COLI* (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 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. This pathology was initially observed in human EPEC strains that had been epidemiologically incriminated in severe outbreaks of infantile diarrhea occurring worldwide and that belonged to certain defined classical EPEC serotypes (Moon et al. 1983; Levine 1987). The pathogenic mechanisms of human EPEC have subsequently been extensively studied and several models have been proposed (Nataro and Kaper 1998).

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), in rabbit EPEC (REPEC) (Tauschek et al. 2002; Zhu et al. 2001), and in dog EPEC (DEPEC) (An et al. 1997; Goffaux et al. 1999). In the O127:H6 human EPEC strain E2348/69, the LEE is inserted in the *selC* locus at about 82 minutes on the *E. coli* K12 chromosome, and it is 35 kb long, varying slightly from that of the LEE_{O157} (see section on STEC above for explanation of the functioning of the LEE locus). As also observed for STEC, one of the LEE genes (*eae*) encodes intimin, a 94 kDa outer-membrane protein involved in intimate attachment to host cells (Nataro and Kaper 1998). Another encodes a translocated intimin receptor called Tir, which interacts with intimin and allows the intimate attachment of the bacteria to the epithelial cells. Other LEE genes encode the secreted proteins EspA, EspB, EspD, and EspF, which are responsible for signal transduction in epithelial cells (Nataro and Kaper 1998; McNamara et al. 2001) and are secreted through the TTSS encoded in the LEE. Several major intimin subtypes have been described to date, based on the antigenic variations of their C-terminal end, and include intimins named α, β, γ, δ, and ϵ (Adu-Bobie et al. 1998; Oswald et al. 2000). Intimin binds a 55-amino-acid region within the extracellular loop of Tir, triggering actin nucleation beneath the adherent bacteria (Kenny 1999). Homologous sequences that may also bind intimin exist in integrins, and certain molecules, such as β1-chain integrins, are proposed as alternative candidate receptors for intimin (Frankel et al. 1996; Liu et al. 1999).

The recently identified EspC enterotoxin located within a second PAI at 60 minutes on the *E. coli* chromosome may be an accessory virulence factor in some EPEC (Mellies et al. 2001). In addition, the *paa* (for porcine A/E associated) 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 on the bacterial surface, appears to contribute to the AE process, and may be involved in the initial bacterial adherence. Similarly, 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).

PATHOGENESIS

EPEC attach loosely to the intestinal epithelial cells (fig. 16.4, bacterial cell A), probably by means of

specific adhesins such as AF/R1, AF/R2, and Ral in the rabbit, Bfp in the dog, and Paa in all species. 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 EspA, EspB, EspD, EspF, and Tir. The signal activity 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 possibly of other host-cell receptors by the bacterial intimin, and cytoskeletal changes such as an accumulation of polymerized actin directly beneath the adherent bacteria (fig. 16.4, 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 that can extend from the epithelial cell in a pseudopod-like structure. The signaling activity also results in an influx of polymorphonuclear neutrophils at the site of bacterial adherence.

The mechanisms by which EPEC induce diarrhea are not yet 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, to a localized inflammatory response at the lesion site, or chloride secretion following polymorphonuclear leukocyte transmigration.

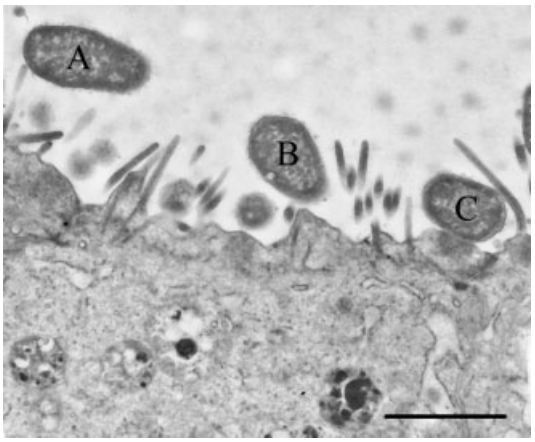


Figure 16.4. Attachment and effacement of PEPEC to the ileal epithelial cells of a weaned pig, showing (*A*) initial loose bacterial attachment, (*B*) subsequent pedestal formation, and (*C*) more intimate bacterial adherence with electron-dense actin polymerization beneath the bacterium and microvillus effacement. Bar = 2μm. (Courtesy of Francis Girard, The *Escherichia coli* Laboratory, Faculté de médécine vétérinaire, Université de Montréal)

Pig

PEPEC are associated with postweaning diarrhea in pigs (Helie et al. 1991; Zhu et al. 1994, 1995) and often belong to serogroups O45 or O103. PEPEC attach to and efface the microvilli of the small and large intestinal mucosa and have been associated with diarrhea in postweaning pigs. The mechanisms by which they cause diarrhea and the role of coinfection with other pathogens are not yet known. However, the similarity to human EPEC 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. 16.4).

Dog

DEPEC are the pathogenic *E. coli* most commonly associated with diarrhea in dogs (Beaudry et al. 1996; Beutin 1999; Drolet et al. 1994; Goffaux et al. 2000; Janke et al. 1989; Wadas et al. 1996). 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 bacteriological evidence of AEEC (Drolet et al. 1994; Janke et al. 1989; 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.

Rabbit

REPEC is 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 (Peeters 1994). 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 (Blanco et al. 1997; Milon et al. 1999; Peeters 1994). 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 serotypes tend to cause a mild diarrhea with possible weight loss.

Any factor that 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 to 12 days of age, with mortality of up to 100% within a litter. In weanling rabbits, diarrhea is observed at 5 to 14 days after weaning, with mortality of 5% to 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 tested 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). RPEC 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 (Fiederling et al. 1997; Pillien et al. 1996), or Ral (Adams et al. 1997; 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).

EXTRAINTESTINAL *E. COLI* (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.

Septicemic Disease

SEPEC are found in the normal intestinal microflora and belong to a limited number of serotypes (Ørskov and Ørskov 1992). In certain circumstances, such as in young animals that 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 septicemic *E. coli* 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 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 the presence of P fimbriae (Tewari et al. 1994) and certain O antigens (Burns and Hull 1999), also contribute to the ability of septicemic *E.* coli to avoid phagocytosis. Type 1 fimbriae promote bacterial adhesion to phagocytic cells by 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 (Maiti et al. 1994**;** 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 (Olsen et al. 1993; tables 16.3 and 16.4). 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* (Girardeau et al. 2003; Dezfulian et al. 2003).

Septicemic *E. coli* 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 hours, 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 16.3. 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 polymorphonuclear 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" is required for resistance to serum and to phagocytosis by polymorphonuclear leukocytes (Ngeleka et al. 1992, 1994).

Septicemic *E. coli* in Calves

E. coli septicemia occurs in calves in the first few days of life and in lambs at 2 to 3 weeks of age (Gay and Besser 1994). 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 enlargement of the spleen and pulmonary edema and hemorrhage. Fibrinous polyarthritis and meningitis may be observed in chronic cases.

The predominant serogroups and virotypes associated with septicemia in calves are shown in table 16.4. Bovine SEPEC often carries 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 septicemic *E. coli* to translocate from the intestine and survive in the blood in certain circumstances, but may not be sufficient to induce septicemia and death.

Septicemic *E. coli* in Chickens

Respiratory tract infection with avian pathogenic *E. coli* (APEC) results in depression and fever in birds

of 4 to 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. 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 lachrymal 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. The predominant serotypes and virotypes of APEC are shown in table 16.5.

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 (Cheville and Arp 1978; Ackermann and Cheville 1991; Pourbakhsh et al. 1997a) 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). 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. Curli promote binding to the major histocompatibility complex class I (MHC-I), extracellular matrix and serum proteins, and to 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), 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. Recent studies with isogenic mutants have confirmed the role of K1 capsule as well as that of the O78 lipopolysaccharide 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 fecal 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 et al. 1986). *In vivo* experiments showed that APEC were present in macrophages, but they 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.

Avian Cellulitis

APEC are also associated with cellulitis of the lower abdomen and thighs that is not associated with clin-

ical illness. Gross lesions are typically 3 to 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 (Elfadil et al. 1996) but results in the 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 (Leclerc et al. 2003). Cellulitis has been reported to be characterized by seasonal variation (Vaillancourt et al. 1992), and some climatic conditions may be predisposing factors (Gomis et al. 2000).

Urinary Tract Infections

E. coli is the pathogen that is most frequently implicated in urinary tract infections (UTIs) in dogs and cats. Infection is most commonly manifested as cystitis but urethritis and prostatis are also seen. There have been many studies on virulence genes of canine and feline uropathogenic *E. coli* (UPEC) but very few on pathogenesis. However, remarkable similarities between the virulence-associated genes in canine and human UPEC suggest that major aspects of pathogenesis are similar between these species. Indeed, there have been recent suggestions that dogs may be a source of UPEC that causes UTI in humans (Johnson et al. 2001, 2003; Starcic et al. 2002). In one recent 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, it is well established that the intestine is a reservoir for UPEC, and it will be necessary to demonstrate that these *E. coli* can indeed induce diarrhea in dogs.

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 (Yuri et al. 1998; Senior et al. 1992; Feria et al. 2001; Johnson et al. 2003). These genes encode specific O antigens (commonly O1, O2, O4, O6, and O25), type 1 fimbriae, P fimbriae, S fimbriae, alpha hemolysin, CNF1, and iron-sequestering systems. Among the UPEC that encode P fimbriae, *papG* allele III is by far the most frequent. Commonly, genes for P fimbriae, S fimbriae, alpha hemolysin, and CNF1 are found together as they are often encoded by genes on a PAI (Feria et al. 2001). However, various UPEC may possess different combinations of virulence factors. Studies with human UPEC have identified PAI I with the *hly* gene alone or in combination with *pap*, or genes for other adhesins; PAI II with the *hly*, *prs, cnfI*, or *hly, prf*, or *pap, bfrB*; PAI III with *iro*, *sfa*; PAI IV with *ybt*; PAI V with *kps*; and high pathogenicity island (HPI) with *ybt* (Oelschlaeger et al. 2002).

The virulence-related factors likely play important roles as adhesins that mediate adherence to bladder epithelium, iron-scavenging systems that enhance survival in a low-iron environment, and cytotoxic proteins that damage tissues. Catheterization, abnormalities of the urinary tract, illnesses such as diabetes, and chemotherapy predispose to infection with a wide variety of bacteria, including *E. coli* that lack the usual complement of urovirulence factors.

Adherence to the mucosa of the urinary tract is a critical factor in virulence of UPEC. Type 1 fimbriae, which are present on most *E. coli* and therefore not specifically associated with UPEC, bind Tamm Horsfall protein and uroplakins that are found on the surface of the human bladder (reviewed by Wullt et al. 2002). In contrast, P fimbriae are highly associated with UPEC, compared with fecal *E. coli*. The genes for both type 1 and P fimbriae undergo phase variation, but little is known about the factors that affect their expression *in vivo*. Adherence of P fimbriae to epithelial cells involves their Galα1-4 Galβ-containing glycolipid receptor and Toll receptor 4 as coreceptor and results in production of interleukin (IL)-6 and IL-8 under *in vitro* conditions. Binding also causes the release of ceramide and activation of signaling pathways regulated by

ceramide. Almost nothing is known about the role of S fimbriae in UTI.

UPEC appear to be better equipped to grow in urine than are non-UPEC strains. Binding to epithelial cells is a major virulence factor, as removal of bacteria by frequent voiding and exfoliation of epithelial cells is important in innate defense against UTI. Other 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, certain O-polysaccharides, and specific outer-membrane proteins, and production of aerobactin.

Signs of urinary tract infection include frequent passage of urine that may appear cloudy. The major steps in pathogenesis are as follows. 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. Factors that predispose to colonization of the urinary tract include diabetes mellitus, immunocompromised states, and impairment of normal micturition. Following adherence of UPEC, production of cytokines including IL-8 attracts a strong neutrophil response. P fimbriae impart some resistance to phagocytosis, but some UPEC are phagocytosed and killed by neutrophils. Damage to bladder epithelium is largely the result of the inflammatory response including the toxic products of the neutrophils. Infection does not usually ascend to the kidneys.

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. The alterations in epithelial cells allow bacteria to adhere and multiply. *E. coli* is the bacterium most frequently implicated and is isolated from 58–88% of cases (Jarvinen 1981; Fransson et al. 1997). The *E. coli* recovered from the uterus of bitches with pyometra have the same characteristics of UPEC, and in some cases there is simultaneous UTI and pyometra involving the same organism (Dhaliwal et al. 1998; Hagman and Kuhn 2002).

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 has been noted particularly in high-producing animals, in the first two 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 that may be peracute, acute, or chronic. The *E. coli* that are implicated in mastitis are the *E. coli* that are found in the environment, and these organisms do not appear to possess any special virulence factors (Hill 1994). It isn't surprising that host factors play a major role in determining the nature of the mastitis that 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 (Bradley and Green 2001). These researchers suggest that some *E. coli* may have evolved so as to adapt to the bovine mammary gland. One aspect of this adaptive behavior is an ability to invade mammary cells, thereby evading the immune system and promoting persistence in the gland.

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 defense 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 polymorphonuclear 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 (Hill 1994). 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.

Experimental infections have been useful in allowing careful measurements of local and systemic response to infection. Increases in serum haptoglobin and amyloid-A concentrations have been reported, and in severely affected animals, increases in serum TNF-alpha and nitrite/nitrate have been noted.

The response to invasion of the lumen of the mammary gland by *E. coli* may be influenced in some cases 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, notably tumor necrosis factor, interleukin (IL)-1, and IL-6, which result in the movement of PMN through the connective tissue into the lumen of the gland. If the PMN response is rapid, the bacteria may be quickly phagocytosed and destroyed. Failure to rapidly remove the *E. coli* results in their growth and severe local and sometimes systemic 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

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 antifimbriae antibodies, which have been shown to be protective when they are delivered 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). Antibodies to the polysaccharide capsule of ETEC may also be protective. Antifimbriae 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, which is due to a toxemia, 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 has been reported to inhibit colonization of the pig intestine by F18-positive *E. coli* (Sarrazin and Bertschinger 1997).

Recent studies have identified differences in kinetics of infection and immunity in weaned pigs infected with F4-positive and F18-positive ETEC (Verdonck et al. 2002). Infection with F4-positive ETEC resulted in intestinal colonization and induction of anti-F4 antibodies that was more rapid than comparable events for F18-positive ETEC. Experimental 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 F4 antigen (Lee et al. 2001).

Serum antibodies against UPEC do not appear to play a significant role in protection. 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. The recognition of LPS by TLR-4 has been shown to be important in the innate immune response that occurs in the early stages of UTI infection with *E. coli* (Schilling et al. 2003).

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*, has also been used to provide protection against severe mastitis (Tomita et al. 2000). 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 basis for the action of this vaccine has recently been suggested (Dosogne et al. 2002). These researchers suggest 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. 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.

A vaccine that blocks the bacterial enterochelin system for obtaining iron in a low iron environment induced antibodies that inhibited the growth of *E. coli* (Lin et al. 1999). The vaccine consisted of FepA, a protein on the bacterial surface that acts as a receptor for iron-enterobactin complexes used by *E. coli* in the mammary gland to bring iron into the cell. It has been suggested that this vaccine may be effective in protecting cows against *E. coli* mastitis, but it is possible that the *E. coli* may be able to use an alternative system. 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).

CONCLUDING REMARKS

The spectrum of *E. coli* diseases is impressive for its range of species and variety of mechanisms. This versatility is founded on considerable opportunity and facility in acquiring new blocks of DNA. Much has been learned 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 sequence of *E. coli* O157:H7. We know almost nothing about the factors that allow one particular serotype of a pathogenic *E. coli* to dominate its class, as is the case with O157:H7 among EHEC and O149:F4 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 lifestyle. Except for STEC, pathogenic *E. coli* have shown remarkable species specificity, but recent studies have demonstrated significant similarities between canine and human UPEC. Continuing research in this area is needed to clarify host-species specificity and to assess whether *E. coli* is evolving to increase its versatility even further.

REFERENCES

Aarestrup, F. M., S. E. Jorsal, P. Ahrens, N. E. Jensen, and A. Meyling. 1997. Molecular characterization of *Escherichia coli* strains isolated from pigs with edema disease. J. Clin. Microbiol. 35:20–24.

Acheson, D. W., R. Moore, S. De Breucker, L. Lincicome, M. Jacewicz, E. Skutelsky, and G. T. Keusch. 1996. Translocation of Shiga toxin across polarized intestinal cells in tissue culture. Infect. Immun. 64:3294–3300.

Ackermann, M., and N. Cheville. 1991. Ultrastructural studies of the lung of turkeys (*Meleagris gallopavo*) inoculated intratracheally with *Escherichia coli*. Vet. Pathol. 28:283–191.

Adams, L. M., C. P. Simmons, L. Rezmann, R. A. Strugnell, and R. M. Robins-Browne. 1997. Identification and characterization of a K88- and CS31A-like operon of a rabbit enteropathogenic *Escherichia coli* strain which encodes fimbriae involved in the colonization of rabbit intestine. Infect. Immun. 65:5222–5230.

Adu-Bobie, J., G. Frankel, C. Bain, A. G. Goncalves, L. R. Trabulsi, G. Douce, S. Knutton, and G. Dougan. 1998. Detection of intimins alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. J. Clin. Microbiol. 36:662–668.

An, H., D. Dubreuil, J. M. Fairbrother, J. Harel. 1997. Cloning and characterization of the *eae* gene from a dog. FEMS Microbiol. Letts. 148:239–245.

An, H., J. M. Fairbrother, C. Desautels, and J. Harel. 1999. Distribution of a novel locus called Paa (porcine attaching an effacing associated) among enteric *Escherichia coli*. Adv. Exp. Med. Biol. 473:179–184.

Audia, J. P., C. C. Webb, and J.W. Foster. 2001. Breaking through the acid barrier: an orchestrated

response to proton stress by enteric bacteria. Int. J. Med. Microbiol. 291:97–106.

Barua, S., T. Yamashino, T. Hasegawa, K. Yokoyama, K. Torii, and M. Ohta. 2002. Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7. Mol. Microbiol. 43:629–640.

Batisson, I., M-P. Guimond, F. Girard, H. An, C. Zhu, E. Oswald, J.M. Fairbrother, M. Jacques and J. Harel. 2003. Characterization of the novel factor Paa involved in the early steps of the attaching and effacing *Escherichia coli* mechanism. Infect. Immun. 71:4516–4525.

Beaudry, M., C. Zhu, J. M. Fairbrother and J. Harel. 1996. Genotypic and phenotypic characterization of *Escherichia coli* isolates from dogs manifesting attaching and effacing lesions. J. Clin. Microbiol. 34:144–148.

Beausoleil, H. E., F. Lepine, and J. D. Dubreuil. 2002. LC-MS analysis of pig intestine sulfatides: interaction with *Escherichia coli* STb enterotoxin and characterization of molecular species present. FEMS Microbiol. Lett. 209:183–188.

Beausoleil, H. E., V. Labrie, and J. D. Dubreuil. 1999. Is *Escherichia coli* STb enterotoxin sufficient to cause pig diarrhea? Vet. Microbiol.70:281–285.

Benz, I., and M. A. Schmidt. 1992. Isolation and serologic characterization of AIDA-I, the adhesin mediating the diffuse adherence phenotype of the diarrhea-associated *Escherichia coli* strain 2787 (O126:H27). Infect. Immun. 60:13–18.

Bertschinger, H. U. 1999. Postweaning *Escherichia coli* diarrhea and edema disease. In *Diseases of swine*. B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (eds.). Ames: Iowa States University Press, pp. 441–454.

Bertschinger, H. U. and C. L. Gyles. 1994. Oedema disease of pigs. In *Escherichia coli in domestic animals and humans*. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 193–219.

Beutin, L. 1999. *Escherichia coli* as a pathogen in dogs and cats. Vet Res. 30:285–298.

Bilkei, G., A. Bolcskei, T. Goos, C. Hofmann, and O. Szenci. 1994. The prevalence of *Escherichia coli* in urogenital tract infections of sows. Tierarztliche Umschau 49:471–472, 475–476.

Blanco, J. E., M. Blanco, J. Blanco, A. Mora, L. Balaguer, L. Cuervo, C. Balsalobre, and F. Munoa. 1997. Prevalence and characteristics of enteropathogenic *Escherichia coli* with the *eae* gene in diarrhoeic rabbits. Microbiol. Immunol. 41:77–82.

Bradley, A. J., and M. J. Green. 2001. Adaptation of *Escherichia coli* to the bovine mammary gland. J. Clin. Microbiol. 39:1845–1849.

Burns, S. M., and S. I. Hull. 1999. Loss of resistance to ingestion and phagocytic killing by O- and K- mutants of a uropathogenic *Escherichia coli* O75:K5 strain. Infect. Immun. 67:3757–3762.

Carpenter, J. L., N. C. Andelman, F. M. Moore, and N. W. King. 1988. Idiopathic cutaneous and renal glomerular vasculopathy of greyhounds. Vet. Pathol. 25:401–407.

Casey, T. A., C. J. Herring, R. A. Schneider, B. T. Bosworth, and S. C. Whipp. 1998. Expression of heat-stable enterotoxin STb by adherent *Escherichia coli* is not sufficient to cause severe diarrhea in neonatal pigs. Infect. Immun. 66:1270–1272.

Cheville, **N.** F.**,** and N. L. Arp. 1978. Comparative pathologic findings of *Escherichia coli* infection in birds. J. Am. Vet. Med. Ass. 173:584–587.

Choi, C., W. Cho, H. Chung, T. Jung, J. Kim, and C. Chae. 2001. *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene in isolates in weaned pigs with diarrhea and/or edema disease. Vet. Microbiol. 81:65–71.

Cohen, M. B., A. Guarino, R. Shukla, and R. A. Giannella. 1988. Age-related differences in receptors for *Escherichia coli* heat-stable enterotoxin in the small and large intestine of children. Gastroenterology 94:367–373.

Cowan, L. A., D. M. Hertzke, B. W. Fenwick, and C. B. Anderson. 1997. Clinical and clinicopathologic abnormalities in greyhounds with cutaneous and renal glomerular vasculopathy: 18 cases. J. Am. Vet. Med. Assoc. 210:789–793.

Cox, E., Y. Van der Stede, F. Verdonck, V. Snoeck, W. B. Van den Broeck, and B. M. Goddeeris. 2002. Oral immunisation of pigs with fimbrial antigens of enterotoxigenic *E. coli*: an interesting model to study mucosal immune mechanisms. Vet. Immunol. Immunopathol. 87:287–290.

Dean-Nystrom, E. A., and J. E. Samuel. 1994. Age-related resistance to 987P fimbria-mediated colonization correlates with specific glycolipid receptors in intestinal mucus in swine. Infect. Immun. 62:4789–4794.

Dean-Nystrom, E. A., B. T. Bosworth, W. C. Cray, and H. W. Moon. 1997. Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. Infect. Immun. 65:1842–1848.

Deprez, P., C. Van den Hende, E. Muylle, and W. Oyaert. 1986. The influence of the administration of sow's milk on the postweaning excretion of hemolytic *E. coli* in the pig. Vet. Res. Commun.10:469–478.

De Rycke, J., A. Milon, and E. Oswald. 1999. Necrotoxic *Escherichia coli* (NTEC): two emerging

categories of human and animal pathogens. Vet. Res. 30:221–233.

de Sauvage, F. J., T. R. Camerato, and D. V. Goeddel. 1991. Primary structure and functional expression of the human receptor for *Escherichia coli* heat-stable enterotoxin. J. Biol. Chem. 266:17912–17918.

Devenish, J., C. Gyles, and J. LaMarre. 1998. Binding of *Escherichia coli* verotoxins to cell surface protein on wild-type and globotriaosylceramide-deficient Vero cells. Can. J. Microbiol. 44:28–34.

Dezfulian, H., I. Batisson, J. M. Fairbrother, P. C. K. Lau, A. Nassar, G. Szatmari, and J. Harel. 2003. Virulence genotypes and characterization of septicemic *Escherichia coli* strains from calves and pigs bearing pathogenicity island-like domains. J. Clin. Microbiol. 41:1375–1385.

Dhaliwal, G. K., C. Wray, and D. E. Noakes. 1998. Uterine bacterial flora and uterine lesions in bitches with cystic endometrial hyperplasia (pyometra). Vet. Rec.143:659–661.

Dho, M., and J. Lafont. 1984. Adhesive properties and iron uptake ability in *Escherichia coli* lethal and nonlethal for chicks. Av. Dis. 28: 1016–1025.

Dho-Moulin, M., and J. M. Fairbrother. 1999. Avian pathogenic E. *coli* (APEC). Vet. Res. 30:299–316.

Donnenberg, M. S., J. B. Kaper, and B. B. Finlay. 1997. Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. Trends Microbiol. 5:109–114.

Dorn, C. R., D. H. Francis, E. J. Angrick, J. A. Willgohs, R. A. Wilson, J. E. Collins, B. H. Jenke, and S. J. Shawd. 1993. Characteristics of Vero cytotoxin producing *Escherichia coli* associated with intestinal colonization and diarrhea in calves. Vet. Microbiol. 36:149–59.

Dosogne, H., F. Vangroenweghe, and C. Burvenich. 2002. Potential mechanism of action of J5 vaccine in protection against severe bovine coliform mastitis. Vet. Res. 33:1–12.

Doughty, S., J. Sloan, V. Bennett-Wood, M. Robertson, R. M. Robins-Browne, and E. L. Hartland. 2002. Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of enterocyte effacement-negative strains of enterohemorrhagic *Escherichia coli*. Infect. Immun. 70:6761–6769.

Dozois, C. M., N. Chanteloup, M. Dho-Moulin, and A. Brée, C. Desautels, and J. M. Fairbrother. 1994. Bacterial colonization and in vivo expression of F1 (type 1) fimbrial antigens in chickens experimentally infected with pathogenic *Escherichia coli.* Avian Dis. 38:231–239.

Dozois, C. M., S. Clément, C. Desautels, E. Oswald, and J. M. Fairbrother. 1997. Expression of P, S, and F1C adhesins by cytotoxic necrotizing factor 1 (CNF1)-producing *Escherichia coli* from septicaemic and diarrheic pigs. FEMS Microbiol. Letts. 152:307–312.

Dozois, C. M., M. Dho-Moulin, A. Brée, J. M. Fairbrother, C. Desautels, and R. Curtiss, III. 2000. Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the *tsh* genetic region. Infect. Immun. 68:4145–4154.

Dreau, D., J. P. Lalles, V. Philouze-Rome, R. Toullec, and H. Salmon. 1994. Local and systemic immune responses to soybean protein ingestion in early-weaned pigs. J. Anim. Sci. 72:2090–2098.

Dreyfus, L. A., B. Harville, D. E. Howard, R. Shaban, D. M. Beatty, and S. J. Morris. 1993. Calcium influx mediated by the *Escherichia coli* heat-stable enterotoxin B (STB). Proc. Natl. Acad. Sci. U S A 90:3202–3206.

Dreyfus, L. A., L. Jaso-Friedmann, and D. C. Robertson. 1984. Characterization of the mechanism of action of *Escherichia coli* heat-stable enterotoxin. Infect. Immun. 44:493–501.

Drolet, R., J. M. Fairbrother, J. Harel, and P. Helie. 1994. Attaching and effacing and enterotoxigenic *Escherichia coli* associated with enteric colibacillosis in the dog. Can. J. Vet. Res. 58:87–92.

Dubreuil, J. D. 1997. *Escherichia coli* STb enterotoxin. Microbiology 143 (Pt.6):1783–1795.

Elfadil, A. A., J. P. Vaillancourt, and A. H. Meek. 1996. Farm management risk factors associated with cellulitis in broiler chickens in southern Ontario. Avian Dis. 40:699–706.

Fairbrother, J. M. 1999. Neonatal *Escherichia coli* diarrhea. In Diseases of swine. B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (eds.). Ames: Iowa States University Press, pp. 433–441.

Fairbrother J. M., and M. Ngeleka. 1994. Extraintestinal *Escherichia coli* Infections in Pigs. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 221–236.

Fairbrother, J. M., A. Broes, M. Jacques, and S. Lariviere. 1989. Pathogenicity of *Escherichia coli* O115:K"V165" strains isolated from pigs with diarrhea. Am. J. Vet. Res. 50:1029–1036.

Fairbrother, J. M., I. Batisson, F. Girard, M. Mellata, and S. Pérès. 2002. Original text on *E. coli*. Animal Health and Production Compendium, CD-ROM CAB International.

Fecteau, G., J. M. Fairbrother, R. Higgins, D. C. Van Metre, J. Paré, B. P. Smith, C. A. Holmberg, and S. Jang. 2001. Virulence factors in *Escherichia coli* isolated from the blood of bacteremic neonatal calves. Vet. Microbiol. 78:241–249.

Fenwick, B. W., and L. A. Cowan. 1998. Canine model of hemolytic-uremic syndrome. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. J. B. Kaper and A. D. O'Brien (eds.). Washington DC: ASM Press, pp. 268–277.

Feria, C., J. Machado, J. D. Correia, J. Goncalves, and W. Gaastra. 2001. Virulence genes and P fimbriae PapA subunit diversity in canine and feline uropathogenic *Escherichia coli*. Vet Microbiol. 82:81–89.

Fiederling, F., M. Boury, C. Petit, and A. Milon. 1997. Adhesive factor/rabbit 2, a new fimbrial adhesin and a virulence factor from *Escherichia coli* O103, a serogroup enteropathogenic for rabbits. Infect. Immun. 65:847–851.

Forte, L. R., P. K. Thorne, S. L. EberL, W. J. Krause, R. H. Freeman, S. H. Francis, and J. D. Corbin. 1992. Stimulation of intestinal Cl-transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. Am. J. Physiol. 263(3 Pt 1):C607–615.

Francis, D. H., P. A. Grange, D. H. Zeman, D. R. Baker, R. Sun, and A. K. Erickson. 1998. Expression of mucin-type glycoprotein K88 receptors strongly correlates with piglet susceptibility to K88 (+) enterotoxigenic *Escherichia coli*, but adhesion of this bacterium to brush borders does not. Infect. Immun. 66:4050–4055.

Frankel, G., O. Lider, R. Hershkoviz, A. P. Mould, S. G. Kachalsky, D. C. Candy, L. Cahalon, M. J. Humphries, and G. Dougan. 1996. The cell-binding domain of intimin from enteropathogenic *Escherichia coli* binds to beta1 integrins. J. Biol. Chem. 271:20359–20364.

Fransson, B., A. S. Lagerstedt, E. Hellmen, and P. Jonsson. 1997. Bacteriological findings, blood chemistry profile and plasma endotoxin levels in bitches with pyometra or other uterine diseases. Zentralbl. Veterinarmed. A. 44:417–426.

Fraser, S. A., L. de Haan, A. R. Hearn, H. K. Bone, R. J. Salmond, A. J. Rivett, N. A. Williams, and T. R. Hirst. 2003. Mutant *Escherichia coli* heat-labile toxin B subunit that separates toxoid-mediated signaling and immunomodulatory action from trafficking and delivery functions. Infect. Immun.71:1527–1537.

Gannon, V. P., C. L. Gyles, and R. W. Friendship. 1988. Characteristics of verotoxigenic *Escherichia coli* from pigs. Can. J. Vet. Res. 52:331–337.

Gay, C. C., and T. E. Besser. 1994. *Escherichia coli* septicaemia in calves. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, CAB International, pp. 75–90.

Gill, D. M., J. D. Clements, D. C. Robertson, and R. A. Finkelstein. 1981. Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin. Infect. Immun. 33:677–682.

Girardeau, J. P., L. Lalioui, A. Mohamed Ou Said, C. De Champs, and C. Le Bouguénec. 2003. Extended virulence genotype of pathogenic *Escherichia coli* isolates carrying the *afa*-8 Operon: evidence of similarities between isolates from humans and animals with extraintestinal infections. J. Clin. Microbiol. 41:218–226.

Girardeau, J., M. Vartanian, J. der Ollier, and M. Contrepois. 1988. CS31A, a new K88-related fimbrial antigen on bovine enterotoxigenic and septicemic *Escherichia coli* strains. Infect. Immun. 56:2180–2188.

Giron, J. A., A. S. Ho, and G. K. Schoolnik. 1993. Characterization of fimbriae produced by enteropathogenic *Escherichia col*i. J. Bacteriol. 175:7391–7403.

Glunder, G. 2002. Influence of diet on the occurrence of some bacteria in the intestinal flora of wild and pet birds. Dtsch Tierarztl Wochenschr. 109:266–270.

Goffaux, F., B. China, L. Janssen, and J. Mainil. 2000. Genotypic characterization of enteropathogenic *Escherichia coli* (EPEC) isolated in Belgium from dogs and cats. Res. Microbiol. 151:865–71.

Goffaux, F., B. China, L. Janssen, V. Pirson, and J. Mainil. 1999. The locus for enterocyte effacement (LEE) of enteropathogenic *Escherichia coli* (EPEC) from dogs and cats. Adv. Exp. Med. Biol. 473:129–136.

Gomis, S. M., A. I. Gomis, N. U. Horadagoda, T. G. Wijewardene, B. J. Allan, and A. A. Potter. 2000. Studies on cellulitis and other disease syndromes caused by *Escherichia coli* in broilers in Sri Lanka. Trop. Anim. Health Prod. 32:341–351.

Griffiths, E. 1994. Iron acquisition systems. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 533–566.

Gyles, C. L. 1994. *Escherichia coli* enterotoxins. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 337–364.

Hadad, J. J., and C. L. Gyles. 1982. The role of K antigens of enteropathogenic *Escherichia coli* in colonization of the small intestine of calves. Can. J. Comp. Med. 46:21–26.

Hagman, R., and I. Kuhn. 2002. *Escherichia coli* strains isolated from the uterus and urinary bladder of bitches suffering from pyometra: comparison by restriction enzyme digestion and pulsed-field gel electrophoresis. Vet. Microbiol. 84:143–153.

Hammermueller J., S. Kruth, J. Prescott, and C. Gyles. 1995. Detection of toxin genes in

Escherichia coli isolated from normal dogs and dogs with diarrhea. Can. J. Vet. Res. 59:265–270.

Harel, J., M. Jacques, J. Fairbrother, M. Bosse, and C. Forget. 1995. Cloning of determinants encoding F1652 fimbriae from porcine septicaemic *Escherichia coli* confirms their identity as F1C fimbriae. Microbiol. 141:221–228.

Harville, B. A., and L. A. Dreyfus. 1995. Involvement of 5-hydroxytryptamine and prostaglandin E2 in the intestinal secretory action of *Escherichia coli* heat-stable enterotoxin B. Infect. Immun. 63:745–750.

Helie, P., M. Morin, M. Jacques, and J. M. Fairbrother. 1991. Experimental infection of newborn pigs with an attaching and effacing *Escherichia coli* O45:K"E65" strain. Infect Immun. 59:814–821.

Hertzke, D. M., L. A. Cowan, P. Schoning, and B. W. Fenwick. 1995. Glomerular ultrastructural lesions of idiopathic cutaneous and renal glomerular vasculopathy of greyhounds. Vet. Pathol. 32:451–459.

Heyderman, R. S., M. Soriani, and T. R. Hirst. 2001. Is immune cell activation the missing link in the pathogenesis of post-diarrhoeal HUS? Trends Microbiol. 9:262–266.

Hill, A. W. 1994. *Escherichia coli* mastitis. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 117–133.

Hitotsubashi, S., Y. Fujii, and K. Okamoto. 1994. Binding protein for *Escherichia coli* heat-stable enterotoxin II in mouse intestinal membrane. FEMS Microbiol. Lett. 122:297–302.

Hitotsubashi, S., Y. Fujii, H. Yamanaka, and K. Okamoto. 1992. Some properties of purified *Escherichia coli* heat-stable enterotoxin II. Infect. Immun. 60:4468–4474.

Hoey, D. E., C. Currie, R. W. Else, A. Nutikka, C. A. Lingwood, D. L. Gally, and D. G. Smith. 2002. Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium. J. Med. Microbiol. 51:143–149.

Holloway, S., D. Senior, L. Roith, and C. C. Tisher. 1993. Hemolyic uremic syndrome in dogs. J. Vet. Int. Med. 7:220–227.

Horwitz, M. A., and S. C. Silverstein. 1980. Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. J. Clin. Investig. 65:82–94.

Imberechts, H., H. U. Bertschinger, B. Nagy, P. Deprez, and P. Pohl. 1997b. Fimbrial colonisation factors F18ab and F18ac of *Escherichia coli* isolated from pigs with postweaning diarrhea and edema disease. Adv. Exp. Med. Biol. 412:175–183.

Imberechts, H., P. Deprez, E. Van Driessche, and P. Pohl. 1997a. Chicken egg yolk antibodies against F18ab fimbriae of *Escherichia coli* inhibit shedding of F18 positive *E. coli* by experimentally infected pigs. Vet. Microbiol. 54: 328–341.

Isaacson, R. E. 1994. Vaccines against *Escherichia coli* diseases. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 629–647.

Janke, B. H., D. H. Francis, J. E. Collins, M. C. Libal, D. H. Zeman, and D. D. Johnson. 1989. Attaching and effacing *Escherichia coli* infections in calves, pigs, lambs, and dogs. J. Vet. Diagn. Invest. 1989 1:6–11.

Jarvinen, A. K. 1981. Urogenital tract infection in the bitch. Vet. Res. Commun. 4:253–269.

Johansen M., L. O. Andresen, S. E. Jorsal, L. K. Thomsen, T. E. Waddell, and C. L. Gyles. 1997. Prevention of edema disease in pigs by vaccination with verotoxin 2e toxoid. Can. J. Vet. Res. 61:280–285.

Johnson, J. R., A. L. Stell, P. Delavari, A. C. Murray, M. Kuskowski, and W. Gaastra. 2001. Phylogenetic and pathotypic similarities between *Escherichia coli* isolates from urinary tract infections in dogs and extraintestinal infections in humans. J. Infect. Dis. 183:897–906.

Johnson, J. R., N. Kaster, M. A. Kuskowski, and G. V. Ling. 2003. Identification of urovirulence traits in *Escherichia coli* by comparison of urinary and rectal *E. coli* isolates from dogs with urinary tract infection. J. Clin. Microbiol. 41:337–345.

Kaper, J. B., S. Elliott, V. Sperandio, N. T. Perna, G. F. Mayhew, and F. R. Blattner. 1998. Attaching-and-effacing intestinal histopathology and the locus of enterocyte effacement. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. J. B. Kaper and A. D. O'Brien (eds.). Washington, DC: American Society for Microbiology, pp. 163–182.

Keller, R., J. G. Ordonez, R. R. de Oliveira, L. R. Trabulsi, T. J. Baldwin, and S. Knutton. 2002. Afa, a diffuse adherence fibrillar adhesin associated with enteropathogenic *Escherichia coli*. Infect. Immun. 70:2681–2689.

Kenny, B. 1999. Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. Mol. Microbiol. 31:1229–1241.

Kimmitt, P. T., C. R. Harwood, and M. R. Barer. 2000. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. Emerg. Infect. Dis. 6:458–465.

Korth, M., R. Schneider, and S. Moseley. 1991. An F41-K88-related genetic determinant of bovine septicemic *Escherichia coli* mediates expression of CS31A fimbriae and adherence to epithelial cells. Infect. Immun. 59:2333–2340.

Krejany, E. O., T. H. Grant, V. Bennett-Wood, L. M. Adams, and R. M. Robins-Browne. 2000. Contribution of plasmid-encoded fimbriae and intimin to capacity of rabbit-specific enteropathogenic *Escherichia coli* to attach to and colonize rabbit intestine. Infect Immun. 68:6472–6477.

La Ragione, R. M., W. A. Cooley, and M. J. Woodward. 2000. The role of fimbriae and flagella in the adherence of avian strains of *Escherichia coli* O78:K80 to tissue culture cells and tracheal and gut explants. J. Med. Microbiol. 49:327–338.

Leclerc, B, J. M. Fairbrother, M. Boulianne, and S. Messier. 2003. Evaluation of the adhesive capacity of *Escherichia coli* isolates associated with avian cellulitis. Avian Dis. 47:21–31.

Lee, E., R. Platt, S. Kang, J. A. Roth, and G. J. Phillips. 2001. Chromosomal integration and expression of the *Escherichia coli* K88 gene cluster in *Salmonella enterica* ser. Choleraesuis strain 54 (SC54). Vet Microbiol. 83:177–183.

Levine, M. M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 155:377–389.

Lin, J., J. S. Hogan, and K. L. Smith. 1999. Growth responses of coliform bacteria to purified immunoglobulin G from cows immunized with ferric enterobactin receptor FepA. J. Dairy Sci. 82:86–92.

Lingwood, C. A., M. Mylvaganam, S. Arab, A. A. Khine, G. Magnusson, S. Grinstein, and P.-G. Nyholm. 1998. Shiga toxin (Verotoxin) binding to its receptor glycolipid. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. J. B. Kaper and A. D. O'Brien (eds.). Washington, DC: American Society for Microbiology, pp. 129–139.

Lintermans, P., P. Pohl, A. Bertels, G. Charlier, J. Vandekerckhove, J. Damme, J. van Schoup, C. Schlicker, T. Korhonen, H. de Greve, and M. Montagu. 1988. Characterization and purification of the F17 adhesin on the surface of bovine enteropathogenic and septicemic *Escherichia coli*. Am. J. Vet. Res. 49:1794–1799.

Liu, H., L. Magoun, and J. M. Leong. 1999. β1-chain integrins are not essential for intimin-mediated host cell attachment and enteropathogenic *Escherichia coli*-induced actin condensation. Infect. Immun. 67:2045–2049.

MacLeod D. L., C. L. Gyles, and B. P. Wilcock. 1991. Reproduction of edema disease of swine with purified Shiga-like toxin-II variant. Vet. Pathol. 28:66–73.

Mainil, J. G., E. Jacquemin, P. Pohl, A. Kaeckenbeeck, and I. Benz. 2002. DNA sequences coding for the F18 fimbriae and AIDA adhesin are localised on the same plasmid in *Escherichia coli* isolates from piglets. Vet. Microbiol. 86:303–311.

Mainil, J. G., J. Gerardin, and E. Jacquemin. 2000. Identification of the F17 fimbrial subunit- and adhesin-encoding (f17A and f17G) gene variants in necrotoxigenic *Escherichia coli* from cattle, pigs and humans. Vet. Microbiol. 73:327–335.

Maiti, S. N., L. DesGroseillers, J. M. Fairbrother, and J. Harel. 1994. Analysis of genes coding for the major and minor fimbrial subunits of the Prs-like fimbriae F165(1) of porcine septicemic *Escherichia coli* strain 4787. Microb. Pathog. 16:15–25.

McNally, A., A. J. Roe, S. Simpson, F. M. Thomson-Carter, D. E. Hoey, C. Currie, T. Chakraborty, D. G. Smith, and D. L. Gally. 2001. Differences in levels of secreted locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157. Infect. Immun. 69:5107–5114.

McNamara, B. P., A. Koutsouris, C. B. O'Connell, J. P. Nougayrede, M. S. Donnenberg, and G. Hecht. 2001. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. J Clin Invest. 107:621–629.

Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss, III, B. Lehoux, and J. M. Fairbrother. 2003b. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. Infect Immun. 71:494–503.

Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss, III, P. K. Brown, P. Arne, A. Bree, C. Desautels, and J. M. Fairbrother. 2003a. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. Infect Immun. 71:536–40.

Mellies, J. L., F. Navarro-Garcia, I. Okeke, J. Frederickson, J. P. Nataro, and J. B. Kaper. 2001. *espC* pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. Infect. Immun. 69:315–24.

Ménard, L.-P. and J. D. Dubreuil. 2002. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1): a new toxin with an old twist. Crit. Rev. Microbiol. 28:43–60.

Messier, S., S. Quessy, Y. Robinson, L. A. Devriese, J. Hommez, and J. M. Fairbrother. 1993. Focal dermatitis and cellulitis in broiler chickens: bacteriological and pathological findings. Avian Dis. 37:839–44.

Milon, A., E. Oswald, and J. De Rycke. 1999. Rabbit EPEC: a model for the study of enteropathogenic *Escherichia coli*. Vet. Res. 30:203–219.

Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella. 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. 41:1340–1351.

Moxley, R. A., and D. H. Francis. 1986. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect. Immun. 53:339–346.

Nagy, B., and P. Z. Fekete. 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Vet Res. 30:259–284.

Nakamine, M., Y. Kono, S. Abe, C. Hoshino, J. Shirai, and T. Ezaki. 1998. Dual infection with enterotoxigenic *Escherichia coli* and porcine reproductive and respiratory syndrome virus observed in weaning pigs that died suddenly. J. Vet. Med. Sci. 60:555–561.

Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11:142–201.

Naylor, S. W., J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. Smith, and D. L. Gally. 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. Infect. Immun. 71:1505–1512.

Ngeleka, M., and J. M. Fairbrother. 1999. $F165_1$ fimbriae of the P fimbrial family inhibit the oxidative response of porcine neutrophils. FEMS Immunology and Medical Microbiology. 25:265–274.

Ngeleka, M., B. Martineau-Doizé, and J. M. Fairbrother. 1994. Septicemia-inducing *Escherichia coli* O115:K"V165"$F1651_1$, resists killing by porcine polymorphonuclear leukocytes in vitro: role of $F1651_1$ fimbriae and K"V165" O-antigen capsule. Infect. Immun. 62:398–404.

Ngeleka, M., J. Harel, M. Jacques, and J. M. Fairbrother. 1992. Characterization of a polysaccharide capsular antigen of septicemic *Escherichia coli* O115:K"V165": F165 and evaluation of its role in pathogenicity. Infect. Immun. 60:5048–5056.

Ngeleka, M., J. Pritchard, G. Appleyard, D. M. Middleton, and J. M. Fairbrother. 2003. Isolation and association of *E. coli* AIDA-I/STb, rather than EAST1 pathotype with diarrhea in piglets and antibiotic sensitivity of isolates. J. Vet. Diagn. Invest. 15:242–52.

Ngeleka, M., L. Brereton, G. Brown, and J. M. Fairbrother. 2002. Pathotypes of avian *Escherichia coli* as related to *tsh*-, *pap*-, *pil*-, and *iuc*-DNA sequences, and antibiotic sensitivity of isolates from internal tissues and the cloacae of broilers. Avian Dis. 46:143–152.

Ngeleka, M., M. Jacques, B. Martineau-Doizé, F. Daigle, J. Harel and J. M. Fairbrother. 1993. Pathogenicity of an *Escherichia coli* O115:K"V165" mutant negative for $F1651_1$ fimbriae in septicemia of gnotobiotic pigs. Infect. Immun. 61:836–843.

Nicholls, L., T. H. Grant, and R. M. Robins-Browne. 2000. Identification of a novel genetic locus that is required for *in vitro* adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. Mol. Microbiol. 35:275–288.

Nolan, L. K., S. M. Horne, C. W. Giddings, S. L. Foley, T. J. Johnson, A. M. Lynne, and J. Skyberg. 2003. Resistance to serum complement, *iss*, and virulence of avian *Escherichia coli*. Vet. Res. Commun. 27:101–110.

Oelschlaeger, T. A., U. Dobrindt, and J. Hacker. 2002. Pathogenicity islands of uropathogenic *E. coli* and the evolution of virulence. Int. J. Antimicrob. Agents 19:517–521.

O'Loughlin, E. V., and R. M. Robins-Browne. 2001. Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. Microbes Infect. 3:493–507.

Olsen, A., A. Arnqvist, M. Hammar, and S. Normark. 1993. Environmental regulation of curli production in *Escherichia coli*. Infect. Agents Dis. 2:272–274.

Ørskov, F., and I. Ørskov. 1992. *Escherichia coli* serotyping and disease in man and animals. Can. J. Microbiol. 38:699–704.

Oswald, E., H. Schmidt, S. Morabito, H. Karch, O. Marches, and A. Caprioli. 2000. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infect Immun. 68:64–71.

Paton J. C., and A. W. Paton. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11:450–479.

Peeters, J. E. 1994. *Escherichia coli* infections in rabbits, cats, dogs, goats and horses. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 261–283.

Philpott, D. J., C. A. Ackerley, A. J. Kiliaan, M. A. Karmali, M. H. Perdue, and P. M. Sherman. 1997. Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium. Am. J. Physiol. 273:G1349–1358.

Pillien, F., C. Chalareng, M. Boury, C. Tasca, J. de Rycke, and A. Milon. 1996. Role of adhesive factor/rabbit 2 in experimental enteropathogenic *Escherichia coli* O103 diarrhea of weaned rabbit. Vet. Microbiol. 1996. 50:105–115.

Pohl, P., E. Oswald, K. van Muylem, E. Jacquemin, P. Lintermans, and J. Mainil. 1993a. *Escherichia coli* producing CNF1 and CNF2 cytotoxins in animals with different disorders. Vet. Res. 24:311–315.

Pohl, P. H., J. E. Peeters, E. R. Jacquemin, P. F. Lintermans, and J. G. Mainil. 1993b. Identification of *eae* sequences in enteropathogenic *Escherichia coli* strains from rabbits. Infect. Immun. 1993. 61:2203–2206.

Pourbakhsh, S., M. Boulianne, B. Martineau Doize, and J. Fairbrother. 1997a. Virulence mechanisms of avian fimbriated *Escherichia coli* in experimentally inoculated chickens. Vet. Microbiol. 58:195–213.

Pourbakhsh, S. A., M. Dho-Moulin, A. Brée, C. Desautels, B. Martineau-Doize, and J. M. Fairbrother. 1997b. Localization of the *in vivo* expression of P and F1 fimbriae in chickens experimentally inoculated with pathogenic *Escherichia coli*. Microb. Pathog. 22:331–341.

Pruimboom-Brees, I. M., T. W. Morgan, M. R. Ackermann, E. D. Nystrom, J. E. Samuel, N. A. Cornick, and H. W. Moon. 2000. Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. Proc. Natl. Acad. Sci. U S A 97:10325–10329.

Qureshi, N., K. Takayama, and R. Kurtz. 1991. Diphosphoryl lipid A obtained from the nontoxic lipopolysaccharide of *Rhodopseudomonas sphaeroides* is an endotoxin antagonist in mice. Infect. Immun. 59:441–444.

Robins-Browne, R. M., A. M. Tokhi, L. M. Adams, and V. Bennett-Wood. 1994. Host specificity of enteropathogenic *Escherichia coli* from rabbits: lack of correlation between adherence in vitro and pathogenicity for laboratory animals. Infect. Immun. 62:3329–3336.

Rodighiero, C., A. T. Aman, M. J. Kenny, J. Moss, W. I. Lencer, and T. R. Hirst. 1999. Structural basis for the differential toxicity of cholera toxin and *Escherichia coli* heat-labile enterotoxin. Construction of hybrid toxins identifies the A2-domain as the determinant of differential toxicity. J. Biol. Chem. 274:3962–3969.

Salajka, E., J. Cernohous, and Z. Sarmanova. 1975. Association of the colonization of the intestine by pathogenic strains of haemolytic *E. coli* in weaned piglets with withdrawal of antibody contained in the dams' milk. Documenta Veterinaria, Brno 8:43–55.

Sandhu, K. S., and C. L. Gyles. 2002. Pathogenic Shiga toxin-producing *Escherichia coli* in the intestine of calves. Can . J. Vet. Res. 66:65–72.

Sandvig, K., and B. van Deurs. 2002. Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. FEBS Lett. 529:49–53.

Sarrazin, E., and H. U. Bertschinger. 1997. Role of fimbriae F18 for actively acquired immunity against porcine enterotoxigenic *Escherichia coli*. Vet. Microbiol. 54:133–144.

Savarino, S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. Proc. Nat. Acad. Sci. U S A 90:3093–3097.

Schilling, J. D., S. M. Martin, C. S. Hung, R. G. Lorenz, and S. J. Hultgren. 2003. Toll-like receptor 4 on stromal and hematopoietic cells mediates innate resistance to uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. U S A. 100:4203–4208.

Schmidt, H. 2001. Shiga-toxin-converting bacteriophages. Res. Microbiol. 152:687–695.

Senior, D. F., P. deMan, and C. Svanborg. 1992. Serotype, hemolysin production, and adherence characteristics of strains of *Escherichia coli* causing urinary tract infection in dogs. Am. J. Vet. Res. 53:494–498.

Sinclair J. F., and A. D. O'Brien. 2002. Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157:H7. J. Biol. Chem. 277:2876–2885.

Smith, H. W., and M. A. Linggood. 1971. Observations on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. J. Med. Microbiol. 4:467–485.

Smith, H. W., and S. Halls. 1968. The production of oedema disease and diarrhoea in weaned pigs by the oral administration of *Escherichia coli*: factors that influence the course of the experimental disease. J. Med. Microbiol.

Smyth, C. J., M. Marrion, and S. G. J. Smith. 1994. Fimbriae of *Escherichia coli*. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon UK: CAB International, pp. 399–436.

Sojka, W. J. 1965. *Escherichia coli* in domestic animals and poultry. Farnham Royal: Commonwealth Agricultural Bureaux, pp. 104–125.

Srimanote, P., A. W. Paton, and J. C. Paton. 2002. Characterization of a novel type IV pilus locus encoded on the large plasmid of locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia*

coli strains that are virulent for humans. Infect. Immun. 70:3094–100.

Starcic, M., J. R. Johnson, A. L. Stell, J. van der Goot, H. G. Hendriks, C. van Vorstenbosch, L. van Dijk, and W. Gaastra. 2002. Haemolytic *Escherichia coli* isolated from dogs with diarrhea have characteristics of both uropathogenic and necrotoxigenic strains. Vet. Microbiol. 85:361–377.

Stawski, G., L. Nielsen, F. Ørskov, and I. Ørskov. 1990. Serum sensitivity of a diversity of *Escherichia coli* antigenic reference strains. Correlation with an LPS variation phenomenon. Acta Pathol. Microbiol. Immunol. Scandinavia 98:828–838.

Suh, J. K., C. J. Hovde, and J. D. Robertus. 1998. Shiga toxin attacks bacterial ribosomes as effectively as eucaryotic ribosomes. Biochemistry 37:9394–9398.

Svensmark, B., K. Nielsen, P. Willeberg, and S. E. Jorsal. 1989. Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. II. Post-weaning diarrhoea. Acta. Vet. Scand. 30:55–62.

Takemura, K., J. S. Hogan, and K. L. Smith. 2003. Effect of immunoglobulin G from cows immunized with ferric citrate receptor (FecA) on iron uptake by *Escherichia coli*. J. Dairy Sci. 86:133–137.

Takemura, K., J. S. Hogan, J. Lin, and K. L. Smith. 2002. Efficacy of immunization with ferric citrate receptor FecA from *Escherichia coli* on induced coliform mastitis. J. Dairy Sci. 85:774–781.

Tarr, P. I., S. S. Bilge, J. C. Vary, S. Jelacic, R. L. Habeeb, T. R. Ward, M. R. Baylor, and T. E. Besser. 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. Infect. Immun. 68:1400–1407.

Tatsuno, I., M. Horie, H. Abe, T. Miki, K. Makino, H. Shinagawa, H. Taguchi, S. Kamiya, T. Hayashi, and C. Sasakawa. 2001. *toxB* gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. Infect. Immun. 69:6660–6669.

Tauschek, M., R. A. Strugnell, and R. M. Robins-Browne. 2002. Characterization and evidence of mobilization of the LEE pathogenicity island of rabbit-specific strains of enteropathogenic *Escherichia coli*. Mol. Microbiol. 44:1533–1550.

Tewari, R., T. Ikeda, R. Malaviya, J. I. MacGregor, J. R. Little, S. J. Hultgren, and S. N. Abraham. 1994. The PapG tip adhesin of P fimbriae protects *Escherichia coli* from neutrophil bactericidal activity. Infect. Immun. 62:5296–5304.

Tomita, G. M., C. H. Ray, S. C. Nickerson, W. E. Owens, and G. F. Gallo. 2000. A comparison of two commercially available *Escherichia coli* J5 vaccines against *E. coli* intramammary challenge. J. Dairy Sci. 83:2276–2281.

Torres, A. G., J. A. Giron, N. T. Perna, V. Burland, F. R. Blattner, F. Avelino-Flores, and J. B. Kaper. 2002. Identification and characterization of lpfABCC'DE, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. 70:5416–5427.

Toth, T. E., and P. B. Siegel. 1986. Cellular defense of the avian respiratory tract: paucity of free-residing macrophages in the normal chicken. Avian Dis. 30:67–75.

Vaillancourt, J. P., A. Elfadil, and J. R. Bisaillon. 1992. Cellulitis in poultry. Canada Poultryman, pp. 34–37.

van Beers-Schreurs, H. M., L. Vellenga, T. Wensing, and H. J. Breukink. 1992. The pathogenesis of the post-weaning syndrome in weaned piglets: a review. Vet. Q. 14:29–34.

Van Bost, S., S. Roels, and J. Mainil. 2001. Necrotoxigenic *Escherichia coli* type-2 invade and cause diarrhoea during experimental infection in colostrum-restricted newborn calves. Vet. Microbiol. 81:315–329.

Verdonck, F., E. Cox, K. van Gog, Y. Van der Stede, L. Duchateau, P. Deprez, and B. M. Goddeeris. 2002. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain. Vaccine 20:2995–3004.

Von Moll, L. K., and J. R. Cantey. 1997. Peyer's patch adherence of enteropathogenic *Escherichia coli* strains in rabbits. Infect. Immun. 65:3788–3793.

Wada, Y., H. Kondo, Y. Nakaoka, and M. Kubo. 1996. Gastric attaching and effacing *Escherichia coli* lesions in a puppy with naturally occurring enteric colibacillosis and concurrent canine distemper virus infection. Vet. Pathol. 1996. 33:717–720.

Wadas, B., I. Kuhn, A. S. Lagersted, and P. Jonsson. 1996. Biochemical phenotypes of *Escherichia coli* in dogs: comparison of isolates isolated from bitches suffering from pyometra and urinary tract infection with isolates from faeces of healthy dogs. Vet. Microbiol. 52:293–300.

Waddell, T. E., and C. L. Gyles. 1995. Sodium deoxycholate facilitates systemic absorption of verotoxin 2e from pig intestine. Infect. Immun. 63:4953–4956.

Wasteson, Y., O. Olsvik, E. Skancke, C. A. Bopp, and K. Fossum. 1988. Heat-stable-enterotoxin-producing *Escherichia coli* strains isolated from dogs. J. Clin. Microbiol. 26:2564–2566.

Whipp, S. C. 1990. Assay of enterotoxigenic *Escherichia coli* heat-stable toxin b in rats and mice. Infect. Immun. 58:930–934.

Whitfield C., W. J. Keenleyside, and B. R. Clarke. 1994. Structure, function and synthesis of surface polysaccharides in *Escherichia coli*. In *Escherichia coli* in domestic animals and humans. C. L. Gyles (ed.). Oxon, UK: CAB International, pp. 437–494.

Wray, C., I. McLaren, and G. R. Pearson. 1989. Occurrence of "attaching and effacing" lesions in the small intestine of calves experimentally infected with bovine isolates of verocytotoxic *E. coli*. Vet. Rec.125:365–368.

Wullt, B., G. Bergsten, M. Samuelsson, and C. Svanborg. 2002. The role of P fimbriae for *Escherichia coli* establishment and mucosal inflammation in the human urinary tract. Int. J. Antimicrob. Agents 19:522–538.

Yamamoto, T., and M. Nakazawa. 1997. Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. J. Clin. Microbiol. 35:223–227.

Yuri, K., K. Nakata, H. Katae, S. Yamamoto, and A. Hasegawa. 1998. Distribution of uropathogenic virulence factors among *Escherichia coli* strains isolated from dogs and cats. J. Vet. Med. Sci. 60:287–290.

Zhang, W. L., B. Kohler, E. Oswald, L. Beutin, H. Karch, S. Morabito, A. Caprioli, S. Suerbaum, and H. Schmidt. 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. J. Clin. Microbiol. 40:4486–4492.

Zhu, C., J. Harel, M. Jacques, and J. M. Fairbrother. 1995. Interaction with pig ileal explants of *Escherichia coli* O45 isolates from swine with postweaning diarrhea. Can. J. Vet. Res. 59:118–123.

Zhu, C., J. Harel, M. Jacques, C. Desautels, M. S. Donnenberg, M. Beaudry, and J. M. Fairbrother. 1994. Virulence properties and attaching-effacing activity of *Escherichia coli* O45 from swine postweaning diarrhea. Infect. Immun. 62:4153–4159.

Zhu, C., T. S. Agin, S. J. Elliott, L. A. Johnson, T. E. Thate, J. B. Kaper, and E. C. Boedeker. 2001. Complete nucleotide sequence and analysis of the locus of enterocyte effacement from rabbit diarrheagenic *Escherichia coli* RDEC-1. Infect. Immun. 69:2107–2115.

17
Actinobacillus

J. I. MacInnes and J. T. Bossé

Members of the genus *Actinobacillus* are small, gram-negative, pleomorphic, coccobacillary rods that are facultatively anaerobic, indole negative, β-galactosidase and urease positive, and reduce nitrates. Apart from *A. pleuropneumoniae* and some *A. 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 always associated with mucous membranes, and the host range of different species tends to be limited.

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. As of 2002, 18 different species of *Actinobacillus* had been described, all but three associated with mammals or birds. Based on genetic approaches such as DNA-DNA hybridization and 16S rRNA gene sequencing, it has been suggested, however, that some species including *dephinicola*, *indolicus*, *minor*, *muris*, *porcinus*, *scotiae*, *seminis,* and *capsulatus* are not *Actinobacillus sensu stricto* (Christensen et al. 2002a,b). Currently recognized members of the genus Actinobacillus are shown in table 17.1.

Although *Actinobacillus* spp. can be benign commensals of the respiratory, alimentary, and genital tracts, *Actinobacillus equuli, lignieresii*, *pleuropneumoniae*, and *suis* can, under certain conditions, cause important diseases in domestic animals.

ACTINOBACILLUS PLEUROPNEUMONIAE

Epidemiology

There are two biotypes of *A. pleuropneumoniae* differentiated on the basis of their requirement for nicotinamide adenine dinucleotide (NAD) (Taylor 1999a). Biotype 1 strains require NAD, whereas biotype 2 strains can synthesise NAD in the presence of specific pyridine nucleotides or their precursors (Niven and Lávesque 1988). There are 13 serotypes (1 to 12, 15) of biotype 1, and 6 serotypes (2, 4, 7, 9, 13, 14) of biotype 2, based on surface polysaccharide antigens (Schaller et al. 2001; Blackall et al. 2002). Serotypes 1 and 5 have been further differentiated into 1a and 1b, and 5a and 5b, respectively, based on minor differences in the polysaccharide structures. Unfortunately, there have been relatively few recent publications regarding the prevalence of different *A. pleuropneumoniae* serotypes. Based on information mostly dating back from the 1980s, the most prevalent serotypes in Asia and Australia are 1, 2, and 5. In Great Britain and Europe, serotypes 2 and 9 are found most often, whereas in Canada, the United States, and Mexico, serotypes 1 and 5 predominate. Although there are usually 1 to 3 predominant serotypes, in some countries as many as 11 different serotypes have been reported (Dubreuil et al. 2000). Recently "untypeable" strains that do not fall into a single serotype have appeared (Dr B. Fenwick, personal communication). As well, strains that share antigens determinants of more that one serotype have been described (Gottschalk et al. 2000).

Table 17.1. Currently Recognized Taxa in the Genus *Actinobacillus,* Host Predilection and Diseases

Species	Hosts	Associations/Diseases
A. equuli	Horses	Commensal and opportunistic pathogen in foals (sleepy foal disease, joint ill purulent nephritis, and arthritis) and adults (abortion, septicemia, nephritis, endocarditis)
	Swine	Commensal and opportunistic pathogen in piglets (arthritis) and adults (abortion, endocarditis)
A. lignieresii	Cattle	Commensal and opportunistic pathogen (wooden tongue)
	Sheep	Commensal and opportunistic pathogen (suppurative lesions of skin, lungs)
A. pleuropneumoniae	Swine	Pleuropneumonia[1]
A. suis	Swine[2]	Commensal[3] and opportunistic pathogen in piglets (primarily septicemia) and adults (septicemia, pneumonia, arthritis, enteritis)

[1]Some serotypes in some animals may behave as commensals.
[2]May very occasionally cause sporadic disease in other species.
[3]Some strains may behave as a primary pathogen in some animals.

Table 17.2. Apx Toxins Produced by Different Serotypes of *Actinobacillus pleuropneumoniae*

	Operon			Activity		MW (kDa)	Serotype
	Activator	Structural	Export	Haemolytic	Cytotoxic		
ApxI	*apxIC*	*apxIA*	*apxIBD*[1]	Strong	Strong	105-110	1, 5a, 5b, 9, 10, 11
ApxII	*apxIIC*	*apxIIA*	none[1]	Weak	Moderate	103-105	All but 10
ApxIII	*apxIIIC*	*apxIIIA*	*apxIIIBD*	None	Strong	120	2, 3, 4, 6, 8
ApxIV[2]	ORF1[3]	*apxIVA*	none[1]	Weak	ND[4]	200[5]	All

Source: Compiled using data from Frey et al. (1993) and Schaller et al. (2000).
[1]The *apxIBD* genes are found in all serotypes except serotype 3. Secretion of ApxII occurs via ApxIBD. Nothing is yet known regarding secretion of ApxIV.
[2]Unlike the other Apx toxins, ApxIV is only produced *in vivo*.
[3]ORF1 seems to be required for activity of ApxIV, although it shares no homology with ApxIC, ApxIIC, or ApxIIIC.
[4]Not determined.
[5]The molecular mass of ApxIV is predicted from the protein sequence.

All serotypes are capable of causing pleuropneumonia, although some serotypes are more virulent than others. These differences may be attributed, at least in part, to the production of different combinations of the Apx toxins (table 17.2), with the most virulent serotypes producing both ApxI and ApxII (Frey 1995). Other factors, notably the amount of surface polysaccharide present, may also contribute to differences in virulence both between serotypes and between strains within the same serotype Rosendal and MacInnes 1990).

Pleuropneumonia often occurs as an epizootic but is rapidly becoming endemic in many countries. Serodiagnostic tests such as enzyme-linked immunosorbent assays (ELISAs) and the complement fixation test (CFT) (Inzana and Fenwick 2001; Enoe et al. 2002) as well as polymerase-chain-reaction (PCR)-based tests (Schaller et al. 2001; Chiers et al. 2002) can be used to monitor the presence of *A. pleuropneumoniae* in swine herds. This information is important for controlling the spread of the disease through herd management practices and vaccination.

Transmission and Disease

Actinobacillus pleuropneumoniae is considered an obligate parasite of the porcine respiratory tract (Taylor 1999a). The organism can be isolated from nasal cavities, tonsils, middle ear, 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 (Jensen et al. 1999). There are no other known natural hosts, and the organism does not survive for long in the environment (Taylor 1999a). Transmission is by aerosols or direct contact with infected pigs. Pleuropneumonia can occur in pigs of all ages. There is no known association with predisposing viral or bacterial infections, but increased incidence of pleuropneumonia is associated with conditions such as overcrowding, poor ventilation, and large temperature fluctuations (Rosendal and Mitchell 1983). The pace of disease can range from peracute to chronic depending on the serotype, the immune status of the host, and the number of bacteria reaching the lung (Cruijsen et al. 1995b). 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 (Taylor 1999a).

Pulmonary lesions associated with peracute and acute disease are characterized by severe edema, inflammation, hemorrhage, and necrosis. The thoracic cavity is often filled with serosanguinous fluid and fibrin clots, and diffuse fibrinous pleuritis and pericarditis are common (Rosendal et al. 1985). In the early stages of disease, marked neutrophil (PMN) infiltration, edema, and fibrinous exudate are apparent (Liggett et al. 1987). In the later stages, macrophage infiltration is more apparent, and necrotic areas are surrounded with dense bands of degenerating leukocytes. Severe necrotizing vasculitis leads to hemorrhage in the lung (Serebrin et al. 1991). *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 (Ajito et al. 1996). *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 (Min and Chae 1998).

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 (Rosendal et al. 1985; Liggett et al. 1987). Chronically infected animals may also harbor *A. pleuropneumoniae* in tonsillar crypts (Sidibe et al. 1993).

Pathogenesis of *A. pleuropneumoniae* Infection

There are three basic stages in the pathogenesis of 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, factors known to contribute to each of these stages are discussed below.

Colonization

Colonization is often a necessary prerequisite to the production of disease. Although *A. pleuropneumoniae* can be isolated from the tonsils and nasal cavities of pigs (Sidibe et al. 1993; Chiers et al. 1999), the bacteria bind preferentially to cells of the lower respiratory tract (Jacques et al. 1991; Dom et al. 1994), but 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.

Although there have been claims that lipopolysaccharide (LPS) plays a role in adhesion, further experiments have shown that this is not the case (Labrie et al. 2002). Recent evidence indicates that more than one adhesin is involved in the binding of *A. pleuropneumoniae* to lung alveolar epithelial cells *in vitro*, and that protein(s) are the major contributor. According to van Overbeke et al. (2002), expression of both fimbriae and a 55 kDa outer membrane protein (OMP) are associated with high levels of adherence. Treatment with proteolytic enzymes greatly reduces this adherence whereas incubation with sodium metaperiodate (which cleaves C-C bonds between neighboring hydroxyl groups in sugar molecules) reduces adherence to a lesser degree. Growth under NAD-restricted conditions is necessary for optimal adherence and expression of both fimbriae and the 55 kDa OMP in serotypes 5a, 9, and 10. In contrast, a serotype 2 strain showed equal adherence and expression of fimbriae and the 55 kDa OMP under both NAD-restricted and NAD-rich conditions. Although the role of the 55 kDa protein is uncertain, the fimbriae are likely involved in adherence of *A. pleuropneumoniae.* These fimbriae have been characterized as type 4, a type shown to mediate adherence of

various mucosal pathogens to epithelial cells (Zhang et al. 2000)

Following adherence to host cells, 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. In particular, mechanisms for overcoming iron-restriction have been shown to be important for many bacterial pathogens. *A. pleuropneumoniae* is capable of utilizing heme compounds (including free heme, hemin, hematin, and hemoglobin) as well as porcine transferrin, but not lactoferrin, for growth (Deneer and Potter 1989; Niven et al. 1989; d'Silva et al. 1995). In addition, *A. pleuropneumoniae* can utilize exogenously supplied hydroxamate siderophores (produced by other bacteria or fungi), and may also liberate an as yet uncharacterized siderophore (Diarra et al., 1996; Mikael et al. 2002). All serotypes of *A. pleuropneumoniae* are capable of obtaining heme products via production of hemolysins (Frey et al. 1993). In addition, all serotypes appear to secrete a high molecular mass protease complex (>200 kDa), which has weak activity against porcine hemoglobin *in vitro* (Negrete-Abascal et al. 1994; Garcia-Cuellar et al. 2000). Other bacterial pathogens have been shown to degrade hemoglobin proteolytically to release heme that is subsequently bound by heme/hemoglobin receptors and transported into the periplasm (Genco and White Dixon. 2001). It is not known whether the >200 kDa protease complex of *A. pleuropneumoniae* serves this function *in vivo*.

In gram-negative bacteria, the outer membrane (OM) forms a permeability barrier that prevents free passage of the large iron-containing molecules. High-affinity receptors localized in the OM bind the various iron-chelates at the cell surface. Two OMPs, TbpA (Tbp1, TfbB), and TbpB (Tbp2, TfbA), form a complex required by *A. pleuropneumoniae* for high-affinity binding of porcine transferrin (Gerlach et al. 1992; Fuller et al. 1998). An OM receptor for ferric hydroxamate siderophores (FhuA) has also been identified (Mikael et al. 2002) and, very recently, the gene encoding a 104 kDa hemoglobin-binding protein (HgbA) has been cloned (A. Khamessan, unpublished data). The gene product has high homology with hemoglobin binding proteins of other *Pasteurellaceae* and can be demonstrated in the reference strains of serotypes 1 to 12. It is not known whether other heme compounds bind to their own specific OM receptors. There is evidence that hemin may bind to TbpB (Gerlach et al. 1992), although it has not been confirmed that the TbpAB complex is responsible for hemin uptake.

Release of the strongly bound iron-complexes from high-affinity OM receptors and subsequent transport across the OM requires energy (Braun and Killmann 1999). This energy is transduced from the proton motive force (PMF) generated in the cytoplasmic membrane (CM) to the high-affinity receptors via a complex of three proteins, TonB-ExbB-ExbD (Moeck and Coulton 1998; Braun and Killmann 1999). Two integral CM proteins, ExbB and ExbD, anchor TonB, which spans the periplasmic space and interacts with high affinity OM receptors, preferentially when ligand is present (Moeck and Coulton 1998). Through a series of conformational changes, energy from the CM is transduced to the OM receptors and the iron is transported into the periplasm (Moeck and Coulton 1998; Braun and Killman 1999). Whereas most organisms possess just one TonB system, several bacteria have now been shown to possess two or more (Seliger et al. 2001). It has been hypothesized that the two systems have greater affinities for different iron sources (Seliger et al. 2001). Very recently, Beddek et al. (unpublished data) have found that *A. pleuropneumoniae* possesses two distinct TonB systems. The TonB1 system, whose expression is transcriptionally linked to that of the transferrin binding protein genes (Tonpitak et al. 2000), shows specificity solely for transferrin, whereas the TonB2 system, the expression of which is not transcriptionally linked with that of other iron utilization genes, is involved in the uptake of a range of host-bound iron sources (Beddek et al., unpublished data). It is interesting that although both TonB systems contribute to virulence in *A. pleuropneumoniae* (Fuller et al. 2000b; Baltes et al. 2001; Sheehan et al., unpublished data), TonB2 mutants are more highly attenuated than TonB1 mutants (Beddek et al. unpublished data).

Once iron-carrier complexes have been transported across the OM of gram-negative bacteria, passage to the cytoplasm is generally mediated by periplasmic binding-protein-dependent transport systems, the ABC-transport systems or permeases (Genco and White Dixon 2001). In *A. pleuropneumoniae*, two distinct ABC-transport systems have been reported to be involved in uptake of iron across the CM, and it is likely that other iron-complex spe-

cific permeases will be found. An ABC-transport system (FhuBCD) specific for ferric hydroxamate has been described (Mikael et al. 2002), and the genes (*afuABC*) encoding another periplasmic binding-protein-dependent iron transport system have been identified (Chin et al. 1996). Homologues of the proteins encoded by *afuABC* are apparently involved in transport of unchelated Fe^{3+} across the CM (Braun and Killmann 1999), although this remains to be confirmed in *A. pleuropneumoniae*. Where and how iron is released from various carrier proteins is not clear, but permeases specific for both unchelated Fe^{3+} as well as various iron complexes have been described (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. Recently, an ABC-transport operon (*cbiKLMQO*) that appears to be required for high-affinity nickel uptake has 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 (Nieboer 1992). 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 virulence of *A. pleuropneumoniae* is likely via its role in urease activity since a mutation within 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 within this bacterium. Urease activity could play a role in nutrient acquisition mechanism producing ammonia, a preferred nitrogen source for many bacteria.

Nutrient uptake systems such as ABC-transporters are frequently identified by signature-tagged mutagenesis (STM) studies. Recent STM studies of *A. pleuropneumoniae* have revealed several ABC-transport homologues required for survival within pig lungs, indicating the requirement for uptake of polyamines, certain sugars, and trace metals (Fuller et al. 2000a). Other putative transport proteins have also been identified in STM studies, but further study is needed to determine the role of these proteins in the pathogenesis of *A. pleuropneumoniae*.

Avoidance of Host Clearance Mechanisms

Effective host defense against bacterial infections in the lung depends on rapid clearance of bacteria from the respiratory tract. The respiratory immune system can be divided into nonspecific (mucociliary function, complement, phagocytes) and specific lymphocyte-mediated mechanisms. The mucociliary clearance mechanism is very important in protecting lungs from infection. Narita et al. (1995) found that suppression of mucus production and ciliary activity by treatment with atropine and xylocaine, respectively, greatly increased the severity of disease when pigs were inoculated intrabronchially with low doses of *A. pleuropneumoniae*. The effects of *A. pleuropneumoniae* infection per se on mucociliary activity has not yet been investigated, but it is possible that the 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 PMNs phagocytose *A. pleuropneumoniae* in the presence of convalescent pig serum (see Specific Clearance Mechanism below) (Cruijsen et al. 1992; 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 minutes within macrophages, during which time liberation of Apx toxins may result in lysis of these phagocytes (Cruijsen et al. 1992).

A. pleuropneumoniae 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 of free toxic oxygen radicals (Ward et al. 1998; Bilinski et al. 1991, Rioux et al. 2000). The periplasmic location of SodC suggests a role in dismutation of superoxide radicals in phagocytic cells (Langford et al. 1996). Isogenic *sodC* mutants, however, are still capable of causing acute pleuropneumonia in experimental infections (Sheehan et al. 2000). Like urease activity, the contribution of SodC to virulence of *A. pleuropneumoniae* may be seen with less-severe challenge doses. The genes encoding two stress response proteins, DnaK and Trigger Factor, were recently identified by STM as essential for survival of *A. pleuropneumoniae* within the porcine respiratory tract, suggesting that they may be important for survival within macrophages (Fuller et al. 2000). Ammonia produced as the result of urea hydrolysis may also play a role in intracellular survival (Bossé and MacInnes 2000) since ammonia inhibits phagosome-lysosome fusion as well as elevates intralysosomal pH in

macrophages, resulting in depression of acid hydrolase activity (Gordon et al. 1980).

The major factors involved in impairment of phagocytic function of both macrophages and PMNs are three RTX-toxins (ApxI, ApxII, and ApxIII) produced in various combinations by the different serotypes of *A. pleuropneumoniae* (table 17.2; Frey 1993). At sublytic doses, these toxins impair macrophage chemotactic and phagocytic function, whereas macrophage and PMN oxidative metabolism is stimulated (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 below) (Kamp et al. 1991; Rycroft et al. 1991; Frey 1993). However, damage to cultured alveolar macrophages by an ApxII and ApxIII-deficient mutant of *A. pleuropneumoniae* serotype 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 antipolysaccharide 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* serotype 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 serotype 1 capsular mutant created by transposon mutagenesis appeared as resistant as its parental strain to killing by normal pig serum (Rioux et al. 2000). In a different transposon mutagenesis study, expression of LPS O-side chains was shown to be responsible for serum resistance in serotype 1 (Paradis et al. 1999).

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 has not been fully resolved, although a positive correlation was observed between delayed-type hypersensitivity response (DTH, a measure of CMIR) to Apx toxins, with 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 of CD4:CD8 ratio was found to correlate with protection against pleuropneumonia (Katinger et al. 1999; Appleyard et al. 2002). However, passive transfer of serum antibodies was shown to be sufficient for protection against pleuropneumonia, showing that CMIR is not absolutely essential (Bossé et al. 1992).

Antibodies against the Apx toxins, capsule, LPS, and certain outer-membrane proteins 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 have been shown to correlate strongly with (Cruijsen et al. 1995a; Furesz et al. 1997), and be 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 (Madsen et al. 1995; Chiers et al. 1998; van Overbeke et al. 2001) indicates that there may be differences in the level of protection obtained depending on the method of preparation 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* (Bossé et al. 1992; Hensel et al. 1995). However, it appears that IgG, and in particular IgG1-anti-Apx toxin antibodies, are the most important in conferring protection (Bossé et al. 1992; Furesz et al. 1998). It is not clear whether *A. pleuropneumoniae* produces an immunoglobulin protease that could interfere with opsonophagocytosis (Kilian et al. 1976; Mulks et al. 1984; Negrete-Abascal et al. 1998)

Damage to Host Tissues

Most of the pathological changes of porcine pleuropneumonia can be attributed the Apx toxins, which have a direct cytotoxic effect on various cell

types as well as indirectly stimulating release of inflammatory mediators from activated phagocytes (Frey 1995).

Activation of alveolar and intravascular macrophages, largely through Apx toxins and LPS, leads to release of toxic oxygen metabolites (including superoxide anion, hydrogen peroxide, and hydroxyl radical), as well as proteolytic enzymes, and various cytokines (Sibille and Reynolds 1990; Pabst 1996). Infection of pigs with *A. pleuropneumoniae* leads to rapid local production of the proinflammatory cytokines IL1-alpha, IL-beta, and IL-6, as well as the potent PMN chemoattractant IL-8 (Choi et al. 1999; Huang et al. 1999; Baarsch et al. 2000). Whether there is also a significant rise in TNF-alpha in infected lung tissue is unclear. In addition, activation of the alternative complement cascade by LPS results in release of C3a and C5a, which attract and activate PMNs and macrophages (Udeze et al. 1987; Bertram 1988). The rapid recruitment and activation of PMNs leads to release of cyclooxygenase-dependent inflammatory mediators, resulting in further PMN and platelet activation, vasodilation, and constriction of pulmonary airways (Bertram 1988; Liggett et al. 1987; Dom et al. 1992). In addition to producing more oxygen radicals and proteolytic enzymes than macrophages, PMNs also release myeloperoxidase, which converts the relatively less-toxic hydrogen peroxide into hypochlorous acid, the most potent cytotoxic oxidant generated during neutrophilic inflammation (Sibille and Reynolds 1990).

Damage to endothelial cells by Apx toxins, as well as direct activation of factor XII by LPS, initiates the coagulation, fibrinolysis, and kinin systems (Udeze et al. 1987; Serebrin et al. 1991). 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 (Bertram, 1988).

Although many of the pathological consequences of *A. pleuropneumoniae* infection have been attributed to LPS, extremely 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). Furthermore, pigs infected with a mutant of a serotype 1 strain of *A. pleuropneumoniae* lacking ApxI and ApxII but with normal LPS did not develop clinical disease or significant lung lesions (Tascon et al. 1994). This indicates that the contribution of LPS to lesion development may be minimal in the absence of Apx toxins.

The critical role of Apx toxins in development of clinical disease and tissue damage has been confirmed using recombinant toxins (rApxI, rApxII, and rApxIII) (Kamp et al. 1997). Endobronchial inoculation of pigs with either rApxI or rApxIII resulted 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, resulted in few or no clinical signs of disease and only mild lung lesions. Thus, ApxII appears to contribute only minimally to lesion formation, despite the fact that serotype 7 strains, which produce only ApxII, are capable of causing severe disease with typical lung lesions (Frey 1995; Kamp et al. 1997). In addition, mutants of serotype 1 and 5 strains, devoid of ApxI but still producing ApxII, also caused typical severe clinical disease and lung lesions (Tascon et al. 1994; Reimer et al. 1995). These results indicate that there may be other cell-associated toxin(s) or virulence determinant(s) contributing to the severe pulmonary lesions caused by these strains.

Recently, a fourth RTX toxin (ApxIV) was reported to be produced by all serotypes of *A. pleuropneumoniae* (Schaller et al. 1999). This toxin is antigenically distinct from the other Apx toxins and is only produced *in vivo*. *E. coli* expressing cloned ApxIV along with an associated upstream ORF was found to have weak hemolytic and cohemolytic (CAMP) activities (Schaller et al. 1999). The contribution of this RTX toxin to pathogenesis of *A. pleuropneumoniae* remains to be elucidated.

Apart from producing Apx toxins and LPS, *A. pleuropneumoniae* also secretes proteases into the culture medium that may contribute to the pathogenesis of infection (Negrete-Abascal et al. 1998; Garcia-Cuellar et al. 2000). Recently, a 24 kDa zinc metalloprotease that is present in all *A. pleuropneumoniae* serotypes was cloned and expressed in *E. coli*. The oligomeric, but not the monomeric, form of recombinant polypeptide cleaved azocoll, gelatin, and actin *in vitro*. This protease is probably different from the >200 kDa metalloprotease complex described by Negrete-Abascal et al. (1998) that also degrades gelatin, since antibodies against this protein failed to recognize the recombinant 24 kDa protease. Although the presence in convalescent antiserum of antibodies specific to the proteases indicates that they are produced *in vivo* (Negrete-Abascal et al. 1998; Garcia-Cuellar et al. 2000), their possible contribution to development of pathology has yet to be investigated.

Prevention and Control

A major control mechanism for reducing incidence of pleuropneumonia is the maintenance of good management practices (Maes et al. 2001). These include providing adequate air quality, ventilation, and temperature; avoiding overcrowding; maintaining a strict all-in/all-out production with thorough cleaning between groups; and obtaining pigs only from herds with similar or higher health status (see Epidemiology above for serological and PCR tests).

In the event of infection, treatment of pigs with acute pleuropneumonia requires direct injection of a highly efficacious antibiotic (ideally selected after antibiotic resistance 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. Evidence from field and experimental studies indicates that infection with one serotype of *A. pleuropneumoniae* provides complete protection against subsequent infection with the homologous serotype, and at least partial protection against heterologous infection (Nielsen 1979; Cruijsen et al. 1995a; Haesebrouck et al. 1996), suggesting that vaccination might be a feasible alternative to antibiotic treatment.

Development of an effective vaccine has been hampered by the antigenic diversity observed between the 15 different serotypes of *A. pleuropneumoniae.* Existing whole-cell vaccines, even those incorporating purified Apx toxins, provide only limited, serotype-specific protection (Cruijsen et al. 1995b). Attenuated strains that secrete active or detoxified Apx toxins may be useful as live attenuated vaccines (Prideaux et al. 1999; Fuller et al. 2000; Garside et al. 2002). Recently, a defined acapsular mutant (Ward et al. 1998) has been licensed for commercial use in the United States. Environmental regulations, however, may limit the use of this vaccine, which carries an antibiotic resistance marker, in other countries. A method of creating unmarked deletion mutations in *A. pleuropneumoniae* such as the *sacB* system described by Oswald et al.(1999) may overcome this barrier. Alternatively, genetically inactivated strains (bacterial ghosts) created via expression of a cloned phage lysis gene are also being explored as possible vaccines against pleuropneumonia (Huter et al. 2000).

In herds with established infection, colostrum from immune sows can induce passive protection in piglets up to 8 weeks of age (Gardner et al. 1991). Since maternal antibodies may reduce protection following vaccination, serological screening is necessary to determine the best time for vaccination. Traditionally, the only method to completely eliminate *A. pleuropneumoniae* from an infected herd was via depopulation and repopulation with known *A. pleuropneumoniae*-free pigs. However, more recently, Segregated Early Weaning (SEW) or Medicated Early Weaning (MEW) has been used as an alternative means of achieving *A. pleuropneumoniae*-free weaners (Dritz et al. 1996).

ACTINOBACILLUS SUIS

Epidemiology

Since its original description in 1962, sporadic cases of *Actinobacillus suis* or *A. sui*s-like organisms have been reported in a variety of birds and mammals. Recent studies suggest that it is likely that many isolates from nonporcine sources are 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). Beginning in the early 1990s, there were increasing numbers of reports of severe outbreaks of *A. suis* in Canada, the United States, and more recently Australia (MacInnes and Desrosiers 1999; Taylor 1999b; Wilson and McOrist 2000).

The 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*. They further demonstrated 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,b). Chemical characterization of the surface polysaccharides of representative strains revealed that the O1 antigen is (1-6)-beta-D-glucan and the O2 antigen is a (Glc, Gal2, GlcNac) branched tetrasaccharide (Monteiro et al. 2000). Several different capsular types have also been identified. The K1 capsule is (1-6)-beta-D-glucan, and the K2 and K3 capsules contain sialic acid, but their precise structure remains to be determined.

Transmission and Disease

A. 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 1999b). *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 1999b). 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 1999b). In neonates and sucking pigs, *A. suis* can cause an acute and rapidly fatal septicemia where death occurs within 15 hours. 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 *Actinobacillus pleuropneumoniae,* relatively little is known about the pathogenesis of *Actinobacillus suis,* in part because there have been no realistic experimental challenge systems available (Slavic 2002b). Despite superficial similarities, *A. pleuropneumoniae* and *A. suis* share only 50% DNA-DNA homology (Borr et al. 1991). They 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. To date, no attachment factors have been described for *A. suis*. In signature tagged mutagenesis studies, homologues of two known adhesins (PfhaB1 and OMP5) were identified in a screen for colonization, but their role needs to be established (Ojha and MacInnes, unpublished data). 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., personal communication).

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 complement depleted (Slavic et al. 2000b). The RTX toxins, *apxI* and *apxII*, which 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). In contrast to *A. pleuropneumoniae*, *A. suis* lacks the *apxIV* genes (Schaller et al. 1999).

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 (Taylor 1999b). Theoretically, *A. pleuropneumoniae* vaccines, especially those with Apx toxins and outer membrane proteins, could provide some cross-protection (MacInnes and Rosendal 1987; Devenish and Rosendal 1989). Unlike *A. pleuropneumoniae*, *A. suis* has no commercially available serodiagnostic tests although Lapointe et al. (2001) used an ELISA based on a saline extract of boiled formalinized whole cells of a field strain to measure antibody levels in response to vaccination. Like many other bacteria, *A. suis* isolates are becoming increasingly resistant to antibiotics, although, given the very sudden onset of disease, it is difficult to initiate treatment in sufficient time.

ACTINOBACILLUS EQUULI

Epidemiology

A. equuli has been associated with sporadic cases of disease in horses worldwide. In 2002, Christensen

et al. (2002a) proposed a new classification scheme for equine isolates of *A. equuli,* which had previously been variously reported as *Actinobacillus 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 heterogenous. These findings are consistent with early reports by Kim (1976) where more than 28 different heat-stable antigenic groups were reported. In light of more recent 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

A. equuli is a common resident of the oral cavity of adult horses although it is sometimes difficult to culture (Phillips 1984; Sternberg and Brandstorm 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*. In young foals, *A. equuli* can cause an acute septicemia also known as sleepy foal disease (Rycroft and Garside 2000). In foals less than 1 month of age, *A. equuli* suppurative nephritis, arthritis, pneumonia, pleuritis, or enteritis is frequently seen in association with septicemia.

Pathogenesis

Virtually nothing is known about the virulence 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. 2003). 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*. Berthoud et al. (2002) found that the *aqxCABD* operon was present in all hemolytic strains of *A. equuli* (which they designated *A. equuli*-like) including two strains from rabbits. In contrast, these genes were absent in nonhemolytic *A. equuli*. It is tempting to speculate that the *aqxA* toxin may have a role in virulence, but this remains to be tested.

Prevention and Treatment

It has been generally thought that all *A. equuli* have equal 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. Unfortunately, hemolytic activity was not evaluated. That 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.

ACTINOBACILLUS LIGNIERESII

Epidemiology

A. lignieresii is the causative agent of actinobacillosis, or wooden tongue, a chronic disease in cattle that can be confused with actinomycosis (caused by *Actinomyces bovis*). 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. *A. lignieresii* has been reported in the oral cavity and pharynx of healthy sheep and cattle, although in some early reports, isolates identified as *A. lignieresii* were misclassified (Bisgaard et al. 1986). Similarly, *A. lignieresii* reported in other species (e.g., rat, dog, duck) were likely other members of the family *Pasteurellaceae*. Recent studies of equine strains of *A. lignieresii* reveal that these organisms are 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 (Christiansen et al. 2002b). Six serotypes of *A. lignieresii* have been described (one of which shares cross-reactive epitopes with *A. pleuropneumoniae*; Lebrun et al. 1999), but there have been no reports of the use of serotyping in epidemiological studies. 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. Damage to mucous membranes predispose 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 windpipe 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 (Rycroft and Garside 2000). Despite the fact that *A. lignieresii* and *A. pleuropneumoniae* share almost 70% DNA-DNA homology (the usual cutoff for classification of organisms of the same genus; Borr et al. 1991) the diseases caused by these organisms are very different. The virulence factors of *A. lignieresii* are unknown. *A. lignieresii* carries homologues of the *A. pleuropneumoniae apxICA* genes, although toxin is not expressed (Schaller et al. 2000).

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*. 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).

CONCLUSIONS AND FUTURE PROSPECTS

Although Christensen and others have done much in recent years to begin to rationalize the taxonomy of members of the genus *Actinobacillus*, there are still problems, especially within the species *equuli* and *lignieresii*. Analysis of additional strains using both genetic and phenotypic approaches will help to clarify the uncertainty. However, it is possible that the genus *Actinobacillus* is made up of many members that, depending on the approach used, will have affiliations with more than one species and defy easy classification.

With the exception of *A. pleuropneumoniae,* where the structures of the O and K antigens have been determined, serotyping of many *Actinobacillus* species remains problematic. In the case of *A. suis,* some progress has been made but a comprehensive serotyping system is not yet available. That said, simple serological techniques that are currently being used such as slide agglutination will likely be replaced by DNA-based testing even for routine epidemiological studies.

Although understanding of pathogenesis of *Actinobacillus* spp. has improved in recent years, many questions remain. This is particularly true with regard to the early stages of disease including both the host and the bacterial side of the attachment process and the elucidation of factors involved in the shift from a commensal lifestyle to that of a pathogen. Current signature tagged mutagenesis studies and the growing availability of genome sequences will increase understanding of these organisms.

REFERENCES

Ajito, T., Y. Haga, S. Homma, M. Goryo, and K. Okada. 1996. Immunohistological evaluation on respiratory lesions of pigs intranasally inoculated with *Actinobacillus pleuropneumoniae* serotype 1. J. Vet. Med. Sci. 58:297–303.

Appleyard, G. D., S. E. Furesz, and B. N. Wilkie. 2002. Blood lymphocyte subsets in pigs vaccinated and challenged with *Actinobacillus pleuropneumoniae*. Vet. Immunol. Immunopathol. 86:221–228.

Baarsch, M. J., D. L. Foss, and M. P. Murtaugh. 2000. Pathophysiologic correlates of acute porcine pleuropneumonia. Am. J. Vet. Res. 61:684–90.

Bada, R., K. R. Mittal, and R. Higgins. 1996. Biochemical and antigenic relationships between porcine and equine isolates of Actinobacillus suis. Vet. Microbiol. 51:393–396.

Baltes, N., W. Tonpitak, G. F. Gerlach, I. Hennig-Pauka, A. Hoffmann-Moujahid, M. Ganter, and H. J. Rothkotter. 2001. *Actinobacillus pleuropneumoniae* iron transport and urease activity: effects on bacterial virulence and host immune response. Infect. Immun. 69:472–478.

Berthoud, H., J. Frey, and P. Kuhnert. 2002. Characterization of Aqx and its operon: the hemolytic RTX determinant of *Actinobacillus equuli*. Vet Microbiol. 87:159–174.

Bertram, T. A. 1988. Pathobiology of acute pulmonary lesions in swine infected with

Haemophilus (*Actinobacillus*) *pleuropneumoniae*. Can. Vet. J. 29:574–577.

Bilinski, T. 1991. Oxygen toxicity and microbial evolution. Biosystems 24:305–312.

Bisgaard, M., J. E. Phillips, and W. Mannheim. 1986. Characterization and identification of bovine and ovine Pasteurellaceae isolated from the oral cavity and rumen of apparently normal cattle and sheep. Acta Pathol. Microbiol. Immunol. Scand. [B]. 94:9–17.

Blackall, P. J., H. L. B. M. Klassen, H. Van Den Bosch, P. Kuhnert, and J. Frey. 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet. Microbiol. 84:47–52.

Borr, J. D., D. A. J. Ryan, and J. I. MacInnes. 1991. Analysis of *Actinobacillus pleuropneumoniae* and related organisms by DNA-DNA hybridization and restriction endonuclease fingerprinting. Int. J. Syst. Bacteriol. 41:121–129.

Bossé, J. T., and J. I. MacInnes. 2000. Urease activity may contribute to the ability of *Actinobacillus pleuropneumoniae* to establish infection. Can. J. Vet. Res. 64:145–150.

Bossé, J. T., H. D. Gilmour, and J. I. MacInnes. 2001. Novel genes affecting urease activity in *Actinobacillus pleuropneumoniae*. J. Bacteriol. 183:1242–1247.

Bossé, J. T., R. P. Johnson, M. Nemec, and S. Rosendal. 1992. Protective local and systemic antibody responses of swine exposed to an aerosol of *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. 60:479–484.

Braun, V., and H. Killmann. 1999. Bacterial solutions to the iron-supply problem. Trends Biochem. Sci. 24:104–109.

Campbell, S. G., T. R. H. Whitlock, J. F. Timoney, and A. M. Underwood. 1975. An unusual epizootic of actinobacillosis in dairy heifers. J. Am. Vet. Med. Assoc. 166:604–606.

Chiers, K., E. Donne, I. Van Overbeke, R. Ducatelle, and F. Haesebrouck. 2002. *Actinobacillus pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. Vet. Microbiol. 85:343–352.

Chiers, K., F. Haesebrouck, I. van Overbeke, G. Charlier, R. Ducatelle. 1999. Early in vivo interactions of *Actinobacillus pleuropneumoniae* with tonsils of pigs. Vet Microbiol. 68:301–306.

Chiers, K., I. van Overbeke, P. De Laender, R. Ducatelle, S. Carel, and F. Haesebrouck. 1998. Effects of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with inactivated vaccines containing the Apx toxins. Vet. Q. 20:65–69.

Chin, N., J. Frey, C. F. Chang, and Y. F. Chang. 1996. Identification of a locus involved in the utilization of iron by *Actinobacillus pleuropneumoniae.* FEMS Microbiol. Lett. 143:1–6.

Choi, C., D. Kwon, K. Min, and C. Chae. 1999. In-situ hybridization for the detection of inflammatory cytokines (IL-1, TNF-α and IL-6) in pigs naturally infected with *Actinobacillus pleuropneumoniae*. J. Comp. Pathol. 121:349–356.

Christensen, H., M. Bisgaard, and J. E. Olsen. 2002a. Reclassification of equine isolates previously reported as *Actinobacillus equuli*, variants of *A. equuli*, *Actinobacillus suis* or Bisgaard taxon11 and proposal of *A. equuli* subsp. *equuli* subsp. nov. and *A. equuli* subsp. *haemolyticus* subsp. nov. Int. J. Syst. Evol. Microbiol. 52:1569–1576.

Christensen, H., M. Bisgaard, O. Angen, and J. E. Olsen. 2002b. Final classification of Bisgaard taxon 9 as *Actinobacillus arthritidis* sp. nov. and recognition of a novel genomospecies for equine strains of *Actinobacillus lignieresii*. Int. J. Syst. Evol. Microbiol. 52:1239–1246

Cruijsen, T., L. A. van Leengoed, M. Ham-Hoffies, and J. H. Verheijden. 1995a. Convalescent pigs are protected completely against infection with a homologous *Actinobacillus pleuropneumoniae* strain but incompletely against a heterologous-serotype strain. Infect. Immun. 63:2341–2343.

Cruijsen, T., L. A. van Leengoed**,** E. M. Kamp, A. Bartelse, A. Korevaar, and J. H. Verheijden. 1995b. Susceptibility to *Actinobacillus pleuropneumoniae* infection in pigs from an endemically infected herd is related to the presence of toxin-neutralizing antibodies. Vet. Microbiol. 47:219–228.

Cruijsen, T. L., L. A. Van Leengoed, T. C. Dekker-Nooren, E. J. Schoevers, and J. H. Verheijden. 1992. Phagocytosis and killing of *Actinobacillus pleuropneumoniae* by alveolar macrophages and polymorphonuclear leukocytes isolated from pigs. Infect. Immun. 60:4867–4871.

Cullen, J. M., and A. N. Rycroft. 1994. Phagocytosis by pig alveolar macrophages of *Actinobacillus pleuropneumoniae* serotype 2 mutant strains defective in haemolysin II (ApxII) and pleurotoxin (ApxIII). Microbiology 140:237–244.

Deneer, H. G., and A. A. Potter. 1989. Effect of iron restriction on the outer membrane proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*. Infect. Immun. 57:798–804.

Devenish, J., and S. Rosendal. 1989. Identification of the heat-labile hemolysin of *Actinobacillus pleuropneumoniae* serotype 1. Can. J. Vet. Res. 53:251–254.

Devenish, J., S. Rosendal, and J. T. Bossé. 1990. Humoral antibody response and protective immunity in swine following immunization with the 104-kilodalton hemolysin of *Actinobacillus pleuropneumoniae*. Infect. Immun. 58:3829–3832.

Diarra, M. S., J. A. Dolence, E. K. Dolence, I. Darwish, M. J. Miller, F. Malouin, and M. Jacques. 1996. Growth of *Actinobacillus pleuropneumoniae* is promoted by exogenous hydroxamate and catechol siderophores. Appl. Environ. Microbiol. 62:853–859.

Dom, P., F. Haesebrouck, and P. De Baetselier. 1992. Stimulation and suppression of the oxygenation activity of porcine pulmonary alveolar macrophages by *Actinobacillus pleuropneumoniae* and its metabolites. Am. J. Vet. Res. 53:1113–1118.

Dom, P., F. Haesebrouck , R. Ducatelle, and G. Charlier G. 1994. In vivo association of *Actinobacillus pleuropneumoniae* serotype 2 with the respiratory epithelium of pigs. Infect Immun. 2:1262–1267.

Dritz, S. S., M. M. Chengappa, J. L. Nelssen, M. D. Tokach, R. D. N. Goodband, J. C. Nietfeld**,** and J. J. Staats. 1996. Growth and microbial flora of nonmedicated, segregated, early weaned pigs from a commercial swine operation. J. Am. Vet. Med. Assoc. 208:711–715.

D'Silva, C. G., F. S. Archibald, and D. F. Niven. 1995. Comparative study of iron acquisition by biotype 1 and biotype 2 strains of *Actinobacillus pleuropneumoniae.* Vet. Microbiol. 44:11–23.

Dubreuil, J. .D., M. Jacques, K. R. Mittal, and M. Gottschalk. 2000. *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis and immunogenicity. Anim. Health. Res. Rev. 1:73–93.

Enoe, C., J. Mousing, A. Schirmer, and P. Willeberg. 2002. Infectious and rearing-system related risk factors for chronic pleuritis in slaughter pigs. Prev. Vet. Med. 54:337–349.

Fenwick, B. W., B. I. Osburn, and H. J. Olander. 1986. Isolation and biological characterization of two lipopolysaccharides and a capsular-enriched polysaccharide preparation from *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. 47:1433–1441.

Frey, J. 1995. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol. 3:257–261.

Frey, J., J. T. Bossé, Y. F. Chang, J. M. Cullen, B. Fenwick, G. F. Gerlach, D. Gygi, F. Haesebrouck, T. J. Inzana, and R. Jansen. 1993. *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. J. Gen. Microbiol. 139:1723–1728.

Fuller, C. A., R. Yu, S. W. Irwin, and A. B. Schryvers. 1998. Biochemical evidence for a conserved interaction between bacterial transferrin binding protein A and transferrin binding protein B. Microb. Pathog. 24:75–87.

Fuller, T. E., B. J. Thacker, C. O. Duran, and M. H. Mulks. 2000a. A genetically-defined riboflavin auxotroph of *Actinobacillus pleuropneumoniae* as a live attenuated vaccine. Vaccine 18:2867–2877.

Fuller, T. E., S. Martin, J. F. Teel, G. R. Alaniz, M. J. Kennedy, and D. E. Lowery. 2000b. Identification of *Actinobacillus pleuropneumoniae* virulence genes using signature-tagged mutagenesis in a swine infection model. Microb. Pathog. 29:39–51.

Furesz, S. E., B. A. Mallard, J. T. Bosse, S. Rosendal, B. N. Wilkie, and J. I. MacInnes. 1997. Antibody- and cell-mediated immune responses of *Actinobacillus pleuropneumoniae*-infected and bacterin-vaccinated pigs. Infect. Immun. 65:358–365.

Furesz, S. E., B. N. Wilkie, B. A. Mallard, S. Rosendal, and J. I. MacInnes. 1998. Anti-haemolysin IgG1 to IgG2 ratios correlate with haemolysin neutralization titres and lung lesion scores in *Actinobacillus pleuropneumoniae* infected pigs. Vaccine 16:1971–5.

Garcia-Cuellar, C., C. Montanez, V. Tenorio, J. Reyes-Esparza, M. J. Duran, E. Negrete, A. Guerrero, and M. de la Garza. 2000. A 24-kDa cloned zinc metalloprotease from *Actinobacillus pleuropneumoniae* is common to all serotypes and cleaves actin in vitro. Can. J. Vet. Res. 64:88–95.

Gardner, I. A., J. T. Bosse, R. F. Sheldrake, S. Rosendal, and R. P. Johnson. 1991. Serological response to *Actinobacillus pleuropneumoniae* serovar 7 infection in a commercial pig herd. Aust. Vet. J. 68:349–352.

Garside, L. H., M. Collins, P. R. Langford, and A. N. Rycroft. 2002. *Actinobacillus pleuropneumoniae* serotype 1 carrying the defined *aroA* mutation is fully avirulent in the pig. Res. Vet. Sci. 72:163–167.

Genco, C. A., and D. White Dixon. 2001. Emerging strategies in microbial haem capture. Mol. Microbiol. 39:1–11.

Gerlach, G. F., C. Anderson, S. Klashinsky, A. Rossi-Campos, A. A. Potter, and P. J. Willson. 1993. Molecular characterization of a protective outer membrane lipoprotein (OmlA) from *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. 61:565–72.

Gerlach, G. F., S. Klashinsky, C. Anderson, A. A. Potter, and P. J. Willson. 1992. Characterization of two genes encoding distinct transferrin-binding proteins in different *Actinobacillus pleuropneumoniae* isolates. Infect. Immun. 60:3253–3261.

Gordon, A. H., P. D. Hart, and M. R. Young. 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature 286:79–80.

Gottschalk, M., A. Lebrun, S. Lacouture, J. Harel, C. Forget, and K. R. Mittal. 2000. Atypical *Actinobacillus pleuropneumoniae* isolates that share antigenic determinants with both serotypes 1 and 7. J. Vet. Diagn. Invest. 12:444–449.

Haesebrouck, F., A. Van de Kerkhof, P. Dom, K. Chiers, and R. Ducatelle. 1996. Cross-protection between *Actinobacillus pleuropneumoniae* biotypes-serotypes in pigs. Vet. Microbiol. 52:277–284.

Haga, Y., S. Ogino, S. Ohashi, T. Ajito, K. Hashimoto, and T. Sawada. 1997. Protective efficacy of an affinity-purified hemolysin vaccine against experimental swine pleuropneumonia. J. Vet. Med. Sci. 59:115–120.

Hensel, A., N. Stockhofe-Zurwieden, M. Ganter, and K. Petzoldt. 1995. Aerosol exposure of pigs to viable or inactivated *Actinobacillus pleuropneumoniae* serotype 9 induces antibodies in bronchoalveolar lining fluids and serum, and protects against homologous challenge. Vet. Microbiol. 47:27–41.

Huang, H., A. A. Potter, M. Campos, F. A. Leighton, P. J. Willson, D. M. Haines, and W. D. Yates. 1999. Pathogenesis of porcine *Actinobacillus pleuropneumonia*, part II: roles of proinflammatory cytokines. Can. J. Vet. Res. 63:69–78.

Huter, V., A. Hensel, E. Brand, and W. Lubitz. 2000. Improved protection against lung colonization by *Actinobacillus pleuropneumoniae* ghosts: characterization of a genetically inactivated vaccine. J. Biotechnol. 83:161–172.

Inzana, T. J., and B. Fenwick. 2001. Serologic detection of *Actinobacillus pleuropneumoniae* in swine by capsular polysaccharide-biotin-streptavidinenzyme-linked immunosorbent assay. J. Clin. Microbiol. 39:1279–1282.

Inzana, T. J., J. Ma, T. Workman, R. P. Gogolewski, and P. Anderson. 1988. Virulence properties and protective efficacy of the capsular polymer of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* serotype 5. Infect. Immun. 56:1880–1889.

Jacques, M., M. Belanger, G. Roy , and B. Foiry. 1991. Adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal epithelial cells and frozen lung sections. Vet. Microbiol. 27:133–143.

Jeannotte, M.-E., D. Slavic, J. Frey, P. Kuhnert, and J. I. MacInnes. 2002. Analysis of non-porcine isolates of *Actinobacillus suis*. Vet. Microbiol. 85:83–93.

Jensen, T. K., M. Boye, T. Hagedorn-Olsen, H. J. Riising, and O. Angen. 1999. *Actinobacillus pleuropneumoniae* osteomyelitis in pigs demonstrated by fluorescent in situ hybridization. Vet. Pathol. 36:258–261.

Kamp, E. M., J. K. Popma, J. Anakotta, and M. A. Smits. 1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. Infect. Immun. 59:3079–3085.

Kamp, E. M., N. Stockhofe-Zurwieden, L. A. M. G. van Leengoed, and M. A. Smits. 1997. Endobronchial inoculation with Apx toxins of *Actinobacillus pleuropneumoniae* leads to pleuropneumonia in pigs. Infect. Immun. 65:4350–4354.

Katinger, A., W. Lubitz, M. P. Szostak, M. Stadler, R. Klein, A. Indra, V. Huter, and A. Hensel. 1999. Pigs aerogenously immunized with genetically inactivated (ghosts) or irradiated *Actinobacillus pleuropneumoniae* are protected against a homologous aerosol challenge despite differing in pulmonary cellular and antibody responses. J. Biotechnol. 73:251–260.

Kilian, M. 1976. The haemolytic activity of *Haemophilus* species. Acta Pathol Microbiol Scand [B] 84B:339–341.

Kim, B. H., J. E. Phillips, and J. G. Atherton. 1976. A*ctinobacillus suis* in the horse. Vet. Rec. 98:239.

Kuhnert, P., H. Berthoud, R. Straub, and J. Frey. 2003. Host cell specific activity of RTX toxins from haemolytic *Actinobacillus equuli* and *Actinobacillus suis*. Vet. Microbiol. 92:161–167.

Labrie, J., S. Rioux, M. M. Wade, F. R. Champlin, S. C. Holman, W. W. Wilson, C. Savoye , M. Kobisch, M. Sirois, C. Galarneau, and M. Jacques. 2002. Identification of genes involved in biosynthesis of *Actinobacillus pleuropneumoniae* serotype 1 O-antigen and biological properties of rough mutants. J. Endotoxin. Res. 8:27–38.

Langford, P. R., B. M. Loynds, and J. S. Kroll. 1996. Cloning and molecular characterization of Cu, Zn superoxide dismutase from *Actinobacillus pleuropneumoniae*. Infect. Immun. 64:5035–5041.

Lapointe, L., S. D'Allaire, S. Lacouture, and M. Gottschalk. 2001. Serologic profile of a cohort of pigs and antibody response to an autogenous vaccine for *Actinobacillus suis*. Vet. Res. 32:175–183.

Lebrun, A., S. Lacouture, D. Cote, K. R. Mittal, and M. Gottschalk. 1999. Identification of *Actinobacillus pleuropneumoniae* strains of serotypes 7 and 4 using monoclonal antibodies: demonstration of common LPS O-chain epitopes with *Actinobacillus lignieresii*. Vet. Microbiol. 65:271–282.

Liggett, A. D., L. R. Harrison, and R. L. Farrell. 1987. Sequential study of lesion development in experimental *Haemophilus pleuropneumonia*. Res. Vet. Sci. 42:204–212.

Macfadyen, L. P., and R. J. Redfield. 1996. Life in mucus: sugar metabolism in *Haemophilus influenzae*. Res. Microbiol. 147:541–551.

MacInnes, J. I., and R. Desrosiers. 1999. Agents of the "suis-ide diseases" of swine: *Actinobacillus suis*, *Haemophilus parasuis*, and *Streptococcus suis*. Can. J. Vet. Res. 63:83–89.

MacInnes, J. I., and S. Rosendal. 1987. Analysis of major antigens of *Haemophilus* (*Actinobacillus*)

pleuropneumoniae and related organisms. Infect. Immun. 55:1626–1634.

Madsen, M. E., K. G. Carnahan, and R. N. Thwaits. 1995. Evaluation of pig lungs following an experimental challenge with *Actinobacillus pleuropneumoniae* serotype 1 and 5 in pigs inoculated with either hemolysin protein and/or outer membrane proteins. FEMS Microbiol. Lett. 131:329–335.

Maes, D., K. Chiers, F. Haesebrouck, H. Laevens, M. Verdonck, and A. de Kruif. 2001. Herd factors associated with the seroprevalences of *Actinobacillus pleuropneumoniae* serovars 2, 3 and 9 in slaughter pigs from farrow-to-finish pig herds. Vet. Rec. 151:206–210.

Mannheim, W., G. R. Carter, M. Kilian, E. L. Biberstein, and J. E. Phillips. 1984. Family III *Pasteurellaceae*. In Bergey's manual of systematic bacteriology, vol. I. N. R. Kreig and J. G. Holt (eds.). Baltimore, MD: Williams and Wilkins Co., pp. 550–575.

Mikael, L. G., P. D. Pawelek, J. Labrie, M. Sirois, J. W. Coulton, and M. Jacques. 2002. Molecular cloning and characterization of the ferric hydroxamate uptake (*fhu*) operon in *Actinobacillus pleuropneumoniae*. Microbiology 48:2869–2882.

Min, K., and C. Chae. 1998. Detection and distribution of DNA of *Actinobacillus pleuropneumoniae* in the lungs of naturally infected pigs by in-situ hybridization. J. Comp. Pathol. 119:169–175.

Moeck, G. S., and J. W. Coulton. 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol. Microbiol. 28:675–681.

Monteiro, M. A., D. Slavic, F. St. Michael, J-R. Brisson, J. I. MacInnes, and M. B. Perry. 2000. The first description of (1-6)-beta-D-glucan in prokaryotes: (1-6)-beta-D-glucan is a common component of *Actinobacillus suis* and is the basis for a serotyping system. Carbohydrate Res. 329:77–86.

Mulks, M. H., E. R. Moxon, J. Bricker, A. Wright, and A. G. Plaut. 1984. Examination of *Haemophilus pleuropneumoniae* for immunoglobulin A protease activity. Infect. Immun. 45:276–287.

Narita, M., K. Kawashima, T. Morozumi, and H. Takashima. 1995. Effect of physical defenses of the respiratory tract on the development of pneumonia in pigs inoculated endobronchially with *Actinobacillus pleuropneumoniae*. J. Vet. Med. Sci. 57:839–844.

Negrete-Abascal, E., V. R. Tenorio, A. L. Guerrero, R. M. Garcia, M. E. Reyes, and M. de la Garza. 1998. Purification and characterization of a protease from *Actinobacillus pleuropneumoniae* serotype 1, an antigen common to all the serotypes. Can. J. Vet. Res. 62:183–190.

Negrete-Abascal, E., V. R. Tenorio, J. J. Serrano, C. Garcia, and M. de la Garza M. 1994. Secreted proteases from *Actinobacillus pleuropneumoniae* serotype 1 degrade porcine gelatin, hemoglobin and immunoglobulin A. Can. J. Vet. Res. 58:83–86.

Nieboer, E. 1992. Absorption and excretion of nickel. In Nickel and human health: current perspectives. E. Nieboer and J. O. Nriagu (eds.). New York: John Wiley and Sons, pp. 49–68.

Nielsen, R. 1979. *Haemophilus parahaemolyticus* serotypes. Pathogenicity and cross immunity. Nord. Vet. Med. 31:407–413.

Niven, D. F., and M. Lávesque. 1988. V-factor-dependent growth of *Actinobacillus pleuropneumoniae* biotype 2 (Bertschinger 2008/76). Int. J. Sys. Bacteriol. 38:319–320.

Niven, D. F., J. Donga, and F. S. Archibald. 1989. Responses of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. Mol. Microbiol. 3:1083–1089.

Oswald, W., W. Tonpitak, G. Ohrt, and G. Gerlach. 1999. A single-step transconjugation system for the introduction of unmarked deletions into *Actinobacillus pleuropneumoniae* serotype 7 using a sucrose sensitivity marker. FEMS Microbiol. Lett. 179:153–160.

Pabst, R. 1996. The respiratory immune system of pigs. Vet. Immunol. Immunopathol. 54:191–195.

Paradis, S. E., J. D. Dubreuil, M. Gottschalk, M. Archambault, and M. Jacques. 1999. Inhibition of adherence of *Actinobacillus pleuropneumoniae* to porcine respiratory tract cells by monoclonal antibodies directed against LPS and partial characterization of the LPS receptors. Curr. Microbiol. 39:313–320.

Phillips, J. E. 1984. Genus III. *Actinobacillus* Brumpt 1910, 849[AL]. In Bergey's manual of systematic bacteriology, vol. 1. N. R. Kreig and J. G. Holt (eds.). Baltimore, MD: Williams and Wilkins Co., pp. 540–575.

Prideaux, C. T., C. Lenghaus, J. Krywult, and A. L. Hodgson. 1999. Vaccination and protection of pigs against pleuropneumonia with a vaccine strain of *Actinobacillus pleuropneumoniae* produced by site-specific mutagenesis of the ApxII operon. Infect. Immun. 67:1962–1966.

Reimer, D., J. Frey, R. Jansen, H. P. Veit, and T. J. Inzana. 1995. Molecular investigation of the role of ApxI and ApxII in the virulence of *Actinobacillus pleuropneumoniae* serotype 5. Microb. Pathog. 18:197–209.

Rioux, S., C. Galarneau, J. Harel, M. Kobisch, J. Frey, M. Gottschalk, and M. Jacques. 2000. Isolation and characterization of a capsule-deficient

mutant of *Actinobacillus pleuropneumoniae* serotype 1. Microb. Pathog. 28:279–289.

Rioux, S., C. Girard, J. D. Dubreuil, and M. Jacques. 1998. Evaluation of the protective efficacy of *Actinobacillus pleuropneumoniae* serotype 1 detoxified lipopolysaccharides or O-polysaccharide-protein conjugate in pigs. Res. Vet. Sci. 65:165–167.

Rosendal, S., and J. I. MacInnes. 1990. Characterization of an attenuated strain of *Actinobacillus pleuropneumoniae*, serotype 1. Am. J. Vet. Res. 51:711–717.

Rosendal, S., and W. R. Mitchell. 1983. Epidemiology of *Haemophilus pleuropneumoniae* infection in pigs. Can. J. Comp. Med. 47:1–5.

Rosendal, S., D. A. Boyd, and K. A. Gilbride. 1985. Comparative virulence of porcine *Haemophilus* bacteria. Can. J. Comp. Med. 49:68–74.

Rossi-Campos, A., C. Anderson, G. F. Gerlach, S. Klashinsky, A. A. Potter, and P. J. Willson. 1992. Immunization of pigs against *Actinobacillus pleuropneumoniae* with two recombinant protein preparations. Vaccine 10:512–518.

Rycroft, A. N., and J. M. Cullen. 1990. Complement resistance in *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* infection of swine. Am. J. Vet. Res. 51:1449–1453.

Rycroft, A. N., and L. H. Garside. 2000. *Actinobacillus* species and their role in animal disease. Vet J. 159:18–36.

Rycroft, A. N., D. Williams, J. M. Cullen, and J. Macdonald. 1991. The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. J. Gen. Microbiol. 137:561–568.

Schaller, A., P. Kuhnert, V. A. de la Puente-Redondo, J. Nicolet, and J. Frey. 2000. Apx toxins in *Pasteurellaceae* species from animals. Vet. Microbiol. 74:365–376.

Schaller, A., R. Kuhn, P. Kuhnert, J. Nicolet, T. J. Anderson, J. I. MacInnes, R. P. Segers, and J. Frey. 1999. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 145:2105–2116.

Schaller, A., S. P. Djordjevic, G. J. Eamens, W. A. Forbes, R. Kuhn, P. Kuhnert, M. Gottschalk, J. Nicolet, and J. Frey. 2001. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apxIVA*. Vet. Microbiol. 79:47–62.

Seliger, S. S., A. R. Mey, A. M. Valle, and S. M. Payne. 2001. The two TonB systems of *Vibrio cholerae*: redundant and specific functions. Mol. Microbiol. 39:801–812.

Serebrin, S., S. Rosendal, A. Valdivieso-Garcia, and P. B. Little. 1991. Endothelial cytotoxicity of *Actinobacillus pleuropneumoniae*. Res. Vet. Sci. 50:18–22.

Sheehan, B. J., P. R. Langford, A. N. Rycroft, and J. S. Kroll. 2000. [Cu,Zn]-superoxide dismutase mutants of the swine pathogen *Actinobacillus pleuropneumoniae* are unattenuated in infections of the natural host. Infect. Immun. 68:4778–4781.

Sibille, Y., and H. Y. Reynolds. 1990. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am. Rev. Respir. Dis. 141:471–501.

Sidibe, M., S. Messier, S. Lariviere, M. Gottschalk, and K. R. Mittal. 1993. Detection of *Actinobacillus pleuropneumoniae* in the porcine upper respiratory tract as a complement to serological tests. Can. J. Vet. Res. 57:204–208.

Slavic, D., J. DeLay, M. A. Hayes, and J. I. MacInnes. 2000a. Comparative pathogenicity of different *Actinobacillus suis* O/K serotypes. Can. J. Vet. Res. 64:81–87.

Slavic, D., T. L. Toffner, M. A. Monteiro, M. B. Perry, and J. I. MacInnes. 2000b. Prevalence of O1/K1 and O2/K3-reactive *Actinobacillus suis* in healthy and diseased swine. J. Clin. Microbiol. 38:3759–3762.

Sternberg, S., and Brandstrom, B. 1999. Biochemical fingerprinting and ribotyping of isolates of *Actinobacillus equuli* from healthy and diseased horses. Vet. Microbiol. 66:53–65.

Tarigan, S., R. F. Slocombe, G. F. Browning, and W. Kimpton. 1994. Functional and structural changes of porcine alveolar macrophages induced by sublytic doses of a heat-labile, hemolytic, cytotoxic substance produced by *Actinobacillus pleuropneumoniae*. Am. J. Vet. Res. 55:1548–1557.

Tascon, R. I., J. A. Vazquez-Boland, C. B. Gutierrez-Martin, I. Rodriguez-Barbosa, and E. F. Rodriguez-Ferri. 1994. The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*: evidence from mutational analysis. Mol. Microbiol. 14:207–216.

Taylor, D. J. 1999a. *Actinobacillus pleuropneumoniae*. In Diseases of swine, 8th ed. B. E. Shaw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (ed.). Oxford, UK: Blackwell Science, pp. 343–354.

———. 1999b. *Actinobacillus suis*. In Diseases of swine, 8th ed. B. E. Shaw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (eds.). Oxford, UK: Blackwell Science, pp. 624–627.

Thwaits, R. N., and S. Kadis. 1993. Purification of surface-exposed integral outer membrane proteins of *Actinobacillus pleuropneumoniae* and their role in opsonophagocytosis. Am. J. Vet. Res. 54:1462–1470.

Tonpitak W., S. Thiede, W. Oswald, N. Baltes, G. F. Gerlach. 2000. *Actinobacillus pleuropneumoniae* iron transport: a set of *exbBD* genes is transcriptionally linked to the *tbpB* gene and required for utilization of transferrin-bound iron. Infect. Immun. 68:1164–1170.

Udeze, F. A., K. S. Latimer, and S. Kadis. 1987. Role of *Haemophilus pleuropneumoniae* lipopolysaccharide endotoxin in the pathogenesis of porcine *Haemophilus* pleuropneumonia. Am. J. Vet. Res. 48:768–73.

van Ostaaijen, J., J. Frey, S. Rosendal, and J. I. MacInnes. 1997. *Actinobacillus suis* strains isolated from healthy and disease swine are clonal and carry *apxICABD* and *apxIICA* homologues. J. Clin. Microbiol. 35:1131–1137.

van Overbeke, I., C. K. Ducatelle, and R. Haesebrouck. 2001. Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing Apx toxins and transferrin-binding proteins. J. Vet. Med. B Infect. Dis. Vet. Public Health 48:15–20.

van Overbeke, I., K. Chiers, G. Charlier, I. Vandenberghe, J. Van Beeumen, R. Ducatelle, and F. Haesebrouck. 2002. Characterization of the in vitro adhesion of *Actinobacillus pleuropneumoniae* to swine alveolar epithelial cells. Vet. Microbiol. 88:59–74.

Ward, C. K., and T. J. Inzana. 1994. Resistance of *Actinobacillus pleuropneumoniae* to bactericidal antibody and complement is mediated by capsular polysaccharide and blocking antibody specific for lipopolysaccharide. J. Immunol. 153:2110–2121.

Ward, C. K., M. L. Lawrence, H. P. Veit, and T. J. Inzana. 1998. Cloning and mutagenesis of a serotype-specific DNA region involved in encapsulation and virulence of *Actinobacillus pleuropneumoniae* serotype 5a: concomitant expression of serotype 5a and 1 capsular polysaccharides in recombinant *A. pleuropneumoniae* serotype 1. Infect. Immun. 66:3326–3336.

Wilson, R. J., and S. McOrist. 2000. *Actinobacillus suis* infection in pigs in Australia. Aust. Vet. J. 78:317–319.

Zhang, Y., J. M. Tennent, A. Ingham, G. Beddome, C. Prideaux, and W. P. Michalski. 2000. Identification of type 4 fimbriae in *Actinobacillus pleuropneumoniae*. FEMS Microbiol. Lett. 189:15–18.

18
Haemophilus

T. J. Inzana and L. Corbeil

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 NAD and protoporphyrin or protoheme compounds, are required for growth. *Haemophilus somnus*, an organism recently reclassified as *Histophilus somni* from *Haemophilus*, is an exception to this rule that will be discussed in this chapter since 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 species establish a commensal relationship with their host, usually inhabiting the upper respiratory tract but in some cases the urogenital tract. However, some species have the capability to disseminate via the bloodstream and cause serious systemic illness or pneumonia. In most cases, infections occur as a result of inadequate immunity, intercurrent infection, severe stress, or a combination of factors. This chapter will focus on the current understanding of those bacterial properties that enable members in the genus *Haemophilus* to evade host defenses and cause disease.

Table 18.1 lists the *Haemophilus* spp. of animals currently recognized, their normal hosts, and any disease(s) they may cause.

Many species of *Haemophilus* are also commensals or pathogens in the respiratory tract or urogenital tract of humans, including the type species *H. 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 that has been reported with this pathogen. Another human pathogen that has been examined extensively is *H. ducreyi*, the etiologic agent of the sexually transmitted disease chancroid. In addition to the species listed above, it is likely there are many *Haemophilus* spp. that are primarily commensal inhabitants of many animals, that have not been identified because they have not, or have only rarely, been 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 are capable of fermenting carbohydrates and reducing nitrates, and are facultatively anaerobic (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, defined media for *H. influenzae* and *H. somnus* have been devised (Inzana and Corbeil 1987). Colonies are nonpigmented to gray or slightly yellow.

Most species of pathogenic importance are encapsulated. The cell wall is typical of that 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 that have been examined (Hitchcock et al. 1986). Six to eight proteins predominate in the outer membrane. However, in the

Table 18.1. *Haemophilus* Species in Animals

Host	Species	Normal habitat	Associated disease(s)
Pigs	*Haemophilus parasuis*	URT[1]	Polyserositis (Glässer's disease) respiratory disease, septicemia, arthritis
Poultry	*H. paragallinarum*	URT[1]	Fowl coryza
Cats	*H. felis*	URT[1]	Upper respiratory infection (rare)
Dogs	*H. haemoglobinophilus*	UGT[2]	Neonatal mortality (rare)
Cattle, sheep	*Haemophilus somnus (Histophilus somni Haemophilus agni, Histophilus ovis)*	URT[1], UGT[2]	Cattle: Thrombotic meningoencephalitis (TME), pneumonia (shipping fever), reproductive failure, septicemia, myocarditis, arthritis Sheep: Epididymitis, pneumonia, septicemia, meningitis, arthritis, myositis, and mastitis
Horses	*Taylorella (H) equigenitalis*	UGT[2]	Abortion and infertility

[1]URT—upper respiratory tract.
[2]UGT—urogenital tract.

absence of available iron, additional iron-regulated proteins are synthesized in the outer membrane that are capable of binding specifically to transferrin of only the natural host (Ogunnariwo et al. 1990; Yu et al. 1992). These transferrin-binding proteins are thought to bind host transferrin for the purpose of transporting required iron into the bacterium within the iron-deficient matrix of the host. The specificity for host transferrin, along with a variety of adherence factors that are required for colonization, 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, as discussed below.

HABITAT AND TRANSMISSION

Each *Haemophilus* spp. is an obligate parasite of the mucosal surfaces of a single mammalian host or closely related species. These bacteria are fastidious and cannot survive for long periods in the environment. *Haemophilus* spp. colonize a new host by direct contact with another host that may have clinical disease, that is a postclinical carrier of the bacterium, or that is an asymptomatic carrier, through contaminated fomites or by inhalation of contaminated aerosol particles. Such transmission may occur over relatively long distances. For example, transmission of the related host-specific bacterium *Actinobacillus pleuropneumoniae* has been shown to occur via air vents over a distance of at least 2.5 meters (Jobert et al. 2000).

Under most circumstances, these bacteria live 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 the result of a combination of virulence of the pathogen and of compromised host defenses, which may occur due to stress from crowding or shipping, previous viral, mycoplasma, or other infection, or other conditions that would suppress innate and adapted host immunity. This may explain, in part, why the predominant pathogenic *Haemophilus* species occur in animals that are subjected to intense management practices. In addition, some species or strains are particularly well adapted to escape host defense mechanisms. The remainder of this chapter will

focus on the virulence factors of the three predominant *Haemophilus* pathogens of animals (*H. parasuis*, *H. paragallinarum*, and *H. somnus*) that contribute to the disease process or enable the bacteria to avoid host defense mechanisms.

HAEMOPHILUS SOMNUS

Overview

Haemophili that resemble *H. somnus* have been isolated from cattle, sheep, bison, and bighorn sheep (Ward et al. 1995; Ward et al. 1999). The ovine isolates have been variously described as *Histophilus ovis, Haemophilus agni*, and *Haemophilus somnus*. *H. somnus* causes several syndromes in cattle and sheep, including thrombotic meningoencephalitis (TME), upper respiratory infection, pneumonia, bacteremia, abortion, arthritis, myocarditis, infertility, and ovine epididymitis (Corbeil et al. 1995; Inzana 1999). Although the organism is capable of causing any of these conditions, it is often found as a commensal in the genital tract, especially the prepuce of bulls and rams. *H. somnus* can be carried in the upper respiratory tract but is isolated more often from the lower respiratory tract (Corbeil et al. 1995). *Haemophilus agni* and *Histophilus ovi*s are primarily carried in the urogenital tract of sheep, but may cause epidydimitis in young rams, vulvitis, low reproductive rates, mastitis, septicemia, arthritis, meningitis, and pneumonia (Ball et al. 1991; Philbey et al. 1991; McDowell et al. 1994; Corbeil et al. 1995; 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 been subject to debate for many years. DNA-DNA hybridization studies indicate that *H. somnus, Haemophilus agni,* and *Histophilus ovis* should be regarded as the same species. Angen et al. (2003) have proposed that all three species constitute a single species, which should be moved to the genus *Histophilus* and named *Histophilus somni*. These conclusions are based on sequencing of the 16S rDNA and *rpoB* genes, as well as the phenotypic traits of capnophilia, yellowish pigment, and indole production. To avoid confusion, the name *Haemophilus somnus* will be used in this chapter. Although the discussion of *H. somnus* below is based on work with bovine isolates, it is also applicable to ovine isolates.

Host-Parasite Interactions

The portal of entry for *H. somnus* 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 isolates of preputial normal flora and 40 isolates of nasal normal flora on growth of *H. somnus in vitro* showed that the number of isolates that enhance growth of *H. somnus* outnumbered the number of isolates that inhibited growth by 4:1. Only a few isolates did not affect *H. somnus* growth *in vitro* (Corbeil et al. 1985). Therefore, although most normal flora do not defend the host in this way, inhibitory flora can be considered as a first line of defense. The mucociliary elevator is a second line of defense for the respiratory tract against *H. somnus* colonization. Once the organism crosses this barrier, it must attach to epithelial cells and then pass through endothelial cells to invade into the bloodstream. Attachment to bovine turbinate cells, vaginal epithelial cells, and endothelial cells has been demonstrated (Corbeil et al. 1995; Sylte et al. 2001). Subsequent to this attachment, *H. somnus* is capable of causing apoptosis of endothelial cells (Sylte et al. 2001). Apoptosis may be partially responsible for the vasculitis that is characteristic of *H. somnus* disease. The organism compromises not only endothelial cells but also phagocytic cells. Several studies, described below, have shown that *H. somnus* modulates bovine neutrophil and macrophage function, decreasing their bactericidal activity. Most recently, Yang et al. (1998) demonstrated that *H. somnus* also causes apoptosis in bovine neutrophils.

Environmental Factors

It is clear from clinical observations that stress is an important component in susceptibility to *H. somnus* infection, especially as part of the "shipping fever" complex (or bovine respiratory disease complex). The role of initial viral infection in decreasing host defenses is not completely defined, however. Healthy calves prechallenged with bovine herpes virus-1 are more susceptible to *H. somnus*-induced pneumonia and systemic disease (A. Potter, personal communication). Crowding of cattle also plays a role, probably by increasing both the infectious challenge as well as contributing to stress. Anecdotally, cold weather predisposes cattle to *H. somnus*-induced disease, especially sudden decreases in environmental temperatures. *H. somnus* infection often results in bacteremia with localization in sites of predilection (brain, placenta, joints, myocardium, and vessels in the lung). In each case, infiltration with neutrophils at the site of infection is characteristic. The resulting vasculitis and thrombosis, the

hallmarks of *H. somnus* infection, may involve small- or medium-sized arteries, and may be accompanied by hemorrhage and edema. Vasculitis and thrombosis occurs in most tissues infected by *H. somnus*, but the duration of infection determines whether it is detected clinically. In experimental infection, the route may determine whether this "hallmark" is detected. For example, vasculitis is typical in experimental TME induced by intravenous inoculation. However, when *H. somnus* is administered intrathecally (into the CSF), purulent meningitis but not vasculitis is detected (Lees et al. 1994). In experimental *H. somnus* pneumonia, degeneration of alveolar macrophages is a consistent finding 24 hours after intrabronchial inoculation (Gogolewski et al. 1987). This suggests that *H. somnus* may destroy macrophages before they kill the pathogen. In the above study, *H. somnus* bacteria were found either free in alveoli or associated with degenerate alveolar macrophages.

Virulence Factors

Virulence in *H. somnus* 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. somnus* strains. Some strains that live in the prepuce are more serum sensitive than isolates from disease, and lack some outer membrane proteins and LOS factors important to virulence.

LOS

The LOS of *H. somnus* contains phosphorylcholine (ChoP), which is expressed in a phase-variable manner (Howard et al. 2000). In *H. influenzae,* the expression of ChoP is also phase variable, and when expressed enhances colonization of *H. influenzae* in the upper respiratory tract of infant rats (Weiser et al. 1998). *In vitro* studies of adherence of *H. influenzae* to human epithelial cells has shown that ChoP binds to the receptor on epithelial cells for platelet-activating factor (Swords et al. 2000). However, ChoP also binds to C-reactive protein, which results in activation of the complement cascade and bactericidal activity (Weiser et al. 1998). Therefore, it is likely that expression of ChoP enhances adherence of *H. influenzae* in the upper respiratory tract, but in order to disseminate and colonize other tissues, ChoP expression must be turned off. The role of ChoP in adherence of *H. somnus* to bovine mucosal surfaces is not yet clear. However, preliminary experiments in our laboratory have shown that when calves are challenged in the upper respiratory tract with a ChoP-negative phase variant of *H. somnus* (greater than 90% ChoP-negative), after 24 hours greater than 35% of the *H. somnus* population recovered from the respiratory tract are ChoP-positive (unpublished data). Therefore, ChoP may also enhance colonization of *H. somnus* to the bovine respiratory surface. However, we have identified isolates from the normal bovine prepuce that do not phase vary and do not contain detectable ChoP (Inzana et al. 1997; Howard et al. 2000). Therefore, adherence of *H. somnus* to epithelial cells is likely to be multifactorial, and expression of critical factors may vary with the epithelial cell type.

The LOS of *H. somnus* is similar in structure and function to the LOS of *H. influenzae* and *Neisseria gonorrhoeae*. The LOS is microheterogeneous, and lacks the O-side chains present on LPS. The lipid A component has endotoxic activity, and is similar in composition to that of *Escherichia coli* (Inzana et al. 1988). The inner and outer core oligosaccharide is very similar in structure to that of *N. gonorrhoeae*, and contains lacto-*N*-neotetraose or lacto-*N*-tetraose (Cox et al. 1998), oligosaccharides that are also present on mammalian glycosphingolipids. Therefore, the oligosaccharide may be poorly immunogenic, in part due to similarity to host antigens. In addition, the outer core oligosaccharide can undergo a high rate of random, structural and antigenic phase variation. However, phase variation predominately occurs in isolates recovered from disease and not, or only a very low rate, in preputial isolates from healthy animals (Inzana et al. 1992; Inzana et al. 1997). Therefore, although monoclonal antibodies to the LOS of *Neisseria* and other *Haemophilus* spp. react with *H. somnus*, there is also extensive antigenic heterogeneity, which in part is through phase variation.

The molecular basis of phase variation in *H. influenzae* is due, at least in part, to microsatellites of repeating DNA motifs in the open reading frame (ORF). The most characterized phase variable loci are *lic1*, *lic2*, and *lic3* (Weiser et al. 1989). The first gene in each locus has microsatellites of 5'-CAAT-3' sequences immediately downstream of the start codon(s). During replication, slip strand mispairing may occur resulting in the loss or gain of one 5'-CAAT-3' repeat. This variation can place the ORF in or out of frame with the start codon, resulting in a translational switch. We have identified five genes with homology to glycosyl transferases in *H. somnus*. Two genes, *lob1* and *lob2A,* are putative

galactose and N-acetylglucosamine transferases, respectively (McQuiston et al. 2000; Wu et al. 2000). The *lob1* contains microsatellites of 5'-CAAT-3' immediately downstream of the start codons, but does not have homology to any of the *lic* genes. In contrast, *lob2A* contains microsatellites of 5'-GA-3' near the 3' end of the ORF. In addition, *lob2BCD* occur immediately downstream and appear to form a locus with *lob2A*. The *lob2B* is almost identical in sequence to *lob2A* and also contains repetitive sequences of 5'-GA-3' in the same location. The *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 a mutant with a lower rate of phase variation and diminished virulence in a mouse model for *H. somnus*. Sequencing of the *H. somnus* genome is likely to identify additional genes responsible for phase variation and additional mechanisms of virulence. The current sequence can be accessed at: http://microgen.ouhsc.edu/h_somnus/h_somnus_home.htm.

H. influenzae, H. ducreyi, and apparently *H. somnus* are capable of catabolizing neuraminic (sialic) acid (NeuAc), and can therefore utilize NeuAc as a nutrient or incorporate it into their LOS through metabolic pathways (Vimr et al. 2000; Schilling et al. 2001; Inzana et al. 2002). Some strains of *H. somnus* can endogenously sialylate their LOS, although the addition of NeuAc enhances sialylation, whereas other strains can only sialylate their LOS when grown in the presence of NeuAc. Analysis of the partially sequenced *H. somnus* genome has identified at least two putative sialyltransferases and a sialylsynthetase in the genome of disease isolate 2336, but not in preputial isolate 129Pt (unpublished data). Furthermore, NeuAc has not been detected on LOS in isolates recovered from the normal prepuce. The addition of NeuAc onto the LOS inhibits antibody binding to *H. somnus* and enhances the serum resistance of the bacteria (Vimr et al. 2000; Schilling et al. 2001; Inzana et al. 2002). Therefore, *H. somnus* 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 and/or NeuAc.

H. somnus and its LOS are capable of causing apoptosis of bovine pulmonary and brain vascular endothelial cells. Apoptosis is dependent on caspase-8 activation of caspase-3. An inhibitor specific for caspase-8 significantly inhibits apoptosis due to LOS (Sylte et al. 2001). Furthermore, LOS induces activation of $P2X_7$, which may transduce the apoptotic signal from LOS (Sylte et al. 2002). However, treatment of endothelial cells with IL-1β diminishes LOS-mediated apoptosis of bovine pulmonary artery endothelial cells (Sylte et al. 2002).

Exopolysaccharide

At least some strains of most pathogenic *Haemophilus* spp. are encapsulated. However, there are conflicting reports regarding the presence of a capsule on *H. somnus*. We have purified a polysaccharide from *H. somnus* culture supernatant and used this antigen in an ELISA to detect antibodies to the antigen in cattle. Compositional analysis indicated the polysaccharide was composed of galactose and mannose, but other components may also be present. Analysis of the genome identified putative genes with homology to mannose biosynthesis genes, such as phosphomannomutase. Immunoelectron microscopy of cells removed gently from agar plates and not washed demonstrated that the polysaccharide did not remain adherent to the cells but was readily shed into the matrix (fig. 18.1). The role of this exopolysaccharide in virulence and pathogenesis is under investigation. However, we have also determined that *H. somnus* produces a biofilm similar to that produced by *Pseudomonas aeruginosa* (unpublished data). It is possible that this exopolysaccharide contributes, at least in part, to biofilm formation. Biofilms, in turn, enhance colonization of the bacteria at particular sites and promote communication between bacteria.

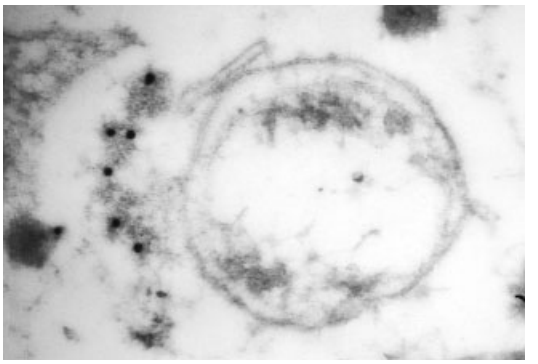


Figure 18.1. Scanning immuno-electron microscopy of *H. somnus* strain 6519 cells that were grown anaerobically and not washed. The gently suspended bacteria were incubated with IgG to purified exopolysaccharide, followed by gold-labeled protein A. Note that gold-labeled protein A bound only to extracellular material shed from the cell. Magnification 25,000X.

Therefore, the exopolysaccharide may be important for colonization of *H. somnus* to particular host sites.

Protein Virulence Factors

Several surface proteins of *H. somnus* have been shown to be important in virulence. Convalescent phase serum that recognizes a 40-kDa outer membrane protein (OMP) was shown to passively protect calves against experimental *H. somnus*-induced pneumonia (Gogolewski et al. 1988). This OMP is surface exposed and often induces the strongest antibody response of all the *H. somnus* antigens. Theisen et al. (1992, 1993) cloned and characterized two 40-kDa proteins of *H. somnus*. Since both of these OMPs were lipid modified, they were designated LppA and LppB. Other OMPs implicated in virulence include a major outer membrane protein (MOMP) of 41-kDa, a heat modifiable OMP (which is 28-kDa at 60°C and 37-kDa at 100°C) and a 17.5-kDa OMP (reviewed in Corbeil et al. 1995). The 41-kDa MOMP, or porin, undergoes antigenic variation, so even though the immune response to this MOMP is not great, it may be a factor in evasion of the host response (Tagawa et al. 2000). A 31-kDa *H. somnus* protein has also been characterized. When the gene for this protein was expressed in *E. coli,* the recombinant strains lysed bovine erythrocytes, suggesting that the 31-kDa protein 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. One of the iron-acquisition mechanisms of *H. somnus* involves binding of transferrin to a surface receptor. Two *H. somnus* 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. somnus*. TbpA is predicted to be an integral (membrane spanning) OMP and may act as a gated pore for iron (Gray-Owen and Schryvers 1996), whereas TbpB is predicted to be a peripheral (non-membrane-spanning) OMP. TbpB has been shown to preferentially bind iron-loaded transferrin, suggesting a mechanism for optimization of iron acquisition (Retzer et al. 1998).

Hemolysin

Some strains of *H. somnus* are weakly hemolytic on blood agar after more than 24 hours incubation at 37°C. However, if some of these strains are incubated for additional time at 4°C, more intense and distinct β-hemolysis is evident. However, an hemolysin has not been purified or characterized from *H. somnus*. Transposon (Tn) mutagenesis of *H. somnus* with transposomes resulted in chromosomal insertion of the Tn, and some of these insertion sites had partial homology to hemolysin genes. However, these mutants remained hemolytic (unpublished results). It is evident that hemolytic activity is not necessary for pathogenesis because the *H. somnus* isolate used to reproduce pneumonia in calves (strain 2336) is not hemolytic.

Ig-Binding Proteins

The immunoglobulin-binding proteins (IgBPs) of *H. somnus* are unique virulence factors related to other large surface proteins of gram-negative bacteria causing respiratory or genital infections. The IgBPs bind bovine IgG2 by the Fc portion and are composed of a peripheral membrane protein, p76, and a surface fibrillar network composed of peptides of varying sizes (Corbeil et al. 1997). Some strains from asymptomatic carriers lack the entire coding region for both sets of proteins (Cole 1992). These strains are killed by bovine complement, whereas IgBP-positive virulent strains are resistant to complement-mediated killing. This resistance is critical to viability in the blood and hence is necessary for septicemia and its sequelae to occur. Recent evidence indicates that the *H. somnus* p76 IgBP has homology with a new family of cysteine proteinases, including the conserved catalytic domain (Shao et al. 2002). The cleavage of either bound IgG2 or of complement components by the proteinase may explain resistance to complement-mediated killing. The IgBPs may also play a role in endothelial cell adherence, since a mutant strain expressing truncated high molecular weight IgBPs and no p76 adhered less well than the wild type to bovine endothelial cells. This correlated with lower virulence of the mutant for mice (Sanders et al. 2003).

Other Virulence Factors

H. somnus modulates phagocyte function and can survive intracellularly. Chiang et al. (1986) showed that *H. somnus* produced suppressive factors for neutrophils, which were identified as purine and pyrimidine bases as well as ribonucleotides and a ribonucleoside. Howard et al. (2002) have recently demonstrated significant inhibition of superoxide anion (O_2^-) produced by phorbol 12-myristate 13-acetate (PMA)-stimulated bovine macrophages previously infected with live pathogenic strains of *H. somnus*, but not following infection with *E. coli* or *H. influenzae*. The inhibition of O_2^- was time- and dose-dependent, and required contact with, but not phagocytosis of, live *H. somnus.*

It is interesting that *H. somnus* also has been shown to produce histamine, probably through decarboxylation of histidine (Ruby et al. 2002). Secretion was enhanced under conditions of high CO_2 concentrations, which may approximate those in the bronchi. As a result the production of histamine, which is a mediator of the inflammatory process, may contribute to the pathogenesis of respiratory disease.

IMMUNITY

Vaccines have been available to protect against TME for many years and are also used to prevent *H. somnus*-induced respiratory disease. However, the mechanisms of protection are not well understood. Several studies provide insight into possible mechanisms. Passive immunization with convalescent serum has demonstrated protection. This suggests that antibody is sufficient for immune defense and also provides more evidence that *H. somnus* is not a typical facultative intracellular parasite. Protective convalescent phase serum recognized predominantly the 78- and 40-kDa OMPs and the IgBPs described above. Subsequent studies showed that monospecific antibody to the 40-kDa OMP was 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. Many studies implied that IgG2 of appropriate specificity is most critical for protection. Passive protection with purified bovine IgG2 or IgG1 antibody specific for the 40-kDa OMP tended 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. somnus*. First, Bastida-Corcuera et al. (1999b) showed that $IgG2^b$, but not $IgG2^a$, binds to *H. somnus* IgBPs by the Fc region. Second, Bastida-Corcuera et al. (1999a) also showed that $IgG2^b$ had twice the activity in activation of the complement cascade compared to $IgG2^a$. Earlier it had been shown that complement was critical for defense against *H. somnus*. Thus, the inherited IgG2 allotype of an animal may contribute to susceptibility to *H. somnus* infection. Interestingly, $IgG2^b$ is expressed later in neonatal calves than $IgG2^a$ by several weeks (Corbeil et al. 1997), possibly explaining the greater susceptibility of some young calves to *H. somnus* pneumonia than others. 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. somnus* (Gogolewski et al. 1989). Ruby et al. (2000) reported the presence of *H. somnus*-specific IgE antibodies in the serum of calves vaccinated with *H. somnus* bacterins. They suggested these IgE antibodies may be related to more severe clinical disease rather than protection. There are probably several other factors that are important in immune defenses against *H. somnus*. The role of T-cell-mediated immunity is yet to be defined. However, bovine IgG2 is associated with a Th1-type response in cattle, so it may be that IgG2 antibody and T-cell-mediated immunity are both involved in protection.

The ability of *H. somnus* to evade these defense mechanisms also affects the outcome of the disease process. *H. somnus* accomplishes this in several ways. 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 IgBPs and expresses oligosaccharides identical to glycosphingolipids on various cell types. This may result in the organism being recognized as self, delaying the immune response. The IgBPs 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. somnus* disease. The balance between evasive mechanisms and host defenses determines whether or not infection with *H. somnus* progresses to a disease state.

CONCLUSION

It is now clear that virulent strains of *H. somnus* produce a multitude of virulence factors primarily aimed at colonization of their host and evasion of host defenses. Relatively avirulent strains that reside primarily in the male urogenital tract lack many of these factors, and therefore are restricted to colonization of a particular niche within the host. Virulent strains appear to cause disease primarily

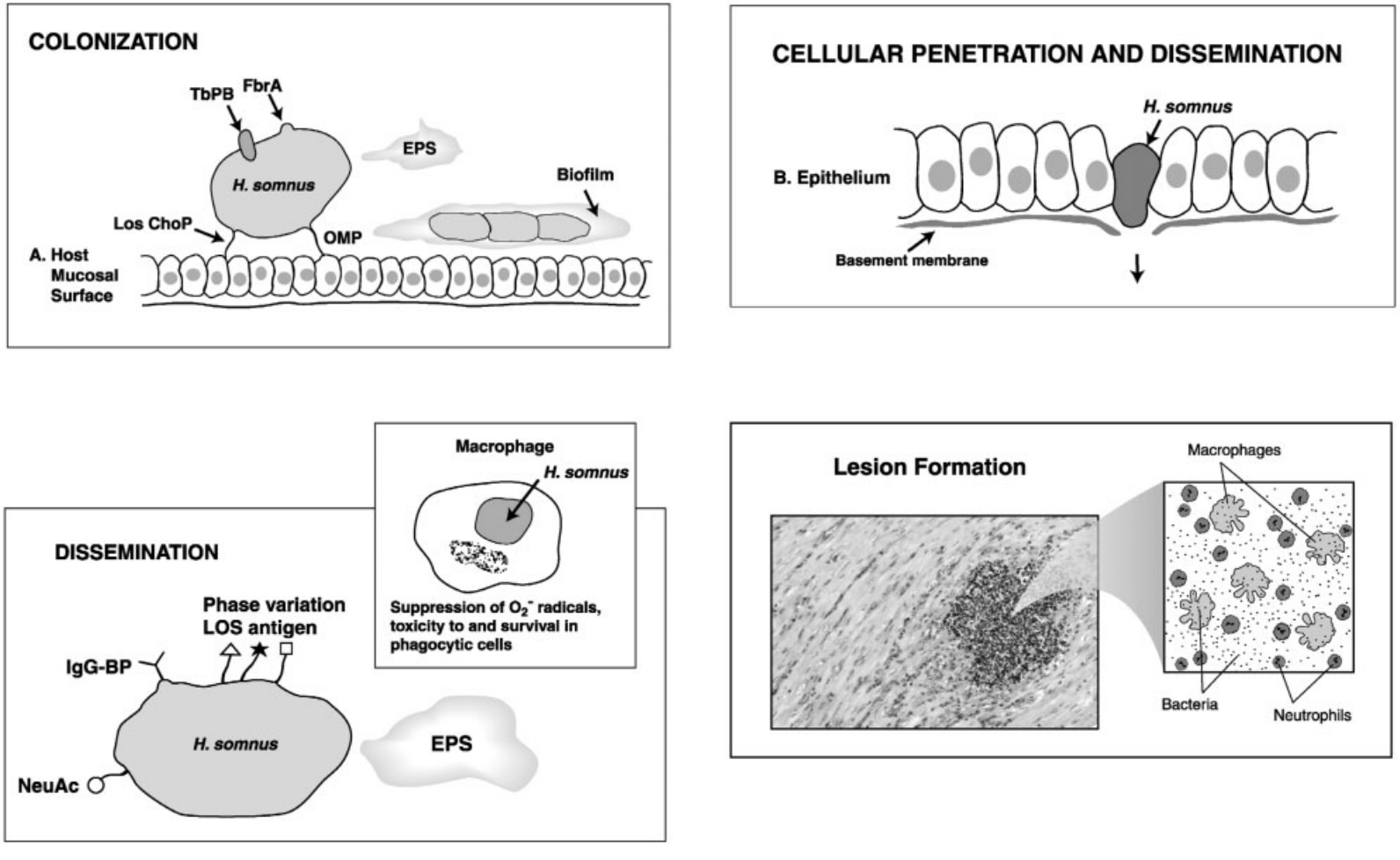


Figure 18.2. Illustration of the pathogenic process leading to disease by *H. somnus*: (*A*) colonization, (*B*) endothelial cell penetration and dissemination, (*C*) evasion of host defenses (through various cell surface features [*large box*] or through impairing macrophage superoxide killing processes [*inset box*]), and (*D*) lesion formation.

through colonization of multiple tissue sites (fig. 18.2A), the capacity to cause apoptosis, to penetrate the endothelial cell lining, and to disseminate (fig. 18.2B) and resist host defenses designed to eliminate the organism (fig. 18.2C). Disease likely occurs as a result of apoptosis and host inflammation, due in part to the presence of endotoxin and the cascading but compromised inflammatory cycle. This cascade results in the infiltration of polymorphonuclear leukocytes (neutrophils) and macrophages to sites of bacterial infection, resulting in lesion formation (fig. 18.2D).

HAEMOPHILUS PARASUIS

Overview

H. parasuis is an obligate upper respiratory tract inhabitant and opportunistic pathogen of swine, and the agent of swine polyserositis (Glässer's disease). Previous infection, particularly by swine influenza virus, predisposes the animal to fibrinous, lobular pneumonia and death due to *H. parasuis*. Glässer's disease (fibrinous inflammation of serous surfaces) may affect the pericardium, pleura, peritoneum, joints, and in severe cases the meninges, most often in young, weaned pigs. Clinical signs include swollen joints and lameness, fever, pleuritis, and possibly meningitis. Piglets that most often develop clinical symptoms are those that receive insufficient colostral antibodies or are severely stressed before natural immunity develops. Outbreaks may occur in pigs that are simultaneously stressed through severe environmental conditions, such as transit or cold weather shortly after weaning. The animal is not predisposed to Glässer's disease, unlike respiratory disease, by viral infection, but rather by stress, such as weaning and transport. Transport also provides ideal circumstances for transmission of infection, particularly if nonimmune animals are mixed with immune shedding carriers. The disease is becoming increasingly important in specific-pathogen-free herds, since infection can result in greater morbidity and mortality through the lack of any immunity to the organism (Miniats et al. 1991a; MacInnes and Desrosiers 1999; Smart 1999). Different isolates representing different serovars vary in virulence for pigs (Miniats et al. 1991a; Nielsen 1993). However, it is not clear whether this variation is strain specific or serovar specific.

Virulence Factors

There is little information regarding specific virulence factors of *H. parasuis*. Below is a brief discussion of those factors that have been investigated.

Capsule

There are at least 15 serovars of *H. parasuis* based on immunodiffusion testing with heat-stable antigens, although some isolates are untypeable (Kielstein and Rapp-Gabrielson 1992; Rapp-Gabrielson and Gabrielson 1992). Seven serovars account for almost 80% of *H. parasuis* isolates in North America, but more than 15% are untypeable. The identity of the serotype-specific antigen has not been confirmed, but is probably the capsular polysaccharide (CP). The presence of CP on *H. parasuis* has been confirmed by Morozumi and Nicolet (1986), but a CP has not been purified or chemically characterized. Like typeable *H. influenzae*, and unlike *H. somnus*, *H. parasuis* appears to form a typical CP. The role of the CP in virulence has not been specifically investigated, but probably acts to protect the bacterium from host defenses. The CPs are high molecular weight carbohydrate polymers, usually repeating polymers of two or three monosaccharide residues. Phosphate, carboxyl, or other acidic groups give the CPs a negative charge. The CP is shed by the bacteria 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. The CP forms a protective coat around the bacterium and allows it to evade host defenses. CPs usually do not activate the complement system or other host defenses, and in the absence of specific antibody enables the bacteria to evade killing by antibody and complement, and phagocytosis by phagocytic cells. Each serotype produces a chemically and antigenically distinct CP, and antibody to one CP serotype usually will not protect against another serotype (Robbins et al. 1980).

Lipooligosaccharide

Like *H. influenzae* and *H. somnus*, *H. parasuis* makes a lipooligosaccharide (LOS)-type endotoxin (Hitchcock et al. 1986). Analysis thus far has been based only on gel electrophoresis (Zucker et al. 1994; Ogikubo et al. 1999). In our analysis, three strains of *H. parasuis* did not undergo structural phase variation following *in vitro* passage, unlike *H. influenzae* and *H. somnus* (fig. 18.3). Furthermore, Zucker et al. (1994) reported that only 7 LOS electrophoretic types were identified from 231 isolates, although the stability of the LOS profile of each isolate was not examined. In addition, eight *H. parasuis* strains could be divided into four serogroups based on reactivity with polyclonal rabbit antisera,

and LOS-specific monoclonal antibodies reacted with all strains of the homologous serogroup but not to any strains of heterologous serogroups. Antigenic variation was not observed after up to ten passages on chocolate agar (Zucker et al. 1994). These results suggest that *H. parasuis* LOS does not undergo phase variation or does so only at a very low rate. In addition, monoclonal antibodies to ChoP failed to react with any of five *H. parasui*s strains by colony immunoblotting, indicating that *H. parasuis,* unlike *H. influenzae* and *H. somnus*, may not express ChoP on its LOS or elsewhere (unpublished data). The biological activity of *H. parasuis* LOS is comparable to *E. coli* LPS in regard to clotting of *Limulus* amoebocyte lysate, mitogenic activity of mouse spleen cells, and enhanced production of TNF-α and nitric oxide (Zucker et al. 1994; Ogikubo et al. 1999). However, unlike *A. pleuropneumoniae* but like *Pasteurella multocida, H. parasuis* produces sialidase, and growth is enhanced by the addition of sialic acid to the growth medium, indicating that sialic acid is metabolized (Lichtensteiger and Vimr 1997). The role of sialidase in virulence is not clear, however.

Protein Antigens

There is little information on proteins of importance in virulence of *H. parasuis*. MacInnes and Desrosiers (1999) reported that there are no known toxins. Schaller et al. (2000) showed that *H. parasuis* was devoid of Apx I, Apx II, and Apx III toxin genes. According to MacInnes and Desrosiers (1999), fimbriae and a neuraminidase have been described. Their relationship to virulence is not clear, but certain OMP profiles seem to be related to virulence (Ruiz et al. 2001). One OMP has been studied in more detail. The *tbpA* gene for a transferrin-binding protein (Tbp1) has been identified and shown to be species specific. Restriction fragment length polymorphism analysis revealed considerable heterogeneity within the ORF so that it is useful for typing and for epidemiologic tracing (De la Puente Redondo et al. 2003). In *A. pleuropneumoniae*, Tbp1 is an integral outer membrane protein that is involved in uptake of iron. Assuming that the protein has a similar location and function in the closely related *H. parasuis,* Tbp1 qualifies as a virulence factor in acquisition of iron from transferrin.

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) (Solano 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 others (Miniats et al. 1991b; Takahashi et al. 2001; Bak and Riising 2002).

HAEMOPHILUS PARAGALLINARUM

Overview

H. paragallinarum is the etiologic agent of fowl coryza, a disease of the upper respiratory tract of chickens worldwide; turkeys and pigeons are resistant to infection. Fowl coryza was first described in the late 1920s and shown to be associated with *H. paragallinarum*. Clinical signs include facial edema, lacrimation, anorexia, and rhinitis resulting from inflammation of the turbinates and sinus epithelium, and acute air sacculitis. Although *H. paragallinarum* can cause primary disease, the infection is usually more severe following viral or mycoplasma infection. The disease is economically important due to culling of severely affected birds and decreased egg production. In the nasal passages, there is marked loss of cilia and microvilli in the epithelial cells, infiltration of phagocytic cells, and accumulation of purulent mucus (Blackall 1999).

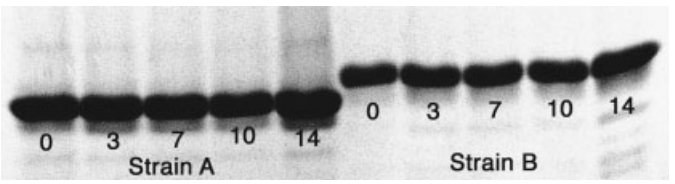


Figure 18.3. Polyacrylamide gel electrophoresis of LOS from two strains of *H. parasuis*. A single colony was subcultured to chocolate agar for 2 weeks. At days 0, 3, 7, 10, and 14, the LOS was isolated from a portion of the population by a micro phenol/water extraction procedure and analyzed. The electrophoretic profile of both isolates lacked heterogeneity, or variation in profile following passage.

VIRULENCE FACTORS

Little work has been done on the identification of specific virulence factors of *H. paragallinarum*, or on how these factors function to contribute to the pathogenesis of disease.

Capsule

The presence of capsule 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 capsule produced (Sawata and Kume 1983). Early reports suggested that the capsule may also be involved in adherence of the bacteria to the host respiratory tract since capsulated bacteria, but not nonencapsulated bacteria, were associated with upper respiratory tract lesions (Sawata et al. 1985).

Hemagglutinin

The hemagglutinins of *H. paragallinarum* that are used in serotyping are important for both pathogenicity and induction of protective immunity. Purified hemagglutinin has been shown to protect against infection with the homologous strain. A modified Kume serotyping scheme relies on hemagglutination inhibition 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). Several studies have shown that serovar-specific immunity is induced by bacterin vaccines. Different serovars are found in isolates from 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.

The hemagglutin of *H. paragallinarum* has been purified and shown to be a 39-kDa outer membrane protein. Recently, Hobb et al. (2002) cloned and sequenced the hemagglutin gene, *hag*, which was found to be similar to the P5 family of outer membrane proteins of other *Pasteurellaceae*. Variation in the amino acid sequence between strains of different serovars was small. This research team is currently investigating the use of a recombinant hemagglutinin vaccine.

NAD Dependence

Another factor that may be linked to virulence is dependence upon nicotinamide adenine dinucleotide (NAD) for growth. Most strains are NAD dependent, but NAD-independent strains have been detected. Serovar C-3 NAD-dependent isolates were shown to be 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 *H. paragallinarum* with plasmid DNA from a NAD-independent C-3 isolate. The resultant transformant was NAD independent and of reduced virulence for chickens. The mechanism for reduced virulence was not clear.

There is little information about the endotoxin of *H. paragallinarum*, but this component is probably responsible for the reported heat-stable toxic activity (Iritani et al. 1981). It has not been confirmed if the endotoxin is an LPS or an LOS. The outer membrane proteins of *H. paragallinarum* have been partially characterized by SDS-PAGE and were found to be similar among 15 isolates, although two 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 by immunoblotting (Blackall et al. 1990). The role of these proteins in virulence, however, has not been determined.

Like other *Haemophilus* spp., *H. paragallinarum* does not produce siderophores but does express four iron-regulated outer membrane proteins (53, 62, 66, and 94 kDa) in response to iron limitation. *H. paragallinarum* can only acquire iron from chicken or turkey transferrin, and not mammalian transferrin, indicating these proteins may be equivalent to transferrin-binding proteins (Ogunnariwo and Schryvers 1992).

CONCLUSIONS

There is enormous scope for investigation of the basis of virulence of *H. parasuis* and *H. paragallinarum*. For example, it is not clear how these opportunistic pathogens cause disease in their specific hosts even though both species are host specific and encapsulated, produce endotoxin and no known exotoxins, and are capable of causing serious disease. Therefore, it appears that these species, like *H. somnus*, are able to evade host defenses and cause disease associated with inflammation. Additional research, perhaps based on acquisition of the genome sequence of virulent isolates, is required to determine what factors contribute to resistance to host defenses, to colonization, and to host-cell damage.

REFERENCES

Angen, Ø., P. Ahrens, P. Kuhnert, H. Christensen, and R. Mutters. 2003. Proposal of *Histophilus somni*

gen. nov. sp. nov. for the three species incertae sedis "*Haemophilus somnus*", "*Haemophilus agni*" and "*Histophilus ovis*". Int. J. Syst. Evolut. Microbiol. (In press)

Bak, H., and H-J. Riising. 2002. Protection of vaccinated pigs against experimental infections with homologous and heterologous *Haemophilus parasuis*. Vet. Rec. 151:502–505.

Ball, H. J., S. Kennedy, and W. A. Ellis. 1991. Experimental reproduction of ovine vulvitis with bacteria of the haemophilus/histophilus group. Res. Vet. Sci. 50:81–85.

Bastida-Corcuera, F. D., J. E. Butler, S. Yahiro, and L. B. Corbeil. 1999a. Differential complement activation by bovine IgG2 allotypes. Vet. Immunol. Immunopathol. 71:115–123.

Bastida-Corcuera, F. D., K. H. Nielsen, and L. B. Corbeil. 1999b. Binding of bovine IgG2a and IgG2b allotypes to protein A, protein G, and *Haemophilus somnus* IgBPs. Vet. Immunol. Immunopathol. 71:143–149.

Blackall, P. J. 1999. Infectious coryza: overview of the disease and new diagnostic options. Clin. Microbiol. Rev. 12:627–632.

Blackall, P. J., D. G. Rogers, and R. Yamamoto. 1990. Outer-membrane proteins of *Haemophilus paragallinarum*. Avian Dis. 34:871–877.

Cassidy, J. P., S. W. McDowell, G. A. Reilly, W. J. McConnell, F. Forster, and D. Lawler. 1997. Thrombotic meningoencephalitis associated with *Histophilus ovis* infection in lambs in Europe. Vet. Rec. 140:193–195.

Chiang, Y.-W., M. L. Kaeberle, and J. A. Roth. 1986. Identification of suppressive components in "*Haemophilus somnus*" fractions which inhibit bovine polymorphonuclear leukocyte function. Infect. Immun. 52:792–797.

Cole, S. P., D. G. Guiney, and L. B. Corbeil 1992. Two linked genes for outer membrane proteins are absent in four non-disease strains of *Haemophilus somnus*. Mol. Microbiol. 6:1895–1902.

Corbeil, L. B., F. D. Bastida-Corcuera, and T. J. Beveridge. 1997. *Haemophilus somnus* immunoglobulin binding proteins and surface fibrils. Infect. Immun. 65:4250–4257.

Corbeil, L. B., R. P. Gogolewski, L. R. Stephens, and T. J. Inzana. 1995. *Haemophilus somnus*: antigen analysis and immune responses. In W. Donachie, F. A. Lainson, and J. C. Hodgson (eds.). Haemophilus, Actinobacillus, and Pasteurella. New York: Plenum Press, pp. 63–73.

Corbeil, L. B., W. Woodward, A. C. Ward, W. D. Mickelsen, and L. Paisley. 1985. Bacterial interactions in bovine respiratory and reproductive infections. J. Clin. Microbiol. 21:803–807.

Cox, A. D., M. D. Howard, J. R. Brisson, M. van der Zwan, P. Thibault, M. B. Perry, and T. J. Inzana. 1998. Structural analysis of the phase-variable lipooligosaccharide from *Haemophilus somnus* strain 738. Eur. J. Biochem. 253:507–516.

De la Puente Redondo, V. A., J. N. Mendez, N. García del Blanco, N. Ladrón Boronat, C. B. Gutiérrez Martín, and E. F. Rodríguez Ferri. 2003. Typing of *Haemophilus parasuis* strains by PCR-RFLP analysis of the *tbpA* gene. Vet. Microbiol. 92:253–262.

Duncan, A. J., C. D. Magee, and T. J. Inzana. 2002. Molecular characterization of a DNA locus involved in lipooligosaccharide biosynthesis in *Haemophilus somnus*. American Society for Microbiology 102nd General Meeting, Salt Lake City, Utah, ASM Press. Abst. D-104

Gogolewski, R. P., C. W. Leathers, H. D. Liggitt, and L. B. Corbeil. 1987. Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. Vet. Pathol. 24:250–256.

Gogolewski, R. P., D. C. Schaefer, S. K. Wasson, R. R. Corbeil, and L. B. Corbeil. 1989. Pulmonary persistence of *Haemophilus somnus* in the presence of specific antibody. J. Clin. Microbiol. 27:1767–1774.

Gogolewski, R. P., S. A. Kania, H. D. Liggitt, and L. B. Corbeil. 1988. Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of *Haemophilus somnus*. Infect. Immun. 56:2307–2316.

Gotschlich, E. C., B. A. Fraser, O. Nishimura, J. B. Robbins, and T. Y. Liu. 1981. Lipid on capsular polysaccharides of gram-negative bacteria. J. Biol. Chem. 256:8915–8921.

Gray-Owen, S. D., and A. B. Schryvers 1996. Bacterial transferrin and lactoferrin receptors. Trends Microbiol. 4:185–191.

Hitchcock, P. J., L. Leive, P. H. Makela, E. T. Rietschel, W. Strittmetter, and D. C. Morrison. 1986. Lipopolysaccharide nomenclature—past, present, and future. J. Bacteriol. 166:699–705.

Hobb, R. I., H-J. Tseng, J. E. Downes, T. D. Terry, P. J. Blackall, M. Takagi, and M. P. Jennings. 2002. Molecular analysis of a hemagglutinin of *Haemophilus paragallinarum*. Microbiol. 148:2171–2179.

Howard, M. D., A. D. Cox, J. N. Weiser, G. G. Schurig, and T. J. Inzana. 2000. Antigenic diversity of *Haemophilus somnus* lipooligosaccharide: phase-variable accessibility of the phosphorylcholine epitope. J. Clin. Microbiol. 38:4412–4419.

Howard, M. D., J. H. Boone, V. Buechner-Maxwell, G. G. Schurig, and T. J. Inzana. (2002). Inhibition

of bovine macrophage superoxide anion production requires cell contact by viable *Haemophilus somnus*. International Pasteurellaceae Society Conference 2002, Banff, Canada.

Inzana, T. J. 1999. The *Haemophilus somnus* complex. In Current veterinary therapy 4: food animal practice. J. L. Howard and R. A. Smith (eds). Philadelphia: W. B. Saunders Co., pp. 358–361.

Inzana, T. J., and L. B. Corbeil. 1987. Development of a defined medium for *Haemophilus somnus* from cattle. Am. J. Vet. Res. 48:366–369.

Inzana, T. J., B. Iritani, R. P. Gogolewski, S. A. Kania, and L. B. Corbeil. 1988. Purification and characterization of lipooligosaccharides from four strains of *Haemophilus somnus*. Infect. Immun. 56:2830–2837.

Inzana, T. J., G. Glindemann, A. D. Cox, W. Wakarchuk, and M. D. Howard. 2002. Incorporation of N-acetylneuraminic acid into *Haemophilus somnus* lipooligosaccharide (LOS): enhancement of resistance to serum and reduction of LOS antibody binding. Infect. Immun. 70:4870–4879.

Inzana, T. J., J. Hensley, J. McQuiston, A. J. Lesse, A. A. Campagnari, S. M. Boyle, and M. A. Apicella. 1997. Phase variation and conservation of lipooligosaccharide epitopes in *Haemophilus somnus*. Infect. Immun. 65:4675–4681.

Inzana, T. J., R. P. Gogolewski, and L. B. Corbeil. 1992. Phenotypic phase variation in *Haemophilus somnus* lipooligosaccharide during bovine pneumonia and after in vitro passage. Infect. Immun. 60:2943–2951.

Iritani, Y., S. Iwaki, and T. Yamaguchi. 1981. Biological activities of crude polysaccharide extracted from two different immunotype strains of *Hemophilus gallinarum* in chickens. Avian Dis. 25:29–37.

Jobert, J. L., C. Savoye, R. Cariolet, M. Kobisch, and F. Madec. 2000. Experimental aerosol transmission of *Actinobacillus pleuropneumoniae* to pigs. Can. J. Vet. Res. 64:21–26.

Kielstein, P., and V. J. Rapp-Gabrielson. 1992. Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. J. Clin. Microbiol. 30:862–865.

Kilian, M., and E. L. Biberstein. 1984. Genus II. Haemophilus Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 561. In Bergey's manual of systematic bacteriology, vol. 1. N. R. Krieg and J. G. Holt (eds.). Baltimore, MD: Williams & Wilkins, pp. 558–569.

Lees, V. W., W. D. Yates, and L. B. Corbeil. 1994. Ovine *Haemophilus somnus*: experimental intracisternal infection and antigenic comparison with bovine *Haemophilus somnus*. Can. J. Vet. Res. 58:202–210.

Lichtensteiger, C. A., and E. R. Vimr. 1997. Neuraminidase (sialidase) activity of *Haemophilus parasuis*. FEMS Microbiol. Lett. 152:269–274.

MacInnes, J. I., and R. Desrosiers. 1999. Agents of the "suis-ide diseases" of swine: *Actinobacillus suis*, *Haemophilus parasuis*, and *Streptococcus suis*. Can. J. Vet. Res. 63:83–89.

McDowell, S. W., J. P. Cassidy, and W. McConnell. 1994. A case of ovine abortion associated with *Histophilus ovis* infection. Vet. Rec. 134:504.

McQuiston, J. H., J. R. McQuiston, A. D. Cox, Y. Wu, S. M. Boyle, and T. J. Inzana. 2000. Characterization of a DNA region containing 5'-CAAT-3' DNA sequences involved in lipooligosaccharide biosynthesis in *Haemophilus somnus*. Microb. Pathogen. 28:301–312.

Miniats, O. P., N. L. Smart, and E. Ewart. 1991a. Vaccination of gnotobiotic primary specific pathogen-free pigs against *Haemophilus parasuis*. Can. J. Vet. Res. 55: 33–36.

Miniats, O. P., N. L. Smart, and S. Rosendal. 1991b. Cross protection among *Haemophilus parasuis* strains in immunized gnotobiotic pigs. Can. J. Vet. Res. 55:37–41.

Morozumi, T., and J. Nicolet. 1986. Morphological variations of *Haemophilus parasuis* strains. J. Clin. Microbiol. 23:138–142.

Nielsen, R. 1993. Pathogenicity and immunity studies of *Haemophilus parasuis* serotypes. Acta Vet. Scand. 34:193–198.

Ogikubo, Y., M. Norimatsu, A. Kojima, Y. Sasaki, and Y. Tamura. 1999. Biological activities of lipopolysaccharides extracted from porcine vaccine strains. J. Vet. Med. Sci. 61:1265–1269.

Ogunnariwo, J. A., and A. B. Schryvers. 1992. Correlation between the ability of *Haemophilus paragallinarum* to acquire ovotransferrin-bound iron and the expression of ovotransferrin-specific receptors. Avian Dis. 36:655–663.

Ogunnariwo, J. A., C. Cheng, J. Ford, and A. B. Schryvers. 1990. Response of *Haemophilus somnus* to iron limitation: expression and identification of a bovine-specific transferrin receptor. Microb. Pathog. 9:397–406.

Philbey, A. W., J. R. Glastonbury, J. T. Rothwell, I. J. Links, and J. E. Searson. 1991. Meningoencephalitis and other conditions associated with *Histophilus ovis* infection in sheep. Aust. Vet. J. 68:387–390.

Rapp-Gabrielson, V. J., and D. A. Gabrielson. 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am. J. Vet. Res. 53:659–664.

Retzer, M. D., R. Yu, Y. Zhang, G. C. Gonzalez, and A. B. Schryvers. 1998. Discrimination between apo and iron-loaded forms of transferrin by transferrin binding protein B and its N-terminal subfragment. Microb. Pathog. 25:175–180.

Robbins, J. B., R. Schneerson, W. B. Eagan, W. Vann, and D. T. Liu. 1980. Virulence properties of bacterial capsular polysaccharides—unanswered questions. In The molecular basis of microbial pathogenicity. M. J. Turner (ed.). Weinheim Verlag Chemie GmbH, pp. 115–132.

Ruby, K. W., R. W. Griffith, and M. L. Kaeberle. 2002. Histamine production by *Haemophilus somnus*. Comp. Immunol. Microbiol. Infect. Dis. 25:13–20.

Ruby, K. W., R. W. Griffith, L. J. Gershwin, and M. L. Kaeberle. 2000. *Haemophilus somnus*-induced IgE in calves vaccinated with commercial monovalent H. somnus bacterins. Vet. Microbiol. 76:373–383.

Ruiz, A., S. Oliveira, M. Torremorell, and C. Pijoan. 2001. Outer membrane proteins and DNA profiles in strains of *Haemophilus parasuis* recovered from systemic and respiratory site. J. Clin. Microbiol. 39:1757–1762.

Sanders, J. D., F. D. Bastida-Corcuera, K. F. Arnold, A. C. Wunderlich, and L. B. Corbeil. 2003. Genetic manipulation of immunoglobulin binding proteins of *Haemophilus somnus*. Microb. Pathog. 34:131–139.

Sawata, A., and K. Kume. 1983. Relationships between virulence and morphological or serological properties of variants dissociated from serotype 1 *Haemophilus paragallinarum* strains. J. Clin. Microbiol. 18:49–55.

Sawata, A., T. Nakai, K. Kume, H. Yoshikawa, and T. Yoshikawa. 1985. Intranasal inoculation of chickens with encapsulated or nonencapsulated variants of *Haemophilus paragallinarum*: electron microscopic evaluation of the nasal mucosa. Am. J. Vet. Res. 46:2346–2353.

———. 1985. Lesions induced in the respiratory tract of chickens by encapsulated or nonencapsulated variants of *Haemophilus paragallinarum*. Am. J. Vet. Res. 46:1185–1191.

Schaller, A., P. Kuhnert, V. A. de la Puente-Redondo, J. Nicolet, and J. Frey. 2000. Apx toxins in Pasteurellaceae species from animals. Vet. Microbiol. 74:365–376.

Schilling, B., S. Goon, N. M. Samuels, S. P. Gaucher, J. A. Leary, C. R. Bertozzi, and B. W. Gibson. 2001. Biosynthesis of sialylated lipooligosaccharides in *Haemophilus ducreyi* is dependent on exogenous sialic acid and not mannosamine. Incorporation studies using N-acylmannosamine analogues, N-glycolylneuraminic acid, and 13C-labeled N-acetylneuraminic acid. Biochem. 40:12666–12677.

Shao, F., P. M. Merritt, Z. Bao, R. W. Innes, and J. E. Dixon. 2002. A *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. Cell 109:575–588.

Smart, N. L. 1999. *Haemophilus parasuis* infection (Glässer's disease). In Current veterinary therapy 4: food animal practice. J. L. Howard and R. A. Smith (eds.). Philadelphia: W. B. Saunders Company, pp. 319–320.

Solano, G. I., E. Bautista, T. W. Molitor, J. Segales, and C. Pijoan. 1998. Effect of porcine reproductive and respiratory syndrome virus infection on the clearance of *Haemophilus parasuis* by porcine alveolar macrophages. Can. J. Vet. Res. 62:251–256.

Soriano, V. E., P. J. Blackall, S. M. Dabo, G. Tellez, G. A. Garcia-Delgado, and R. P. Fernandez. 2001. Serotyping of *Haemophilus paragallinarum* isolates from Mexico by the Kume hemagglutinin scheme. Avian Dis. 45:680–683.

Swords, W. E., B. A. Buscher, I. K. Ver Steeg, A. Preston, W. A. Nichols, J. N. Weiser, B. W. Gibson, and M. A. 2000. Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Mol. Microbiol. 37:13–27.

Sylte, M. J., C. J. Kuckleburg, L. B. Corbeil, T. J. Inzana, and C. J. Czuprynski. 2002. Stimulation of P2X7 enhances *Haemophilus somnus* LOS-mediated apoptosis of endothelial cells. Submitted.

Sylte, M. J., L. B. Corbeil, T. J. Inzana, and C. J. Czuprynski. 2001. *Haemophilus somnus* induces apoptosis in bovine endothelial cells in vitro. Infect. Immun. 69:1650–1660.

Tagawa, Y., F. Bastida-Corcuera, and L. B. Corbeil. 2000. Immunological characterization of the major outer membrane protein of *Haemophilus somnus*. Vet. Microbiol. 71:245–254.

Takahashi, K., S. Ngai, T. Yagihashi, T. Ikehata, Y. Nakano, K. Senna, T. Maruyama, and J. Murofushi. 2001. A cross-protection experiment in pigs vaccinated with *Haemophilus parasuis* serovars 2 and 5 bacterins, and evaluation of bivalent vaccine under laboratory and field conditions. J. Vet. Med. Sci. 63:487–491.

Taole, M., J. Albertyn, E. Van Heerden, R. R. Bragg. 2002. Virulence of South African isolates of *Haemophilus paragallinarum*. Part 3: experimentally produced NAD-independent isolate. Onderstepoort J. Vet. Res. 69:189–196.

Theisen, M., C. R. Rioux, and A. A. Potter. 1992. Molecular cloning, nucleotide sequence, and characterization of a 40,000-molecular-weight lipoprotein of *Haemophilus somnus*. Infect. Immun. 60:826–831.

———. 1993. Molecular cloning, nucleotide sequence, and characterization of lppB, encoding an antigenic 40-kilodalton lipoprotein of *Haemophilus somnus*. Infect. Immun. 61:1793–1798.

Vimr, E., C. Lichtensteiger, and S. Steenbergen. 2000. Sialic acid metabolism's dual function in *Haemophilus influenzae*. Mol. Microbiol. 36:1113–1123.

Ward, A. C., M. D. Jaworski, J. M. Eddow, and L. B. Corbeil. 1995. A comparative study of bovine and ovine *Haemophilus somnus* isolates. Can. J. Vet. Res. 59:173–178.

Ward, A. C., N. W. Dyer, and L. B. Corbeil. 1999. Characterization of putative *Haemophilus somnus* isolates from tonsils of American bison (Bison bison). Can. J. Vet. Res. 63:166–169.

Weiser, J. N., J. M. Love, and E. R. Moxon. 1989. The molecular mechanism of phase variation of *Haemophilus influenzae* lipopolysaccharide. Cell 59:657–665.

Weiser, J. N., N. Pan, K. L. McGowan, D. Musher, A. Martin, and J. Richards. 1998. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. J. Exp. Med. 187:631–640.

Won, J., and R. W. Griffith. 1993. Cloning and sequencing of the gene encoding a 31-kilodalton antigen of *Haemophilus somnus*. Infect. Immun. 61:2813–2821.

Wu, Y., J. H. McQuiston, A. Cox, T. D. Pack, and T. J. Inzana. 2000. Molecular cloning and mutagenesis of a DNA locus involved in lipooligosaccharide biosynthesis in *Haemophilus somnus*. Infect. Immun. 68:310–319.

Yang, Y. F., M. J. Sylte, and C. J. Czuprynski. 1998. Apoptosis: a possible tactic of *Haemophilus somnus* for evasion of killing by bovine neutrophils? Microb. Pathog. 24:351–359.

Yu, R. H., S. D. Gray-Owen, J. Ogunnariwo, and A. B. Schryvers. 1992. Interaction of ruminant transferrins with transferrin receptors in bovine isolates of *Pasteurella haemolytica* and *Haemophilus somnus*. Infect. Immun. 60:2992–2994.

Zucker, B., M. Kruger, E. Rehak, and F. Horsch. 1994. The lipopolysaccharide structure of *Haemophilus parasuis* strains in SDS-PAGE. Berl. Munch. Tierarztl. Wochenschr. 107:78–81.

19
Bordetella

D. A. Bemis and B. Fenwick

Members of the genus *Bordetella* are associated with contagious respiratory diseases. The type species *Bordetella pertussis* causes pertussis (whooping cough) in humans. Similar diseases of domestic animals are caused by *Bordetella bronchiseptica*, *Bordetella avium,* and *Bordetella parapertussis*. *B. bronchiseptica* is associated with diseases known as infectious tracheobronchitis (kennel cough) in dogs, and atrophic rhinitis in pigs and produces disease in several other mammalian species. *Bordetella avium* is primarily associated with respiratory disease in poultry and is especially known as the cause of coryza in turkeys. *Bordetella parapertussis* causes a nonprogressive form of pneumonia in lambs and a mild form of whooping cough in humans.

CHARACTERISTICS OF THE ORGANISM

The genus *Bordetella* is taxonomically classified in the family Alcaligenaceae, within the beta subdivision of Proteobacteria, along with the closely related genera *Achromobacter* and *Alcaligenes* (DeLey et al. 1986). There are currently eight species of *Bordetella*, with the remaining, more recently named species being *Bordetella hinzii*, *Bordetella holmesii*, *Bordetella trematum,* and *Bordetella petrii*. None of these species has been associated with disease in animals; however, *B. hinzii* is commonly isolated from the respiratory tract of poultry. *B. hinzii*, *B. holmesii,* and *B. trematum* are associated with opportunistic infections of various body sites in humans; *B. holmesii* has also been isolated from pertussis-like respiratory infections.

B. pertussis, *B. parapertussis,* and *B. bronchiseptica* represent a single genomospecies (von Wintzingerode et al. 2002). Differences in host specificity and virulence may have arisen through independent evolution of different lineages from a *B. bronchiseptica* progenitor (van der Zee et al. 1997). *B. avium* and *B. petrii* are genetically more divergent species and represent the closest link with the genus *Achromobacter* (von Wintzingerode et al. 2001).

Bordetella species are asaccharolytic, gram-negative rods; all but *B. petrii* are obligately aerobic. *Bordetella* species have relatively simple growth requirements and do not require complex blood-borne nutrients for growth. Only *B. pertussis* is incapable of growth on common peptone-containing media due to the presence of inhibitory substances such as short-chain fatty acids, colloidal sulfur, or secondary metabolites. Various combinations of fresh red blood cells, starch, charcoal, glycerol, ion exchange resins, and cyclodextrin have been incorporated into *B. pertussis* isolation media to absorb or inactivate inhibitory substances. While not necessary for growth of the other *Bordetella*, inclusion of these can be of value in the initial isolation from complex diagnostic samples.

Mature colonies of *Bordetella* appear on most agar media in 2 or 3 days. *B. bronchiseptica* and *B. avium* grow more rapidly and are more resistant to various physical and chemical conditions than *B. pertussis* and *B. parapertussis*. *B. bronchiseptica* and *B. avium* grow on enteric isolation media (e.g., MacConkey agar) and Cetrimide agar. Resistance of *B. bronchiseptica* to the nitrofuran class of antimicrobials has provided a basis for selective isolation of this species from infected animals. Members of the genus *Bordetella* metabolize amino and carboxylic acids (but not carbohydrates) as primary

carbon and energy sources. The alkaline reactions produced by such metabolism are often applied in differential media and biochemical tests for further characterization of *Bordetella* isolates. Clinical microbiologists rely heavily on recognition of the above traits and, in some cases, specific virulence factors to identify *Bordetella* species. Because of the complex phylogenetic relationships among *Bordetella*, *Achromobacter,* and *Alcaligenes*, accurate genus-level identification, based solely on phenotypic characteristics, is impossible (Yabuuchi et al. 1998).

Initial studies suggested that *Bordetella* had a clonal population structure (Musser et al. 1986; Register et al. 1997). However, using more sensitive methods it is now clear that there are significant genotypic differences between strains of the same species, and infections are not caused by a specific clone (Khattak et al. 1992; Keil and Fenwick 1998b).

SOURCES OF THE BACTERIUM

Bordetella (with the exception of *B. petrii*) are closely associated with warm-blooded animals. *B. pertussis*, *B. parapertussis*, *B. bronchiseptica,* and *B. avium* replicate in the respiratory tracts of their respective hosts. Preferred growth substrates such as proline and glutamic acid are abundant in respiratory secretions. These *Bordetella* species may also produce hydroxamate siderophores and binding proteins that aid in mobilizing iron from transferrin, lactoferrin, and heme (Giardina et al. 1995; Pradel et al. 2000). Host defenses of most animals eventually clear the organisms, but acutely infected animals (with or without symptoms) represent the most important sources of infection. *B. hinzii* is a commensal of the respiratory tract of poultry; *B. holmesii* and *B. trematum* may occupy similar niches in humans.

Contaminated fomites such as bedding, litter, or water may serve as indirect vehicles of transmission of animal-associated *Bordetella,* but their presence in these natural environments is apt to be short-lived and directly related to the intensity of infected animals in that particular environment (Stehmann et al. 1991). *Bordetella* species are readily killed by decreased oxidation/reduction potentials, UV irradiation, pH and temperature extremes, and many common chemical disinfectants. The comparatively slow growth rate of *Bordetella* species also makes them poor competitors for nutrients in most natural environments. The "classical" mammalian *Bordetella* species possess a two-component, environmentally induced signal transduction system (BvgAS) that globally regulates the expression of many virulence factors and housekeeping genes (Ezzell et al. 1981; Taylor and Zhulin 1999). The BvgAS system may represent a vestige of evolution from a biphasic environmental and pathogenic existence (von Wintzingerode et al. 2002; Schneider et al. 2002).

It is clear that depending on the strain examined, growth conditions have a marked influence on the type and amount of outer membrane proteins expressed by *B. bronchiseptica* (Keil and Fenwick 1999). *B. bronchiseptica* has been grown in natural fresh and salt water (Porter et al. 1991) and has been isolated from marine mammals (Register et al. 2000; Foley et al. 2002), but isolation of mammalian *Bordetella* species from natural water sources has not been reported. Recently, *B. petrii* and several unnamed *Bordetella*-like organisms were isolated from environmental sources (von Wintzingerode et al. 2001). *B. petrii* is a facultative anaerobe that bears closest resemblance to *B. avium*.

BACTERIAL VIRULENCE FACTORS

Because of their close genetic relatedness, the classical mammalian *Bordetella* species share a number of expressed and silent virulence-factor genes. Less is known about virulence factors of *B. avium* and the opportunistic *Bordetella* pathogens. Virulence factors that promote attachment or colonization in the respiratory tract include fimbriae (FIM), filamentous hemagglutinin (FHA), a family of autotransporter proteins exemplified by pertactin (PTN), and type III secretion system associated proteins. Virulence factors that alter host functions and enable the bacterium to escape destruction in the host include dermonecrotic toxin (DNT), osteotoxin, adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), pertussis toxin (PTX), type III secretion products, lipopolysaccharide (LPS), and factors that promote intracellular survival.

FILAMENTOUS HEMAGGLUTININ

Filamentous hemagglutinin (FHA) is a high molecular weight (220 kDa), secreted outer membrane protein that plays a major role in attachment of *B. pertussis*, *B. parapertussis,* and *B. bronchiseptica*. In a rat model, FHA is required but not sufficient for tracheal colonization by *B. bronchiseptica* (Cotter et al. 1998). Hemagglutinating activity is associated

with virulence in *B. avium*, and recently a gene orthologous to *fhaB* has been detected in this species (Spears et al. 2003). The N-terminal portion (220 kDa) of FHA is proteolytically cleaved from a 370 kDa precursor protein encoded by the *fha*B gene and secreted in a signal peptide-dependent fashion across the cytoplasmic membrane. Secretion requires an accessory protein produced by another gene in the FHA operon, *fha*C. Three functional domains on the mature FHA molecule are involved in binding to eukaryotic cells. The carbohydrate-binding domain mediates attachment to ciliated cells; the *arg-gly-asp* (RGD) tripeptide sequence promotes phagocytosis by cross-linking a leukocyte response integrin with an integrin-associated protein, which leads to an up-regulation of C3-mediated binding of bacteria to cells; and the heparin-binding domain may help in low affinity binding to cell-associated extracellular matrix components (Hannah et al. 1994; Ishibashi et al. 1994; Menozzi et al. 1994). FHA is fully secreted in *B. pertussis* but less-complete FHA is produced by *B. bronchiseptica* strains (Jacob-Dubuisson et al. 2000).

Fimbriae

Fimbriae (FIM) are heteropolymeric filamentous appendages that are involved in attachment and colonization by many gram-negative bacteria. The main structural component of fimbriae consists of a single, repeating, low-MW (18–22 kDa) subunit protein; genes that encode the major fimbrial subunit proteins of *Bordetella* include *fim*2, *fim*3, *fim*X, *fim*A, and *fim*N. FIM2 and FIM3 were initially defined with *B. pertussis* serotype-specific antisera; however, serological cross-reactions occur among *Bordetella* fimbrial proteins. Fimbriae are implicated in attachment of *B. avium,* but the above fimbriae have not been detected in *B. avium* by serological or genetic means (Jackwood and Saif 1987).

Fimbrial biogenesis is a complex process involving a cluster of genes called *fim*ABCD (Willems et al. 1992)). These are located in the FHA operon immediately downstream to *fha*B, the structural gene for FHA. *Fim*B and *fim*C are involved in fimbrial protein transport and assembly. A mutation in *fim*B abolishes the expression of all fimbriae. A gene with similarity to *fimC* is found in *B. avium* (Spears et al. 2003). *Fim*A encodes a major structural subunit that is nonfunctional in *B. pertussis* but is expressed in *B. bronchiseptica* and probably *B. parapertussis* (Boschwitz et al. 1997). *Fim*D is a putative adhesin and tip-binding protein; however, mutation of *fim*D blocks the expression of major fimbrial subunits suggesting that *fim*D may also have chaperone-like activity (Geuijen et al. 1997). Major fimbrial subunit genes other than *fim*A are located at various locations elsewhere in the chromosome. Expression of major fimbrial subunit proteins may also be regulated, "on or off," by base additions or deletions within a cytosine-rich sequence in *Bordetella* fimbrial promoters. This phenomenon, termed "fimbrial type-switching," may enhance adaptation to selective environmental pressures. Fimbrial receptors are composed of sulfated sugars and are ubiquitous in the respiratory tract (Geuijen et al. 1996). The role of specific adhesin receptors in host-species specificity is not known, but early studies demonstrated that lectin and saccharide inhibition of bacterial attachment varied between host species.

Pertactin

Pertactin (PTN) is a nonfimbrial, outer membrane protein adhesin that belongs to a family of autotransporter proteins. Such proteins effect their own proteolytic cleavage, membrane transport, and secretion from a single precursor protein. Mature pertactin contains a single RGD sequence, proline-rich repeat sequences, and leucine-rich repeat sequences that are involved in eukaryotic cell binding (Leininger et al. 1991; Emsley et al. 1996). Pertactin is a 68-kD protein in *B. bronchiseptica*, a 69-kD protein in *B. pertussis,* and a 70-kDa protein in *B. parapertussis*. Genetic and antigenic polymorphisms, attributable to repeat regions, may occur frequently (Mooi et al. 1998; Boursaux-Eude and Guiso 2000; Register 2001).

Other *Bordetella* proteins that share C-terminal amino acid sequence homology with pertactin and contain RGD motifs include tracheal colonization factor (TCF) (Finn and Stevens 1995), BrkA (Rambow et al. 1998), and Vag8 (Finn and Amsbaugh 1998). TCF and Vag8 have only been found in *B. pertussis*; TCF functions as the name implies, but the function of Vag8 is unknown. BrkA confers resistance to serum complement killing in *B. pertussis,* but it does not appear to have this effect in strains of *B. bronchiseptica* that also have this protein.

Pertussis Toxin

Pertussis toxin (PTX) is of major importance in whooping cough. It is a 105 kDa bacterial protein toxin that has ADP-ribosylating activity and a typical A-B bacterial toxin subunit structure composed

of six polypeptides in a ratio of 1:1:1:2:1. The pertussis toxin operon includes the *ptx* structural gene and nine additional *ptl* genes that encode proteins needed for secretion of the toxin across the outer membrane. Entry of PTX into cells causes inactivation of guanine nucleotide-binding proteins and disrupts a number of different cellular activities (Cotter and Miller 2001). Although PTX is secreted only in *B. pertussis*, promoterless versions of the complete structural gene sequence are found in some strains of *B. bronchiseptica* and *B. parapertussis* (Arico and Rappuoli 1987). Return of these sequences to *B. pertussis* and introduction of the full pertussis operon into *B. bronchiseptica* and *B. parapertussis* results in the expression of fully active toxin (Lee et al. 1989; Hausman et al. 1996).

Adenylate Cyclase Toxin

Adenylate cyclase toxin (ACT) is a member of the RTX (repeat in toxin) family of bacterial toxins that contain glycine-aspartic acid-rich repeat sequences. ACT has separable, bifunctional activities as a hemolysin and as a calmodulin-dependent adenylate cyclase toxin (Raptis et al. 1989). ACT is secreted as a 216-kDa protein that may be cleaved into smaller catalytically active fragments. ACT is encoded by a five-gene operon: *cya*A is the structural gene, *cya*C is required for posttranslational acylation that activates the toxin, and *cya*B, *cya*D, and *cya*E are required for secretion (Glaser et al. 1988). Entry into cells via ion-permeable pores is followed by calmodulin activation and accumulation of cyclic AMP. This activity greatly inhibits phagocytic cell functions (Gueirard and Guiso 1993; Harvill et al. 1999b). The carboxy terminal portion of ACT has homology with the alpha hemolysin of *Escherichia coli* and the leukotoxin of *Mannheimia haemolytica*.

Dermonecrotic Toxin

Dermonecrotic toxin (DNT) is a single, 162-kDa intracellular polypeptide produced by *Bordetella* species (Pullinger et al. 1996). It is thermolabile and subject to proteolytic degradation. When injected intradermally into animals, DNT produces nonulcerating, necrotic lesions. High doses of DNT given systemically are lethal for mice and sublethal doses produce marked splenic atrophy as the result of vasoconstrictive effects (Iida and Okonogi 1971). In experimental cellular systems, DNT produces a variety of cytotoxic and mitogenic effects (Walker and Weiss 1994; Lacerda et al. 1997). The mode of action involves deamidation of a glycine residue at amino acid position 63 in small G proteins like Rho, Rac, and Cdc42 leading to their constitutive activation and polymerization of actin (Horiguchi et al. 1997). DNT has structural and functional homology with cytotoxic necrotizing factor I (CNF I) of *E. coli* and the deamidation activities of both toxins are mapped to their C-terminus (Kashimoto et al. 1999; Lerm et al. 1999). DNT can impair osteoblast differentiation and is important in atrophic rhinitis (Brockmeier et al. 2002). A genetically and biologically different DNT has been isolated from *B. avium*.

Another toxin that inhibits osteoblasts but lacks dermonecrotic activity has been isolated from *B. avium* (Gentry-Weeks et al. 1988). It is an 80-kDa homodimeric beta-cystathionase with one molecule of pyridoxal phosphate in each subunit and has been called osteotoxin (Gentry-Weeks et al. 1995).

Tracheal Cytotoxin

Tracheal cytotoxin (TCT) is a muramyl dipeptide (N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanyl-γ-glutamyldiaminopimelyl-alanine) produced by *B. avium* and each of the mammalian respiratory *Bordetella* (Gentry-Weeks et al. 1988; Luker et al. 1995). It is a normal degradative product of bacterial cell-wall synthesis but is unique in *Bordetella* species because it is secreted from the cell. Damage to ciliated tracheal epithelial cells is due to TCT stimulation of IL-1 production in mucus-secreting cells that results in the formation of large amounts of nitric oxide in respiratory tract secretions (Flak and Goldman 1996).

Type III Secretion Products

B. bronchiseptica and *B. parapertussis* possess a functional type III, contact-dependent, secretion system (van den Akker 1997; Yuk et al. 1998). The products introduced into cells by this system have not been identified, but a functional type III secretion system is required for cytotoxicity, apoptosis, and inactivation of the transcription factor, NF-κB, by *B. bronchiseptica* (Yuk et al. 2000). It is proposed that these activities may have a modulating effect on the host immune response.

Lipopolysaccharide

The cell envelopes of *Bordetella* species contain lipopolysaccharides (LPS) with biological activities similar to those attributed to endotoxins of other bacteria. The LPS of *B. pertussis* and ovine strains of *B. parapertussis* lack a terminal O antigen, while *B. bronchiseptica* and human *B. parapertussis*

strains have an O antigen composed of the single sugar, 2,3-dideoxy-di-N-acetyl-galactoseaminuronic acid (Di Fabio et al. 1992). There are also structural variations within the core polysaccharides of these species. A genetic locus, *wlb*, containing 12 genes, is required for LPS biosynthesis (Allen et al. 1998). Mutants with defects within this locus did not colonize the respiratory tracts of mice as well as parent strains (Harvill et al. 2000; Spears et al. 2000; Preston et al. 2003).

Cellular Invasion and Intracellular Survival

Bordetella are primarily extracellular pathogens; however, various degrees of invasion and intracellular survival have been demonstrated *in vitro* (Guzman et al. 1994; Baneman and Gross 1997; Forde et al. 1998; Bassinet et al. 2000; Brockmeier and Register 2000). FHA, but not FIM2, FIM3, or pertactin, promoted invasion of epithelial cells. Increased survival time of *B. bronchiseptica* over that of *B. pertussis* in macrophage was attributed to *B. bronchiseptica*'s ability to tolerate the acidic environment within phagolysosomes. However, in epithelial cells it was demonstrated that the presence of urea and urease activity (an activity most likely to increase pH) increased survival of some *B. bronchiseptica* strains. An aromatic amino acid auxotrophic mutant of *B. bronchiseptica* has reduced ability to invade and survive in a mouse macrophage-like cell line and in the respiratory tract of mice (McArthur et al. 2003). The survival periods (< 4 days), in all cases, have been relatively short compared to well-known facultative intracellular bacteria such as *Mycobacterium* and *Brucella*. There is no evidence that *Bordetella* replicate within cells or spread from cell to cell. It is postulated that invasion and intracellular survival by *Bordetella* may contribute to the persistence and chronicity of infections.

Genetic Regulation of Virulence Factors

B. pertussis, *B. parapertussis,* and *B. bronchiseptica* possess a sensory transduction system that simultaneously activates one set of genes and represses another set. The transcription of many *Bordetella* virulence factors and BvgAS, itself, are positively regulated, *en masse,* by this system (Roy et al.1990; Tejada et al. 1996). The *bvg*AS locus encodes a membrane-spanning, sensor protein and a *trans*-acting transcriptional regulator. Environmental signals, such as mammalian body temperature, physiological levels of sodium or potassium cations, and low sulfate anion or nicotinic acid concentrations (positive signals), trigger a complex histidine kinase phosphorelay system that begins with the autophosphorylation of BvgS and ends with activation of the virulence gene transcriptional regulator, BvgA. A number of uncharacterized BvgA regulated proteins have been called virulence-activated gene-proteins (Vag) (Finn and Amsbaugh 1998). As virulence genes and *vag* are expressed under the positive influence of BvgA, other genes (virulence repressed genes—*vrg*) are simultaneously repressed. Most Vrg proteins are not well characterized (Stenson and Peppler 1995; Schneider et al. 2002). The best example of a *bvg*-repressed protein is flagellin of *B. bronchiseptica*. In some strains, urease and the siderophore alcaligin are also *bvg* repressed (Giardina et al. 1995; McMillan et al. 1996). When environmental signals are reversed, *Vrg* are expressed and *Vag* are not expressed.

In addition to the reversible effects of environmental signals (a phenotypic property referred to as modulation), the *bvg*AS locus is also subject to frame shift mutations due to a stretch of cytosine sequences within its promoter region.

Another locus, *bvg*R, is involved in the *bvg*AS repression of some genes in *B. pertussis* (Merkel et al. 1998). Proteins and virulence traits have recently been reported that require the *bvg*AS regulon but are not expressed under Bgv+ (virulent phase) or Bvg- (avirulent phase) environmental conditions (Cotter and Miller 1997; Register and Ackermann 1997). The state required for expression of the new phenotypes has been called Bvg intermediate phase, Bvg_i. A second two-component sensory transduction system, called *ris*, has also been described in *B. bronchiseptica* (Jungnitz et al. 1998).

The need for such sophisticated virulence-associated regulatory systems in *Bordetella* is not apparent. These systems may be vestiges of evolution from organisms with environmental habitats or perhaps may play a role in interhost survival. A gene homolog of *bvg*S has been detected in *B. avium*.

PATHOGENESIS

Overview—General Disease Patterns

B. pertussis, B. parapertussis, B. bronchiseptica, and *B. avium* are associated with acute, highly contagious respiratory diseases in their natural hosts and have worldwide distribution. Disease is seen most frequently in young animals, but animals of all ages may become infected. The most common

symptom is coughing, but sneezing, dyspnea, oculonasal discharge, torticollis, decreased weight gain, and tracheal collapse may occur. For all but the paroxysmal stage of coughing in human pertussis, bacteria are usually present in large numbers when disease occurs. *Bordetella* infections have high morbidity and low mortality. Most infections are subclinical or only mildly symptomatic. More severe clinical disease is frequently precipitated by coinfection with other pathogens.

Diseases involving sites other than the respiratory tract are rare and occur most commonly in compromised hosts. Zoonotic disease appears to be rare and has only been suggested for *B. bronchiseptica* (Woolfrey and Moody 1991). Reverse zoonosis has not been reported; *B. pertussis* and *B. parapertussis* are restricted to single hosts. Human and ovine strains of *B. parapertussis* are distinct and do not cross hosts. Human *B. bronchiseptica* infections have mostly been of the nonrespiratory, opportunistic type (wound infections, bacteremia, peritonitis). *B. bronchiseptica* has the widest host distribution among *Bordetella*. Dogs, pigs, and guinea pigs are most frequently infected with *B. bronchiseptica,* but infections in cats, horses, and rabbits are also common. Less-frequent isolations are made from rats, wildlife species (opossums, raccoons, ferrets, koalas, lesser bushbabies, hedgehogs, bears, foxes, leopards, seals, sea otters, Dall sheep), and chickens. *B. bronchiseptica* does not appear to be pathogenic in chickens, but isolation from diseased ostrich chicks has been reported (Clubb et al. 1994). Except for *B. parapertussis* in lambs and rare atypical infections (e.g., in exotic species), *Bordetella* species are not commonly associated with domestic ruminants. *B. avium* infects several species of fowl; psittacines, ratites, finches, and domestic songbirds are reported to be susceptible to *B. avium* (Gerlach 1994; Raffel et al. 2002). *B. avium* has been associated with lockjaw syndrome in cockatiels, a respiratory disease with a hallmark symptom of temporomandibular rigidity (Clubb et al. 1994).

Bacterium-Host Interactions

Animals encounter *Bordetella* through direct or indirect contact with other animals. Colonization is initiated in the upper airways of the respiratory tract, and conditions that produce droplet aerosols or airborne dust are likely to facilitate animal-to-animal spread. Once colonization is established, *Bordetella* species may spread to the lower respiratory tract. It is difficult to measure in natural disease, but, conceptually, a minimum infectious dose is required to establish *Bordetella* infection, and this will vary depending on the site of bacterial deposition, natural and acquired host resistance, environmental factors, and virulence of the agent. Observations from controlled experimental studies in laboratory rodents and gnotobiotic piglets suggest that, as a function of infectious dose, *Bordetella,* when introduced into the nasal cavity, may (1) fail to become established, (2) become established with growth limited to the nasal cavity, or (3) become established in the nasal cavity and spread to the trachea and lower respiratory tract (Martineau et al. 1982; Cotter et al. 1998; Harvill et al. 1999a).

Many properties and potential virulence factors of the classical mammalian *Bordetella* species have been characterized by biochemical, genetic, and molecular techniques. However, there is little information on how they function in the pathogenesis of natural infections. The most consistent feature in the pathogenesis of *Bordetella*-induced respiratory disease is marked colonization of ciliated airway epithelium (Bemis and Kennedy 1981; Temple et al. 1998). Bacteria attached to ciliated epithelial cells are prominently seen in specimens from heavily infected tissues (fig. 19.1). Attachment is mediated by FHA, fimbriae, and perhaps other adhesins (Savelkoul et al. 1996; Keil et al. 2000). It is not known if other factors such as enzyme hydrolysis or motility are involved in reaching or preparing the attachment site. Once anchored, factors like LPS, TCF, BrkA, PTX, ACT, and urease enable *Bordetella* to resist killing by mucosal humoral factors, avoid phagocytic uptake, invade cells, and survive intracellularly. Iron acquisition plays an important role in bacterial replication in the respiratory tract. As bacteria replicate, infection can spread by direct extension to adjacent cells and tissue. Flagella may also play a role in bacterial spread within the respiratory tract (Temple et al. 1998). Colonization of the nasal cavity may not require the same complement of adhesins that is required for colonization of the lower respiratory tract (Cotter and Miller 2001). *Bordetella* infections are rarely found outside the respiratory tract. In most animals, colonization does not result in clinical disease.

Mucociliary clearance is impaired in heavily colonized animals. Ciliated cells may have reduced motility or be prematurely expelled from the epithelium as the result of nitric oxides produced by exposure to TCT (Bemis and Kennedy 1981; Flak and Goldman 1996). The products of type III secretion systems, DNT, and osteotoxin may also directly

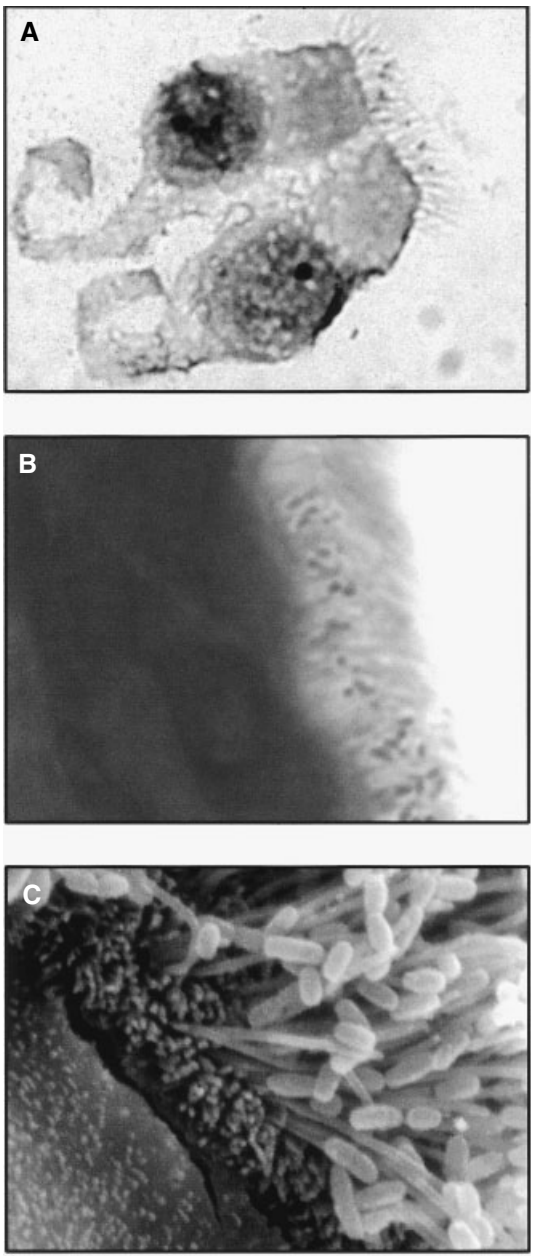


Figure 19.1. *Bordetella bronchiseptica* infection of canine trachea. (*A*) Gram stain of tracheal wash; (*B*) toluidine blue stain of 1 μm sections of glutaraldehyde fixed Epon-Araldite embedded tissue; (*C*) scanning electron micrograph of explant tissue.

damage epithelial cells or underlying submucosal structures such as the nasal turbinate bones. Cytokines produced in response to TCT, LPS, and damaged tissue attract phagocytes that may cause additional damage to infected tissue. PTX and ACT greatly reduce the effectiveness of phagocytic killing and inhibit microbial clearance. The result is an overall reduction in upper respiratory defensive functions, which increase the risk of deep lung infection by other bacteria, the development of bacterial pneumonia with an associated increase in the severity of clinical disease, and a less-optimistic prognosis.

Influence of Environmental Factors

Temperature, moisture, ammonia levels, dust, ventilation, and animal density are management considerations that, if neglected, can promote droplet aerosols, produce nonspecific airway inflammation, and cause various physiological stresses in animals, which lower the infectious dose needed to initiate infection, increase spread of infection within a population, and increase the severity of disease.

Bordetella infection is not dependent on synergistic activities with other organisms. The presence of other pathogens, however, can have an additive effect on the severity of disease. Canine distemper virus, canine parainfluenza virus, and canine adenovirus type II have such effects in dogs (Keil and Fenwick 1998a). Control of these agents by vaccination greatly reduces the risk and severity of disease following *B. bronchiseptica* infection. Colonization of the nasal cavities of pigs with toxigenic strains of *Pasteurella multocida* results in progressive, end-stage atrophic rhinitis (Chanter et al. 1989). *P. multocida* colonization is commonly aided by antecedent *B. bronchiseptica* infection. Multiple external infection pressures are likely to occur in *Bordetella*-associated diseases.

Lesions

Gross morphologic lesions are seldom seen in animals that subsequently recover from *Bordetella* infections. In severe cases, mucopurulent discharge may be seen in affected airways, and in young animals, tracheal collapse may occur (Ford 1995). Bronchopneumonia can sometimes be detected in living animals by radiographic examination. At necropsy, pneumonic lesions appear as grey to red consolidated areas, usually bilaterally distributed, in the anterioventral regions of the lungs. In pigs with atrophic rhinitis, snout distortion, snout shortening, and bleeding from the nasal cavity may be seen when toxigenic *P. multocida* is present (Chanter et al. 1989). Gross morphologic nasal lesions are not usually noticed in live pigs infected with *B. bronchiseptica* alone. However, in pigs, rabbits, and

mice killed at the peak of experimental *B. bronchiseptica* infection, atrophy of the ventral nasal turbinate bones can be seen.

Microscopic changes in the airway epithelium vary from abundant bacteria among the cilia of morphologically normal-appearing epithelial cells, to an epithelium infiltrated with neutrophils, with or without patchy areas of cuboidal epithelial cells devoid of cilia (fig. 19.2), to an epithelium with complete squamous metaplasia and neutrophilic exudates in the airways. Late in infection, the epithelium contains an increased number of mucus-secreting cells.

Submucosal lesions may exist as diffuse or focal areas of suppurative, neutrophilic and mononuclear cell infiltrate with occasional vascular hyperplasia. Late in infection, mucosa-associated lymphoid tissue in the lamina propria often exhibits some degree of hyperplasia. Lesions of nasal turbinate bones consist of focal areas of cartilaginous metaplasia, atrophy, and a lack of osteoblasts.

IMMUNITY

There is good evidence that immunocompetent animals mount an effective immune response to *Bordetella*. Clinical symptoms, if present, are usually self-limiting and resolve in a few days. Most infected animals become culture-negative for *Bordetella* in the trachea and lungs within several weeks and, if challenged, are refractory to reinfection of those sites (with the same strain) for a period of at least several months. The mechanisms of adaptive immunity and identity of protective antigens are less well known.

It is presumed that local antibodies play an important role in protection against reinfection of the lower airways, but recent evidence suggests that serum antibodies may play a role as well. Antibodies against *Bordetella* have been detected in mucosal secretions as early as 4 days following exposure (Bey et al. 1981; Arp and Helwig 1988). Serum antibodies are also produced following infection. Anti-*Bordetella* antibody blocks but does not reverse attachment to ciliated cells and can neutralize the effects of *Bordetella* toxins *in vitro*. Mice that are T- and B-cell deficient develop lethal systemic infections when exposed experimentally to *B. bronchiseptica*; passively transferred *B. bronchiseptica* convalescent serum (but not *B. pertussis* convalescent serum) protected such mice against lethal infection and hastened the clearance of *B. bronchiseptica* from the lungs of wild-type mice following challenge (Harvill et al. 1999a; Kirimanjeswara et al. 2003). The mechanisms of antibody-mediated clearance are not known. Type 1 T-helper cells are selectively activated in humans following *B. pertussis* infection (Ryan et al. 1997), and effective

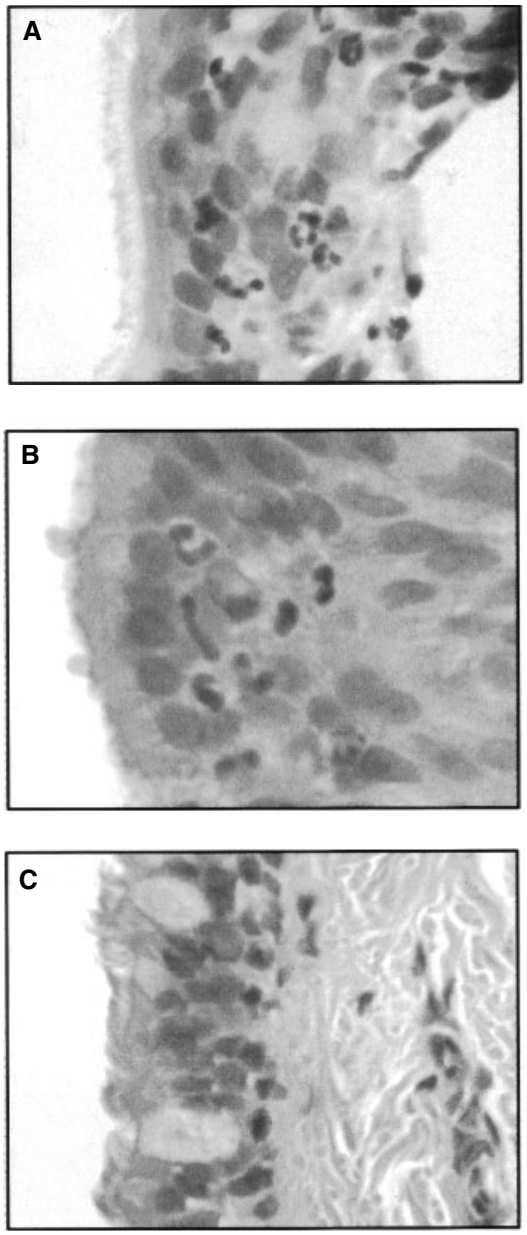


Figure 19.2. Hematoxylin and eosin stained tracheal tissue from *Bordetella bronchiseptica* infected dogs. (*A*) Acute infection with infiltration of epithelium by PMNs and intact cilia; (*B*) acute infection with infiltration of epithelium by PMNs, cilia loss, and epithelial cell extrusion; (*C*) recovery phase with normal cilia and increased mucus-secreting cells.

immunization of mice in a *B. pertussis* respiratory disease model is dependent on the induction of cell-mediated immunity (Mills et al. 1993). Little is known about the mechanisms of cellular immunity that may relate to protection against *Bordetella* infection.

Vaccination provides a degree of protection against disease but may not prevent colonization, especially in the nasal cavity. Mass vaccination with commercial vaccines has been applied against *B. pertussis* in humans, against *B. avium* in turkeys, and against *B. bronchiseptica* in pigs, dogs, cats, and, to some extent, guinea pigs. Types of vaccines used include killed whole-cell bacterins, live attenuated or avirulent cultures, acellular extracts, and purified component vaccines. Live culture vaccines have not been approved for use in humans and purified component vaccines have been used only in humans. Nonviable vaccines are generally accompanied by some form of adjuvant and are administered by intramuscular or subcutaneous injection, while live vaccines are administered by the intranasal route in mammals and in water, or by the intraocular route in poultry. Protection against challenge has been observed as early as 3 days after vaccination with a live intranasal vaccine (Williams et al. 2002). Injectable vaccines may produce higher levels of serum antibodies than intranasal vaccines, especially when used for annual revaccination in seropositive animals (Ellis et al. 2002). In a canine challenge model of *B. bronchiseptica* infection, sequential intranasal and intramuscular vaccination afforded the greatest protection, suggesting that there may be beneficial and separable components of protection produced by both local and systemic immune responses (Ellis et al. 2001).

The principal protective antigens of *Bordetella* (FHA, PTX, ACT, PTN, and FIM) have been used in purified component vaccines against *B. pertussis*. It has been accepted that such vaccines must contain FHA and PTX, at a minimum, for effective protection in humans; however, the presence of additional components results in greater protection. The efficacy of individual purified components in protecting animals against natural disease caused by *B. bronchiseptica* and *B. avium* is not known, but FHA, PTN, and FIM have shown promise in experimental systems (Lee et al. 1986; Kobisch and Novotny 1990; Keil et al. 2000). Correlations of serum antibody levels and measures of systemic cell-mediated immune responses with protective effects of *Bordetella* vaccination have been inconsistent, even when testing with highly purified protective antigens (Hewlett and Halperin 1998; Bruss and Siber 2002; Watanabe et al. 2002). Extensive genomic rearrangements have been detected in natural populations of *B. pertussis* (Stibitz and Yang 1999). It has been suggested that differences in surface antigen profiles of current wild-type *B. bronchiseptica* strains and existing vaccine strains might account for some vaccine protection failures (Keil and Fenwick 1999). It also has been suggested that vaccination, itself, may provide a strong selective pressure to induce genetic changes in pleiotrophic *Bordetella* protective antigens, particularly PTN (Mooi et al. 1998; Boursaux-Eude and Guiso 2000).

CONCLUSION

Beyond their well-established clinical significance, as a group, members of the genus *Bordetella* have much to offer in terms of advancing our fundamental understanding of the complex relationship involved in the normal and pathologic interaction between microorganisms and the mucosal surfaces of their animal hosts. The *Bordetella* organisms are a particularly captivating group to study because of the host specificity of some, the broad host range of others, and the fact that some virulence mechanisms and regulatory systems are widely shared while others are unique to specific species or strains. When taken together, the group presents a combination of microbe and host factors that is particularly attractive for systems-biology investigational approaches in the postgenomic era. Finally, the recent recognition of vaccine-mediated immunity driving the molecular adaptation of protective protein antigens is a subject of both fundamental and practical importance.

REFERENCES

Allen, A. G., R. M. Thomas, J. T. Cadisch, and D. J. Maskell. 1998. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus *wbl* from *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Mol. Microbiol. 29:27–38.

Arico, B., and R. Rappuoli. 1987. *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. 169:2847–2853.

Arp, L. H., and D. H. Hellwig. 1988. Passive immunization versus adhesion of *Bordetella avium* to the tracheal mucosa of turkeys. Avian Dis. 32:494–500.

Banemann, A., and R. Gross. 1997. Phase variation affects long-term survival of *Bordetella bronchiseptica* in professional phagocytes. Infect. Immun. 65:3469–3473.

Bassinet, L., P. Gueirard, B. Maitre, B. Housset, P. Gounon, and N. Guiso. 2000. Role of adhesions and toxins in invasion of human tracheal epithelial cells by *Bordetella pertussis*. Infect. Immun. 68:1934–1941.

Bemis, D. A., and J. R. Kennedy. 1981. An improved system for studying the effect of *Bordetella bronchiseptica* on the ciliary activity of canine tracheal epithelial cells. J. Infect. Dis. 144:349–357.

Bey, R. F., F. J. Shade, R. A. Goodnow, and R. C. Johnson. 1981. Intranasal vaccination of dogs with live avirulent *Bordetella bronchiseptica*: correlation of serum agglutination titer and the formation of secretory IgA with protection against experimentally induced infections tracheobronchitis. Am. J. Vet. Res. 42:1130–1132.

Boschwitz, J. S., H. G. J. van der Heide, F. R. Mooi, and D. A. Relman. 1997. *Bordetella bronchiseptica* expresses the fimbrial structural subunit gene fimA. J. Bacteriol. 179:7882–7885.

Boursaux-Eude, C., and N. Guiso. 2000. Polymorphism of repeated regions of pertactin in *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Infect. Immun. 68:4915–1817.

Brockmeier, S. L., and K. B. Register. 2000. Effect of temperature modulation and Bvg mutation of *Bordetella bronchiseptica* on adhesion, intracellular survival and cytotoxicity for swine alveolar macrophages. Vet. Microbiol. 73:1–12.

Brockmeier, S. L., K. B. Register, T. Magyar, A. J. Lax, G. D. Pullinger, and R. A. Kunkle. 2002. Role of the dermonecrotic toxin of *Bordetella bronchiseptica* in the pathogenesis of respiratory disease in swine. Infect. Immun. 70:481–490.

Bruss, J. B., and G. R. Siber. 2002. Quantitative priming with inactivated pertussis toxoid vaccine in the aerosol challenge model. Infect. Immun. 70:4600–4608.

Chanter, N., T. Magyar, and M. Rutter. 1989. Interactions between *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs. Res. Vet. Sci. 47:48–53.

Clubb, S. L., B. L. Homer, J. Pisani, and C. Head. 1994. Outbreaks of Bordetellosis in psittacines and ostriches. In Main conference proceedings, Assn. Avian Veterinarians, pp. 63–68. Annual Meeting, Reno, NV.

Cotter, P. A., and J. F. Miller. 1997. A mutation in the *Bordetella bronchiseptica bvg*S gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. Mol. Microbiol. 24:671–685.

———. 2001. *Bordetella*. In Principles of bacterial pathogenesis. Edward O. Groisman (ed.). San Diego: Academic Press, pp.621–671.

Cotter, P. A., M. H. Yuk, S. Mattoo, B. J. Akerley, J. Boschwitz, D. A. Relman, and J. F. Miller. 1998. Filamentous hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. Infect. Immun. 66:5921–5929.

DeLey, J., P. Segers, K. Kersters, W. Mannheim, and A. Lievens. 1986. Intra- and intergeneric similarities of the *Bordetella* ribosomal ribonucleic acid cistrons: proposal for a new family, *Alcaligenaceae*. Int. J. Syst. Bacteriol. 36:405–414.

Di Fabio, J. L., M. Caroff, D. Karibian, J. C. Richards, and M. B. Perry. 1992. Characterization of the common antigenic lipopolysaccharide O-chains produced by *Bordetella bronchiseptica* and *Bordetella parapertussis*. FEMS Microbiol Lett. 76:275–281.

Ellis, J. A., D. M. Haines, K. H. West, J. H. Burr, A. Dayton, H. G. G. Townsend, E. W. Kanara, C. Konoby, A. Crichlow, K. Martin, and G. Headrick. 2001. Effect of vaccination on experimental infection with *Bordetella bronchiseptica* in dogs. J. Am. Vet. Med. Assn. 218:367–375.

Ellis, J. A., S. Krakowka, A. D. Dayton, and C. Konoby. 2002. Comparative efficacy of an injectable vaccine and an intranasal vaccine in stimulating *Bordetella bronchiseptica*-reactive antibody responses in seropositive dogs. J. Am. Vet. Med. Assn. 220:43–48.

Emsley, P., I. J. Charles, N. F. Fairweather, and N. W. Isaacs. 1996. Structure of *Bordetella pertussis* virulence factor P.69 pertactin. Nature. 381:90–92.

Ezzell, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark. 1981. Phase-shift markers in the Genus *Bordetella*: loss of cytochrome d-629 in phase IV variants. Microbios. 31:171–181

Finn, T. M., and D. F. Amsbaugh. 1998. Vag8 a *Bordetella pertussis* bvg-regulated protein. Infect. Immun. 66:3985–3989.

Finn, T. M., and L. A. Stevens. 1995. Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. Mol. Microbiol. 16:625–634.

———. 1995. Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. Mol. Microbiol. 16:625–634.

Flak, T. A., and W. E. Goldman. 1996. Autotoxicity of nitric oxide in airway disease. Am. J. Respir. Crit. Care Med. 154:S202–S206.

Foley, J. E., C. Rand, M. J. Bannasch, C. R. Norris, and J. Milan. 2002. Molecular epidemiology of feline bordetellosis in two animal shelters in California, USA. Prev. Vet. Med. 54:141–156.

Ford, R. B. 1995. Infectious tracheobronchitis. In Kirk's current veterinary therapy XII. J. D. Bonagura (ed.). Philadelphia: W. B. Saunders Co., pp. 905–908.

Forde, C. B., R. Parton, and J. G. Coote. 1998. Bioluminescence as a reporter of intracellular survival of *Bordetella bronchiseptica* in murine phagocytes. Infect. Immun. 66:3198–3207.

Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, R. Curtiss, III. 1988. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of *Bordetella avium.* Infect. Immun. 56:1698–1707.

Gentry-Weeks, C. R., J. Spokes, and J. Thompson. 1995. *beta*-Cystathionase from *Bordetella avium*. Role(s) of lysine 214 and cysteine residues in activity and cytotoxicity. J. Biol. Chem. 270:7695–7702.

Gerlach, H. 1994. Bacteria. In Avian medicine: principles and application. B. W. Ritchie, G. J. Harrison, and L. R. Harrison (eds.). Lake Worth: Wingers Publishing Co., pp. 949–983.

Geuijen, C. A. W., R. J. L. Willems, and F. R. Mooi. 1996. The major fimbrial subunit of *Bordetella pertussis* binds to sulfated sugars. Infect. Immun. 64:2657–2665.

Geuijen, C. A. W., R. J. L. Willems, M. Bongaerts, J. Top, H. Gielen, and F. R. Mooi. 1997. The role of *Bordetella pertussis* minor fimbrial subunit FimD in colonization of mouse respiratory tract. Infect. Immun. 65:4222–4228.

Giardina, P. C., L. A. Foster, J .M. Musser, B. J. Akerley, J. F. Miller, and D. W. Dyer. 1995. Bvg repression of alcaligin synthesis in *Bordetella bronchiseptica* is associated with phylogenic lineage. J. Bacteriol. 177:6058–6063.

Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullman, and A. Danchin. 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. Mol. Microbiol. 2:19–30.

Gueirard, P., and N. Guiso. 1993. Virulence of *Bordetella bronchiseptica*: role of adenylate cyclase-hemolysin. Infect. Immun. 61:4072–4078.

Guzman, C. A., M. Rohde, M. Bock, and K. N. Timmis. 1994. Invasion and intracellular survival of *Bordetella bronchiseptica* in mouse dendritic cells. Infect. Immun. 62:5528–5537.

Hannah, J. H., F. D. Menozzi, G. Renaul, C. Locht, and M. J. Brennan. 1994. Sulfated glycoconjugate receptors for the *Bordetella pertussis* adhesin filamentous hemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. Infect. Immun. 62:5010–5019.

Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller. 2000. Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. Infect. Immun. 68:6720–6728.

Harvill, E. T., P. A. Cotter, and J. F. Miller. 1999a. Pregenomic comparative analysis between *Bordetella bronchiseptica* RB50 and *Bordetella pertussis* Tohama I in murine models of respiratory tract infection. Infect. Immun. 67:6109–6118.

Harvill, E. T., P. A. Cotter, M. H. Yuk, and J. F. Miller. 1999b. Probing the function of *Bordetella bronchiseptica* adenylate cyclase toxin by manipulating the host immunity. Infect. Immun. 67:1493–1500.

Hausman, S. Z., J. D. Cherry, U. Heininger, C. H. Wirsing von Konig, and D. L. Burns. 1996. Analysis of proteins encoded by *ptx* and *ptl* genes of *Bordetella bronchiseptica* and *B. parapertussis*. Infect. Immun. 64:4020–4026.

Hewlett, E. L., and S. A. Halperin. 1998. Serological correlates of immunity to *Bordetella pertussis*. Vaccine. 16:1899–1900.

Horiguchi, Y., N. Inoue, M. Masuda, T. Kashimoto, J. Katahira, N. Sugimoto, and M. Matsuda. 1997. *Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln 63 of the GTP-binding protein Rho. Proc. Nat. Acad. Sci. USA. 14:11623–11626.

Iida, T., and T. Okonogi. 1971. Leinotoxicity of *Bordetella pertussis* in mice. J. Med. Microbiol. 4:51–61.

Ishibashi, Y., S. Claus, and D. Relman. 1994. *Bordetella pertussis* filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). J. Exp. Med. 180:1225–1233.

Jackwood, M., and Y. Saif. 1987. Pili of *Bordetella avium*: expression, characterization, and role in in vitro adherence. Avian Dis. 31:277–286.

Jacob-Dubuisson, F. J., B. Kehoe, E. Willery, N. Reveneau, C. Locht, and D. Relman. 2000. Molecular characterization of *Bordetella bronchiseptica* filamentous hemagglutinin and its secretion machinery. Microbiol. 146:1211–1221.

Jungnitz, H., N. P. West, M. J. Walker, G. S. Chhatwal, and C. A. Guzman. 1998. A second two-component regulatory system of *Bordetella bronchiseptica* required for bacterial resistance to oxidative stress, production of acid phosphatase and in vivo persistence. Infect. Immun. 66:4640–4650.

Kashimoto, T., J. Katahira, W.R. Cornejo, M. Masuda, A. Fukuoh, T. Matsuzawa, T. Ohnishi, and Y. Horiguchi. 1999. Identification of functional domains of *Bordetella* dermonecrotizing toxin. Infect. Immun. 67:3727–3732.

Keil, D. J., and B. Fenwick. 1998a. Role of *Bordetella bronchiseptica* in infectious

tracheobronchitis in dogs. J. Am. Vet. Med. Assn. 212:200–207.

———. 1998b. Evaluation of canine *Bordetella bronchiseptica* isolates using randomly amplified polymorphic DNA fingerprinting and ribotyping. Vet. Micro. 66:41–51.

———. 1999. Strain- and growth condition-dependent variability in outer membrane protein expression by *Bordetella bronchiseptica* isolates from dogs. Am. J. Vet. Res. 60:1016–1021.

Keil, D. J., E. H. Burns Jr., W. R. Kisker, D. Bemis, and B. Fenwick. 2000. Cloning and immunologic characterization of a truncated *Bordetella bronchiseptica* filamentous hemagglutinin fusion protein. Vaccine 18:860–867.

Khattak, M. N., R. C. Matthews, and J. P. Burnie. 1992. Is *Bordetella pertussis* clonal? Br. Med. J. 304:813–815.

Kirimanjeswara, G. S., P. B. Mann, and E. T. Harvill. 2003. Role of antibodies in immunity to *Bordetella* infections. Infect. Immun. 71:1719–1724.

Kobisch, M., and P. Novotny. 1990. Identification of a 68 kilodalton outer membrane protein as the major protective antigen of *Bordetella bronchiseptica* by using specific pathogen free piglets. Infect. Immun. 58:352–357.

Lacerda, H. M., G. D. Pullinger, A. J. Lax, and E. Rozengurt. 1997. Cytotoxic necrotizing factor 1 from *Escherichia coli* and dermonecrotic toxin from *Bordetella bronchiseptica* induce p21 (rho)-dependent tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 cells. J. Biol. Chem. 272:9587–9596.

Lee, C. K., A. Roberts, and S. Perrin. 1989. Expression of pertussis toxin in *Bordetella bronchiseptica* and *Bordetella parapertussis* carrying recombinant plasmids. Infect. Immun. 57:1413–1418.

Lee, S., A. Way, and E. Osen. 1986. Purification and subunit heterogeneity of pili of *Bordetella bronchiseptica*. Infect. Immun. 51:586–593.

Leininger, E., M. Roberts, J. Kenimer, I. Charles, N. Fairweather, P. Novotny, and M. Brennan. 1991. Pertactin, an arg-gly-asp containing *Bordetella pertussis* surface protein that promotes adherence to mammalian cells. Proc. Natl. Acad. Sci. U S A 88:345–349.

Lerm, M., G. Schmidt, U. M. Goehring, J. Schirmer, and K. Aktories. 1999. Identification of the region of Rho involved in substrate recognition by *Escherichia coli* cytotoxic necrotizing factor 1 (CNF). J. Biol. Chem. 8:28999–299004.

Luker, K. E., A. N. Tyler, G. R. Marshal, and W. E. Goldman. 1995. Tracheal cytotoxin structural requirements for respiratory epithelial damage in pertussis. Mol. Microbiol. 16:733–743.

Martineau, G .P., A. Broes, and B. Martineau-Doize. 1982. Atrophic rhinitis: Appraisal of infection pressure on gnotobiotic piglets infected with *Bordetella bronchiseptica*. Can. J. Comp. Med. 46:376–381.

McArthur, J. D., N. P. West, J. N. Cole, H. Jungnitz, C. A. Guzman, J. Chin, P. R. Lehrbach, S. P. Djordjevic, and M. J. Walker. 2003. An aromatic amino acid auxotrophic mutant of *Bordetella bronchiseptica* is attenuated and immunogenic in a mouse model of infection. FEMS Microbiol. Lett. 221: 7–16.

McMillan, D. J., M. Shojaei, G. S. Chhatwal, C. A. Guzman, and M. J. Walker. 1996. Molecular analysis of the bvg-repressed urease of *Bordetella bronchiseptica*. Microb. Pathog. 21:379–394.

Menozzi, F. D., P. E. Boucher, G. Riveau, C. Gantiez, and C. Locht. 1994. Surface-associated filamentous hemagglutinin induces autoagglutination of *Bordetella pertussis*. Infect. Immun. 62:4261–4269.

Merkel, T. J., C. Barros, and S. Stibitz. 1998. Characterization of the *bvg*R locus of *Bordetella pertussis*. J. Bacteriol. 180:1682–1690.

Mills, K. H., A. Barnard, J. Watkins, and K. Redhead. 1993. Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect. Immun. 61:399–410.

Mooi, F. R., H. van Oirschot, K. Heuvelman, H. G. van der Hiede, W. Gaastra, and R. J. Willems. 1998. Polymorphism in the *Bordetella pertussis* virulence factors P. 69/pertactin and pertussis toxin in the Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect. Immun. 66:670–675.

Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander. 1986. Genetic diversity and relationships in population of *Bordetella* spp. J. Bacteriol. 166:230–237.

Porter, J. F., R. Parton, and A. C. Wardlaw. 1991. Growth and survival of *Bordetella bronchiseptica* in natural waters and in buffered saline without added nutrients. Appl. Environ. Microbiol. 57:1202–1206.

Pradel E., N. Guiso, F. D. Menozzi, and C. Locht. 2000. *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect. Immun. 68:1919–1927.

Preston, A., E. Maxim, E. Toland, E. J. Pishko, E. T. Harvill, M. Caroff, and D. J. Maskell. 2003. *Bordetella bronchiseptica* PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. Mol. Microbiol. 48:725–736.

Pullinger, G. D., T. E. Adams, P. B. Mullan, T. I. Garrod, and A. J. Lax. 1996. Cloning, expression

and molecular characterization of the dermonecrotic toxin gene of *Bordetella spp*. Infect. Immun. 64:4163–4171.

Raffel, T. R., K. B. Register, S. A. Marks, and L. Temple. 2002. Prevalence of *Bordetella avium* infection in selected wild and domesticated birds in eastern USA. J. Wildl. Dis. 38:40–46.

Rambow, A. A., R. C. Fernandez, and A. A. Weiss. 1998. Characterization of BrkA Expression in *Bordetella bronchiseptica*. Infect. Immun. 66:3978–3980.

Raptis, A., L. Knipling, and J. Wolff. 1989. Dissociation of catalytic and invasive properties of *Bordetella pertussis* adenylate cyclase. Infect. Immun. 57:1725–1730.

Register, K. B.. 2001. Novel genetic and phenotypic heterogeneity in *Bordetella bronchiseptica* pertactin. Infect. Immun. 69:917–1921.

Register, K. B., and M. R. Ackermann. 1997. A highly adherent phenotype associated with virulent Bvg$^+$-phase swine isolates of *Bordetella bronchiseptica* grown under modulating conditions. Infect. Immun. 65:5295–5300.

Register, K. B., A. Boisvert, and M. R. Ackermann. 1997. Use of ribotyping to distinguish *Bordetella broncheseptica* isolates. Int. J. Syst. Bacteriol. 47:678–683.

Register, K. B., R. E. Sacco, and G. Foster. 2000. Ribotyping and restriction endonuclease analysis reveal a novel clone of *Bordetella bronchiseptica* in seals. J. Vet. Diagn. Invest. 12:535–540.

Roy, C. R., J. F. Miller, and S. Falkow. 1990. Autogenous regulation of the *Bordetella pertussis* bvgABC operon. Proc. Natl. Acad. Sci. U S A 87:3763–3767.

Ryan, M., G. Murphy, L. Gothefors, L. Nilsson, J. Storsaeter, and K. H. Mills. 1997. *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type 1 T helper cells. J. Infect. Dis. 175:1246–1250.

Savelkoul, P. H., D. P. DeKerf, R. J. Willems, F. R. Mooi, B. A. van der Zeijst and W. Gaastra. 1996. Characterization of the fim 2 and fim 3 fimbrial subunit genes of *Bordetella bronchiseptica*: roles of fim 2 and fim 3 fimbriae and flagella in adhesion. Infect. Immun. 64:5098–5105.

Schneider, B., D. Stubs, and R. Gross. 2002. Identification and genomic organization of gene loci negatively controlled by the virulence regulatory BvgAS two-component system in *Bordetella bronchiseptica*. Mol. Genet. Genomics 267:526–535.

Spears, P. A., L. M. Temple, and P. E. Orndorff. 2000. A role for lipopolysaccharide in turkey tracheal colonization by *Bordetella avium* as demonstrated in vivo and in vitro. Mol. Microbiol. 36:1425–1435.

Spears, P. A., L. M. Temple, D. M. Miyamoto, D. J. Maskell, and P. E. Orndorff. 2003. Unexpected similarities between *Bordetella avium* and other pathogenic *Bordetellae*. Infect. Immun. 71:2591–2597.

Stehmann, R., G. Mehlhorn, and V. Neuparth. 1991. Characterization of strains of *Bordetella bronchiseptica* isolated from animal housing air and of the airborne infection pressure proceeding from them. Dtsch. Tieraerztl. Wochenschr. 98:448–450.

Stenson, T. H., and M. S. Peppler. 1995. Identification of two *bvg*-repressed surface proteins of *Bordetella pertussis*. Infect. Immun. 63:3780–3789.

Stibitz, S., and M-S. Yang. 1999. Genomic plasticity in natural populations of *Bordetella pertussis*. J. Bacteriol. 181:5512–5515.

Taylor, B. L., and I. B. Zhulin. 1999. PAS domains: internal sensors of oxygen, redox potential and light. Microbiol. Mol. Biol. Rev. 63:479–506.

Tejada, G. M., J. F. Miller, and P. A. Cotter. 1996. Comparative analysis of virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. Mol. Microbiol. 22:895–908.

Temple, L. M., A. A. Weiss, K. E. Walker, H. J. Barnes, V. L. Christensen, D. M. Miyamoto, C. B. Shelton, and P. E. Orndorff. 1998. *Bordetella avium* virulence measured in vivo and in vitro. Infect. Immun. 66:5244–5251.

van den Akker, W. M. 1997. *Bordetella bronchiseptica* has a BvgAS-controlled cytotoxic effect upon interaction with epithelial cells. FEMS Microbiol. Lett. 156:239–244.

van der Zee, A., F. Mooi, J. van Embden, and J. Musser. 1997. Molecular evolution and host adaptation of *Bordetella* spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J. Bacteriol. 179:6609–6617.

von Wintzingerode, F., A. Schattke, R. A. Siddiqui, U. Rosick, U. B. Gobel, and R. Gross. 2001. *Bordetella petrii sp. nov.*, isolated from an anaerobic bioreactor and emended description of the genus *Bordetella*. Int. J. Syst. Evol. Microbiol. 51:1257–1265.

von Wintzingerode, F., G. Gerlach, B. Schneider, and R. Gross. 2002. Phylogenetic relationships and virulence evolution in the Genus *Bordetella*. Curr. Top. Microbiol. Immunol. 264:177–199.

Walker, K. E., and A. A. Weiss. 1994. Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. Infect. Immun. 62:3817–3828.

Watanabe, M., E. Komatsu, T. Sato, and M. Nagai. 2002. Evaluation of efficacy in terms of antibody

levels and cell-mediated immunity of acellular pertussis vaccines in a murine model of respiratory infection. FEMS Immunol. Med. Microbiol. 33:219–225.

Willems, R. J. L., H. G. J. van der Heide, and F. R. Mooi. 1992. Characterization of a *Bordetella pertussis* fimbrial gene cluster which is located directly downstream of the filamentous hemagglutinin gene. Mol. Microbiol. 6:2661–2671.

Williams, J., R. Laris, A. W. Gray, and A. A. Jacobs. 2002. Studies of the efficacy of a novel intranasal vaccine against feline bordetellosis. Vet. Rec. 150:439–442.

Woolfrey, B. F., and J. A. Moody. 1991. Human infections associated with *Bordetella bronchiseptica*. Clin. Microbiol. Rev. 4:243–255.

Yabuuchi, E., Y. Kawamura, Y. Kosako, and T. Ezaki. 1998. Emendation of genus *Achromobacter* and *Achromobacter xylosoxidans* (Yabuuchi and Yano) and proposal of *Achromobacter ruhlandii* (Packer and Vishniac) *comb. nov*., *Achromobacter piechaudii* (Kiredjian et al.) *comb. nov*., and *Achromobacter xylosoxidans subsp*. *denitrificans* (Ruger and Tan) *comb. nov*.. Microbiol. Immunol. 42:429–438.

Yuk, M. H., E. T. Harvill, and J. F. Miller. 1998. The *bvgAS* virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol. Microbiol. 28:945–959.

Yuk, M. H., E. T. Harvill, P. C. Cotter, and J. F. Miller. 2000. Modulation of host immune responses, induction of apoptosis and inhibition of NF-κB activation of the *Bordetella* type III secretion system. Mol. Microbiol. 35:991–1004.

20
Pasteurella and *Mannheimia*

J. D. Boyce, R. Y. C. Lo, I. Wilkie, and B. Adler

PASTEURELLA

Bacteria of the genus *Pasteurella* have been the subject of significant study since the 1880s when Louis Pasteur first recognized that *P. multocida* was the causative agent of a fatal disease in chickens and used a laboratory attenuated strain as the first live bacterial vaccine. It is now known that *Pasteurella* species can cause disease in a wide range of animal species and that many of these diseases are of significant economic importance.

Members of the genus *Pasteurella* are nonmotile, gram-negative coccobacillary- to rod-shaped organisms measuring 0.3–1.0 μm in width and 1.0–2.0 μm in length. They are generally oxidase and catalase positive and nonhemolytic. They can grow under aerobic, microaerophilic or anaerobic conditions and are unable to grow on MacConkey agar or in Simmons citrate medium.

Diseases/Economic Impact

Pasteurella species can cause disease in almost all animals, but are also part of the normal oropharyngeal flora in many species. They are both primary causative agents of disease and opportunistic pathogens. *P. multocida* is the most widely studied member of the genus and is the causative agent of fowl cholera (FC), hemorrhagic septicemia (HS), and a variety of respiratory syndromes such as atrophic rhinitis of swine (AR) and purulent rhinitis of rabbits ("snuffles").

FC is a disease of domesticated and wild birds, causing large losses in commercial chicken, turkey, and duck flocks. It is also responsible for significant mortality in wild waterfowl. HS is an acute, febrile, and rapidly lethal disease of ungulates. It is of greatest economic importance in Asia and Africa where annual losses in a number of Asian countries have been estimated at approximately $US1.0 billion (Verma and Jaiswal 1998). Buffalo (*Bubulus* sp.) are particularly susceptible, but cattle and pigs are also affected. AR is generally a disease of growing swine but also has been reported in rabbits, goats, and cattle. It causes atrophy of the turbinate bones, distortion of the maxilla, and reduced growth rates. *P. multocida* infection in rabbits is also associated with purulent rhinitis, otitis media, sinusitis, genital infections, and pneumonia. Particularly in feeder calves, feedlot cattle, and housed pigs, *P. multocida* may be associated with acute or chronic bronchopneumonia. Other common associations in any species include subcutaneous abscesses or cellulitis (particularly following bite wounds), mastitis, genital infections, and septicemia.

Taxonomy, Nomenclature, and Typing

The genus *Pasteurella* is heterogeneous and the taxonomy is undergoing continuing revision. The genus presently comprises at least nine species (table 20.1) that fall into two separate 16S rRNA phylogenetic groups (Dewhirst et al. 1993). The first group contains the type species *P. multocida* as well as the species *P. canis*, *P. stomatis*, *P. dagmatis,* and *Pasteurella* species B. The second group clusters together with *Haemophilus paragallinarum* and includes *P. avium*, *P. gallinarum*, *P. volantium,* and *Pasteurella* species A (Mutters et al. 2003). Several species have recently been removed from the genus including [*P*]. *aerogenes*, *anatis*, *granulomatis*, *haemolytica*, *testudinis,* and *ureae*. Other species with uncertain taxonomy, but unlikely to constitute part of the genus *Pasteurella*, include [*P*]. *bettyae*, *caballi*, *langaa*, *lymphangitidis*, and *mairii*. Two

Table 20.1. Currently Recognized Taxa in the Genus *Pasteurella,* Host Predilection and Diseases

Species	Hosts	Associations/diseases (common serotypes)
P. multocida	Birds, mammals	AR of pigs (toxigenic serotypes A and D), HS of ungulates (B:2; B:2,5; E:2; E:2,5), FC of birds (A, F, rarely D), snuffles of rabbits (mostly A), bite wound contamination, chronic and acute pneumonias, mastitis, cellulitis (mostly A)
P. avium	Cattle, chickens	Commensal in birds, sinusitis, pneumonia in calves
P. canis	Cattle, dogs, humans, sheep	Commensal in dogs; bite-wound contamination (acute osteomyelitis, cellulitis, septicemia), pneumonia in cattle and sheep
P. dagmatis	Cats, dogs, humans	Commensal in dogs and cats; bite-wound contamination
P. gallinarum	Birds	Probable commensal in birds, occasionally low-grade infections
P. species A	Birds	Sinusitis, conjunctivitis
P. species B	Cats, dogs, humans	Probable commensal in dogs and cats, wound infections
P. stomatis	Cats, dogs, humans	Commensal in dogs and cats, canine bronchitis, wound contamination
P. volantium	Chickens	Commensal in birds, not known to be pathogenic

new species (*P. skyensis* and *P. phocoenarum*) tentatively assigned as members of the genus *Pasteurella* on the basis of 16S rRNA sequence await further analysis (Birkbeck et al. 2002).

The complete genome sequence of the *P. multocida* strain PM70 has been determined (May et al. 2001). It contains a single circular chromosome of 2,257,487 bp and has been annotated to encode 2,015 open reading frames (ORFs), 6 rRNA operons, and 57 tRNAs. *P. multocida* strain PBA100 (serotype A:1) has only 5 rRNA operons and has been estimated to be 2.35 Mbp (Hunt et al. 1998). The genomic G + C content ranges between 37.7 and 45.9% (Mutters et al. 2003). There are no genomic data available for other members of the genus.

P. multocida strains can be differentiated by a number of serological and recombinant DNA methods. The most widely used serological methods are the indirect hemagglutination test, which primarily recognizes capsule antigens, and the gel diffusion precipitin assay, which recognizes LPS types. Using these assays, one can differentiate *P. multocida* serovars into 5 distinct capsular groups (A, B, D, E, and F) (Carter 1967; Rimler and Rhoades 1987) and 16 LPS types (1 to 16) (Heddleston and Rebers 1972). The capsule biosynthetic loci of the 5 capsular groups have been identified and a multiplex PCR capsular typing system developed (Townsend et al. 2001). Certain diseases are often associated with particular capsular groups; HS is generally caused by serogroup B and E; FC by serogroups A, F, and occasionally D; AR by toxin-producing strains, usually of serogroup D; and respiratory tract infections caused by serogroup A. There are no data available on serological typing methods for *Pasteurella* species other than *P. multocida*.

A number of molecular typing methods has been used to identify and differentiate *Pasteurella* serovars and species (Blackall and Miflin 2000). These include restriction endonuclease analysis (REA), multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), ribotyping, pulsed-field gel electrophoresis, repetitive sequence based PCR (rep-PCR), and amplified fragment length polymorphism (AFLP). Combinations of these approaches have proved very useful for strain identification and are excellent tools for analysis of epidemiology of field infections (Blackall and Miflin 2000; Amonsin et al. 2002).

Sources of the Bacteria

P. multocida is a primary pathogen or commensal of a wide range of vertebrate species, but it can also survive for long periods in organic matter and up to a year in water (Bredy and Botzler 1989). Many ani-

mals carry *P. multocida* and other *Pasteurellae* as part of their normal oropharyngeal flora, and this is undoubtedly the source of *Pasteurellae* that are a common cause of subcutaneous infections following animal bites. Maintenance and transmission of FC-causing *P. multocida* is probably via carrier birds. *P. multocida* has been isolated from the cloaca and pharynx of apparently healthy birds in flocks with a previous history of FC and also in apparently healthy flocks (Muhairwa et al. 2000). Wild birds and other animals have been suggested as possible carriers (Christensen et al. 1998; Muhairwa et al. 2001; Petersen et al. 2001), although some studies have found little evidence of cross-species transmission (Carpenter et al. 1991). Transmission within a flock may be via contaminated water or aerosols (Pabs-Garnon and Soltys 1971a; Simensen and Olson 1980). Vertical transmission *in ovo* does not appear to occur. There is circumstantial evidence that *P. multocida* B:2 can be passed from cattle/buffalo to pigs, but the extent of interspecies infection for other strains is unknown. Cattle, buffalo, and deer have been shown to be capable of carrying *P. multocida* serotype B strains asymptomatically in the tonsils and upper respiratory tract lymph nodes (de Alwis 1992; Saharee et al. 1992; Aalbaek et al. 1999). Carrier rates are highest following outbreaks (20–40%) and in endemic areas (de Alwis 1992). Under some as yet uncharacterized conditions, carrier animals may begin to shed infectious organisms.

Bacterial Virulence Factors

For the purposes of this review, we define virulence genes as those encoding factors that are involved in interactions with the host and are directly responsible for pathological damage, while virulence-associated genes are those whose products process or regulate virulence factors, are required for the activity of virulence factors, or are involved in metabolic processes essential to a pathogenic lifestyle (Wassenaar and Gaastra 2001). Since many *Pasteurella* strains cause diseases that are specific to certain hosts, identified virulence factors may only apply to the syndrome for which they were characterized. Using these definitions, only a few true virulence genes from *Pasteurella* species have been defined, and all have been from *P. multocida*.

Some serotype A and D strains of *P. multocida* produce a toxin termed *P. multocida* toxin (PMT). PMT is a 146-kDa cytotoxic protein that acts through complex intracellular-signaling pathways (Zywietz et al. 2001) to stimulate cellular cytoskeletal rearrangements. It is a very potent mitogen for fibroblasts, and acts via osteoblasts to stimulate osteoclasts (Wilson et al. 2000). Purified PMT can initiate pneumonia and pleuritis in rabbits, but it is not known how significant this is in field diseases. However, some strains that produce PMT appear to be avirulent (Rimler and Brogden 1986) and most *P. multocida* strains, including those that cause FC, HS, and pneumonias, do not produce PMT.

Many *P. multocida* strains produce a polysaccharide capsule that differs in its composition depending on the serotype. The serotype A capsule contains hyaluronic acid, while serotype D and F capsules contain heparin and chondroitin, respectively (DeAngelis et al. 2002). No structure has been determined for the capsular polysaccharides of serotypes B and E. The capsule of serotype A strains can contribute significantly to virulence, as genetically modified strains incapable of exporting capsule were avirulent in chickens and significantly more susceptible to the bactericidal activity of complement than isogenic encapsulated strains (Chung et al. 2001). Similarly, acapsular serotype B (HS) strains were highly attenuated in a mouse model of infection, were readily removed from the blood of infected animals, and were significantly more susceptible to macrophage uptake (Boyce and Adler 2000). Capsule thus clearly plays a significant role in protecting the bacteria from host immune mechanisms, although apparently acapsular mutants of some strains remain virulent (Matsumoto and Strain 1993). Furthermore, the capsule of serotype A has been shown to mediate adhesion to turkey air-sac macrophages and turkey peripheral blood monocytes (Pruimboom et al. 1996; Pruimboom et al. 1999).

Analysis of the genome sequence of the *P. multocida* strain PM70 identified a number of genes whose products were predicted (on the basis of amino acid identity to characterized proteins from other species) to be involved in adhesion to host cells. These include the putative filamentous hemagglutinins PfhB1 and PfhB2, the fimbrial subunit PtfA (PM0084), and putative fimbrial subunits, FimA, Flp_1 (PM0855), and Flp_2 and the predicted surface fibrils Hsf_1 and Hsf_2 (May et al. 2001). The contribution of adhesins and fimbriae to virulence in many bacterial diseases is clear, and a number of *P. multocida* strains have been shown to express fimbriae (Rebers et al. 1988; Ruffolo et al. 1997) and to adhere to eukaryotic cells (Jacques et al. 1993; Lee et al. 1994; Al-Haddawi et al. 2000). However, only *pfhB1* and *pfhB2* are known virulence

factors since mutants with transposon insertions in these genes are attenuated for virulence in mice (Fuller et al. 2000). Furthermore, there appears to be poor correlation between possession of fimbriae and ability to cause atrophic rhinitis (Chanter 1990).

Analysis of the genome sequence of the *P. multocida* strain PM70 identified more than one hundred predicted virulence-associated genes (May et al. 2001). *P. multocida* contains many genes (>50) predicted to be involved in the acquisition of iron. Iron is an essential nutrient for bacteria, and soluble iron is usually scarce in host tissues. Thus, *P. multocida* has evolved numerous (probably partially redundant) iron uptake systems to enable it to scavenge iron efficiently from the host during infection. Defined mutants in each of the functionally related iron uptake genes *tonB*, *exbD,* and *exbB* have been constructed and each mutant was attenuated for virulence in mice (Bosch et al. 2002).

A number of large-scale screening methods, including signature-tagged mutagenesis (STM) (Fuller et al. 2000), *in vivo* expression technology (Hunt et al. 2001), and microarray analysis (Boyce et al. 2002), have been applied to *P. multocida* strains in an attempt to identify virulence and virulence-associated genes. These methods have identified many genes (in addition to the genes discussed above) involved in metabolic processes required preferentially during *in vivo* growth. These genes include those involved in amino acid, carbohydrate, and nucleotide biosynthesis and transport. Microarray analysis of *P. multocida* gene expression following growth in infected chickens indicated that the bacteria must alter the regulation of a range of genes to cope with the low levels of nutrients (especially amino acids) available in the blood (Boyce et al. 2002).

Pathogenesis and Pathology

Given that the disease syndromes caused by the various strains of *P. multocida* are distinctly different, it follows that the pathogenesis of these diseases will be more or less specific for each condition although some mechanisms may be common to different diseases. The details of pathogenesis are either unknown or, at best, poorly understood for all *Pasteurella*-induced diseases with the exception of AR. It is not known, for example, how those serogroups commonly associated with mucosa colonize tonsillar tissue or respiratory tract epithelium, or why and how the carrier state proceeds to systemic infection in HS and FC carrier animals. Possession of fimbriae would suggest that adhesion to cell surfaces plays a role in infection, but it is not known what advantages, if any, adhesion might offer in most pasteurelloses. For example, possession of fimbriae does not appear to enhance colonization of pig nasal epithelium by AR-inducing strains (Chanter and Rutter 1990). However, concurrent infection with toxigenic strains of *Bordetella bronchiseptica* does enhance colonization by *P. multocida*. It has been suggested that PMT itself is a colonization factor, though no specific mechanism has been proposed. During an outbreak of disease, infection passes directly to other animals, yet the route(s) and mechanisms by which bacteria enter the body remain speculative, and the site(s) of initial replication are largely unknown. Entry may be a passive process, as in entry via a bite wound, or active as in the transport of bacteria by alveolar macrophages in the lung (Hassanin et al. 1995). *P. multocida* can enter epithelial cells actively *in vitro* by interacting with F-actin (Rabier et al. 1997), but it is not known to what extent, if any, this occurs *in vivo*. Infection may require leukocyte assistance, as it is very difficult to establish infections across intact conjunctival or intestinal epithelia, whereas intratracheal inoculation of virulent FC strains in chickens and HS strains in pigs readily initiates infection. In soft tissues, virtually any serotype or strain of *P. multocida* spreads rapidly along fascial planes, causing an intense cellulitis in which coagulative necrosis of myocytes is prominent.

Although FC and HS are traditionally regarded as "septicemic" diseases, it has never been shown conclusively that the organisms actually multiply in blood. The increasing numbers in blood as disease progresses might only reflect spillover from the primary site(s) of multiplication. Initially, both virulent and avirulent strains are cleared rapidly from chicken and turkey blood (Snipes et al. 1986), and mouse macrophages initially inactivate virulent and avirulent organisms equally *in vitro* (Collins et al. 1983). In FC, primary multiplication sites are likely the lungs, liver, and to a lesser extent, spleen (Pabs-Garnon and Soltys 1971b). Gross and microscopic lesions are most common in these organs and consist of multiple foci of necrosis associated with bacteria, and some inflammatory cell infiltration, depending on the rapidity of progression of the disease. No primary site(s) of bacterial multiplication have been identified for HS. Peripharyngeal swelling and cellulitis are common features of HS field disease, indicating this may be the primary site

for that disease. In peracute experimental cases (death <20 hrs post-challenge), edema, hyperemia and cellulitis around the site of inoculation are usually the only lesion-associated signs.

It has been suggested frequently that the clinical effects in fulminating infections, such as HS and FC, are the result of endotoxin. Endotoxins of FC- and HS-causing strains have been shown to mimic clinical signs of advanced disease in their respective hosts (Rhoades and Rimler 1987; Horadagoda et al. 2002). While LPS may be a major pathogenic factor in advanced disease, its role in early pathogenesis is less certain. The dose of endotoxin required to affect chickens is very high, and LPS from both highly virulent and nonvirulent strains is equally toxic for turkeys (Rhoades and Rimler 1987). Consumptive coagulopathy and alteration of leukocyte function occur in infected turkeys, and LPS-induced coagulopathy combined with endothelial damage is probably responsible for the widespread hemorrhages noted in some HS cases. Widespread petechiation is a common terminal event in many bacterial diseases, but whether or not coagulopathy makes any significant contribution to early pathogenesis is not clear.

The lesions of AR range from mild to severe and consist of destruction of nasal turbinate bones, deviation of the nasal septum, distortion of the maxilla, and retarded growth. Some details of the pathogenesis are known. It is associated with the toxin (PMT) produced by some serogroup D and A strains. Minute doses of PMT stimulate osteoclast activity, an effect mediated via osteoblasts, which are suppressed. The lesions of AR can be reproduced by small parenteral doses of toxin (Mullan and Lax 1998; Lax and Grigoriadis 2001), but the cell-signaling pathways through which PMT operates are still being defined (Ohnishi et al. 1998; Lei et al. 2001; Zywietz et al. 2001). When instilled into the nose of rabbits, PMT can cause rhinitis, turbinate atrophy, pneumonia, pleuritis, acute hepatic necrosis, and atrophy of splenic lymphoid follicles (Chrisp and Foged 1991), but the toxin has not been shown to have a significant role in the pathogenesis of syndromes other than AR.

P. multocida is frequently isolated from acute and chronic bronchopneumonia in many different species. It has long been assumed that the organism in these situations is preconditioned by some other pathogenic mechanism, such as compromise of the mucociliary escalator of bronchioles by *Mycoplasma* spp. in so-called enzootic pneumonia of pigs. Lesions range from acute, suppurative, necrotizing bronchopneumonia with dominant polymorphonuclear cell infiltrates, to more localized, granulomatous lesions in the chronic forms.

Immunity

Immunity to *P. multocida* may be both specific and nonspecific. As for most bacteria, an important primary, nonspecific defense against most disease is an intact epithelial surface. All strains of *P. multocida* are capable of causing cellulitis if introduced into subepithelial tissues. This may progress to a fulminating systemic disease, or be contained, depending on host and strain.

Nonspecific defenses such as complement defensins and phagocytosis help to eliminate early infections of any bacterium (Rose et al. 1988), although no specific roles for complement and defensins have been demonstrated in *P. multocida*-related diseases. Numerous studies have linked virulence with possession of capsules (Hansen and Hirsh 1989; Boyce and Adler 2000; Chung et al. 2001). The amount of capsule clearly reduces the rate of phagocytosis (Harmon et al. 1991; Boyce and Adler 2000) but some apparently acapsular strains retained virulence for turkeys (Matsumoto and Strain 1993). Capsule probably protects the bacteria until they arrive in a suitably protected milieu where growth may proceed. Macrophages from immunologically naïve mice will initially phagocytose and inactivate opsonized virulent *P. multocida* as rapidly as avirulent strains (Collins et al. 1983). Several studies have linked virulence of FC strains with ability to survive in chicken serum. However, some very low-virulence strains can grow and multiply in 90% chicken serum (Diallo and Frost 2000) and both virulent and avirulent FC strains are rapidly cleared from turkey blood (Tsuji and Matsumoto 1989, 1990).

Animals that have survived natural infection with the HS and FC-causing strains of *P. multocida* usually develop solid immunity to future challenge by the same strain and often to heterologous strains as well. Vaccination with killed bacteria (bacterins) derived from *in vitro*-grown cultures stimulates protection only against homologous serotypes, and the duration of immunity is generally short. However, vaccination with bacterins derived from *in vivo*-grown cultures can provide protection against heterologous serotypes (Heddleston and Rebers 1972; Rimler et al. 1979; Ibrahim et al. 2000). Similarly, cross-serotype protection can be induced by administering live, attenuated strains (Homchampa et al. 1997). Thus, it appears that certain antigens

involved in providing cross-serotype protection are only expressed during growth within the host. One of these "cross-protective factors" has recently been isolated, but the gene(s) encoding the product are yet to be identified (Rimler 2001). Since strong protection can be provided by passive immunization, immunity is likely to be mainly due to the humoral system (Heddleston et al. 1975). Cell-mediated immune mechanisms have been demonstrated, but their role in immunity remains unclear (Baba 1984; Verma and Jaiswal 1997).

At present there are commercial vaccines available for a range of diseases caused by *P. multocida*. Bacterins, many with oil adjuvants, are widely used for FC (serotypes A:1,3,4), HS, and AR (Homchampa et al. 1997; Verma and Jaiswal 1998; Magyar et al. 2002). These vaccines suffer from an inability to elicit cross-serotype protection. In addition, the duration of immunity is generally short and animals vaccinated with these bacterins may still suffer disease outbreaks. Commercial live-attenuated vaccines are available for FC (Hopkins and Olson 1997) and live vaccines for HS have been tested (Myint et al. 1987; Myint and Carter 1989). However, the mechanism of attenuation for these strains is unknown and the vaccines have occasionally caused disease outbreaks (Hopkins et al. 1998). Defined attenuated strains capable of stimulating protective immunity have been constructed (Homchampa et al. 1997; Boyce and Adler 2001) with one nearing final vaccine trials (Scott et al. 1999).

Some AR vaccines contain purified recombinant PMT, and peptides of OmpH have provided protection against FC (Luo et al. 1999). However, to date there are no candidate antigens for recombinant subunit vaccines against FC or HS that will give broad and lasting immunity.

Conclusions

More than 120 years have passed since Louis Pasteur proved that *P. multocida* was the causative agent of FC and showed that the disease could be alleviated by vaccination with a laboratory-attenuated strain. *P. multocida* remains a significant pathogen with major economic and wildlife management consequences. Available vaccines are either inefficacious (bacterins) or may cause clinical disease (live vaccines), and understanding of pathogenesis at the molecular level remains far from complete. However, genes of likely importance for virulence have been identified (Fuller et al. 2000; Hunt et al. 2001), and the recent availability of the genome sequence of a *P. multocida* strain has allowed identification of large gene sets critical for *P. multocida* growth *in vivo* and in nutrient-limited media (Paustian et al. 2001; Boyce et al. 2002; Paustian et al. 2002; fig. 20.1). Combined transcriptome and proteome analysis of the organism's response to various conditions should allow the functional characterization of genes. The ability to make directed mutations would significantly increase our ability to characterize putative virulence genes. Complete sequence information for other *Pasteurella* species and the strains associated with specific diseases would allow comparisons that would lead to a deeper understanding of the pathogenesis and host specificity of the unusually wide range of disease syndromes caused by these organisms.

MANNHEIMIA

The genus *Mannheimia* is a member of the family Pasteurellaceae, which traditionally includes the genera *Haemophilus, Actinobacillus,* and *Pasteurella* (Pohl 1981). Most members of the *HAP* family are mucosal commensals of mammals, including humans, and birds; however, many are opportunistic pathogens and can cause diseases under appropriate conditions.

The type member of the genus *Pasteurella* is *Pasteurella haemolytica*, which produces a weak hemolytic phenotype on sheep's blood agar plates. *P. haemolytica* has historically been classified into 16 serotypes based on indirect hemagglutination tests against extractable capsular surface antigens (Biberstein 1978). The genus was further divided into two biotypes, A and T, based on the ability to ferment arabinose and trehalose, respectively (Smith 1961; Lo and Shewen 1992). In total, there were 12 A serotypes and 4 T serotypes. In 1995, Younan and Fodar reported the isolation of *P. haemolytica* serotype (A17). In 1990, using data from DNA-DNA hybridization studies, biochemical properties, and genetic analyses, the T biotypes (serotypes T3, T4, T10, and T15) were reclassified as a separate species named *Pasteurella trehalosi* (Bingham et al. 1990; Sneath and Stevens 1990). In 1999, using data from ribotyping, multilocus enzyme electrophoresis, 16S rRNA sequence comparison, and DNA-DNA hybridization, the *P. haemolytica* A serotypes were reclassified into a new genus *Mannheimia* to distinguish them from *Pasteurella multocida* (Angen et al. 1999b). Hence, the previous A serotypes of *P. haemolytica* (A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17) are now renamed as *M. haemolytica*. The remaining A serotype, A11, which is unrelated to *M. haemolytica,* was renamed *M. glucosida* (Angen et al.

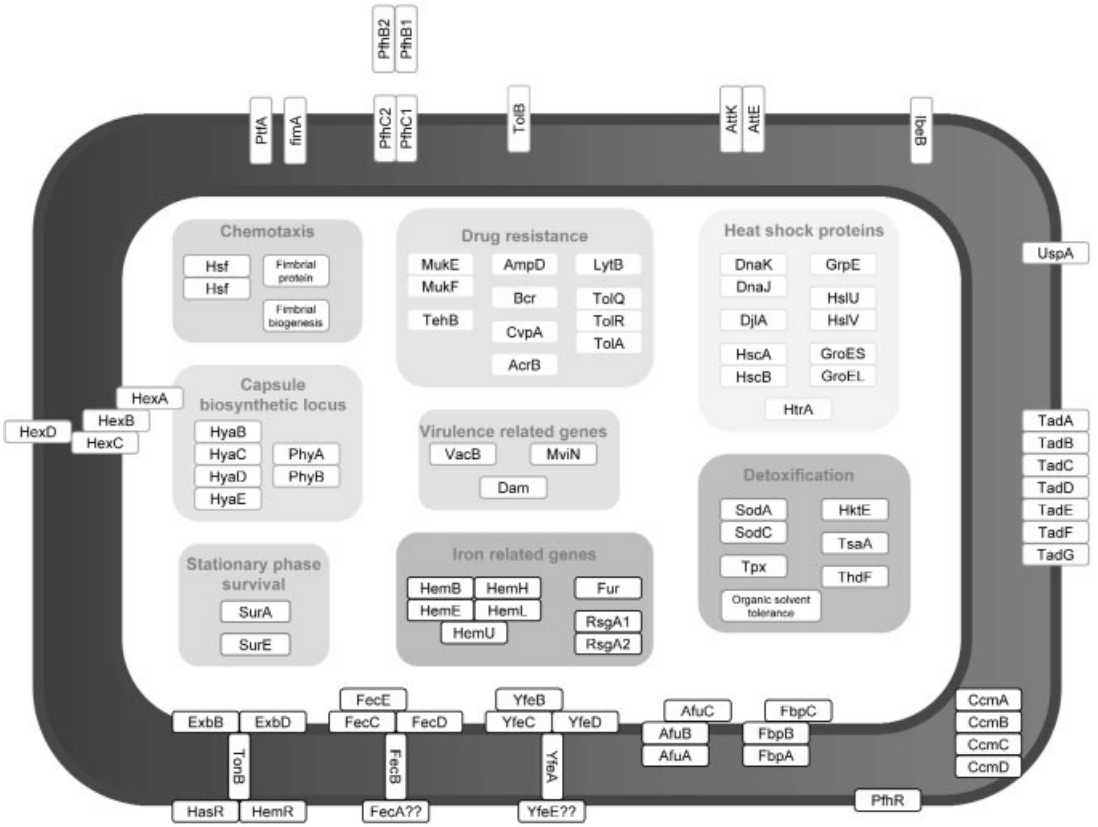


Figure 20.1. *P. multocida* putative virulence factors. (Courtesy of V. Kapur, B. May, and M. Paustian)

1999a). There are currently five species recognized within the genus *Mannheimia* (*M. haemolytica*, *M. glucosida*, *M. granulomatis*, *M. ruminalis,* and *M. varigena*), however, only *M. haemolytica* will be discussed further.

Characteristics of the Organism

M. haemolytica is a bipolar staining, gram-negative facultative anaerobic capsulated, rod-shaped bacterium with average sizes of 1–2 x 0.5 μm. It is nonmotile and catalase and oxidase positive, and it ferments glucose and other carbohydrates, producing acid but no gas. The temperature range for growth is between 22 and 44°C, with the optimum at 37°C. They are negative in the methyl-red, Voges-Proskauer, and gelatinase tests. The genome consists of a single circular double-strand DNA of 2.4 Mbp. The mol % G + C content of the DNA is about 40%. Recently, the complete genome has been determined and the nucleotide sequence data are available at the following web site: http://www.hgsc.bcm.tmc.edu. Occasionally, a small plasmid (approximately 4.2 kb) may be found in some strains. This plasmid is responsible for the streptomycin and sulfonamide resistance phenotype in these strains.

Sources of the Bacterium

M. haemolytica occurs as a commensal on the mucous membranes of the upper respiratory and digestive tracts of healthy animals and birds. *M. haemolytica* can be recovered frequently from cattle, sheep, goats, and occasionally from the upper respiratory tract of chickens and turkeys. It is the principle microorganism responsible for bovine pneumonic pasteurellosis in North America. It is also responsible for pneumonia and septicemia in sheep in the United Kingdom (Gilmour and Gilmour 1989). It has also been shown to cause fatal pneumonia in bighorn sheep in the wild (Foreyt 1989).

Bacterial Virulence Factors

A number of factors that contribute to the virulence of *M. haemolytica* A1 have been identified

and characterized. These are described in table 20.2. Some of the properties and possible role(s) of these virulence factors in pathogenesis have been obtained through molecular analysis of the genetic loci involved in production of these factors. However, the direct involvement of most of these factors in virulence has not been conclusively demonstrated because of difficulties in the construction of defined mutants for animal experimentation. Nevertheless, some of these virulence factors are currently being pursued as vaccine components.

Leukotoxin (Lkt)

Markham and Wilkie (1980) observed that cell-free culture supernatant of *M. haemolytica* A1 can impair phagocytosis at low concentrations and are cytotoxic at high concentrations. Subsequently, it was discovered that the supernatant contains a heat-labile leukotoxin (Lkt) secreted by *M. haemolytica* A1 during exponential growth (Shewen and Wilkie 1985). The genetic element for production of Lkt has been cloned and characterized (Lo et al. 1985, 1987; Strathdee and Lo 1989). It contains four genes (*lktC, A, B, D*) arranged in an operon. The gene *lktA* is the structural gene for the leukotoxin, *lktC* codes for an enzyme, which acylates LktA to the active toxin. The genes *lktB* and *lktD* code for a type II secretion system that secretes the toxin. This *lkt* genetic determinant is highly homologous to the cognate genes of the α-hemolysin operon of *Escherichia coli* (Strathdee and Lo 1987). The encoded proteins are also highly similar and have been shown to be antigenically cross-reactive. These two toxins form the basis for establishing the RTX "repeats in toxin" family due to the presence of a series of glycine-rich nanopeptide repeats in the toxin (Welch et al. 1995). The RTX toxins have been found in all 16 serotypes of *P. haemolytica* (Burrows et al. 1993) and other bacterial pathogens including the human periodontal pathogen *Actinobacillus actinomycetemcomitans* (Kolodrubetz et al. 1989); the swine pathogens *Actinobacillus pleuropneumoniae*, *Actinobacillus lignieresii,* and *Actinobacillus suis* (Burrows 1993); as well as human pathogens *Bordetella pertussis* (Glaser et al. 1988)*, Morganella morganii, Proteus vulgaris* (Koronakis et al. 1987), and *Neisseria meningitidis* (Thompson et al. 1993).

The *M. haemolytica* Lkt has a very narrow target-cell specificity; being cytotoxic against ruminant leukocytes (Shewen and Wilkie 1982; Adlam 1989). It has been shown that the bovine integrin CD18 and CD11a/CD18 molecules are the receptors that mediate the binding of Lkt to the target cells (Li et al. 1999). The cytotoxicity of Lkt on target cells is a result of the insertion of the toxin into the plasma membrane and the formation of transmembrane pores of approximately 0.9 to 3 nm in diameter (Clinkenbeard et al. 1989). The target cells are then rendered permeable to uncontrolled ion and water influx resulting in cell lysis.

Table 20.2. Virulence Factors of *M. haemolytica*

	Activity and role in pathogenesis	References
Leukotoxin	Pore forming cytolysin destroy leukocytes	Shewen and Wilkie 1982
O-sialoglycoprotease	Cleavage of glycoproteins from macrophages or other leukocytes	Abdullah et al. 1992
Neuraminidase	Removal of sialic acid from host glycoproteins	Uhlich et al. 1993
Iron regulated outer-membane proteins	Iron acquisition	Ogunnariwo and Schryvers 1990
Lipopolysaccharide	Modulate leukocyte activities Endotoxic activity	Paulsen et al. 1990; Whiteley et al. 1992
Capsular polysaccharide	Masking of cell surface Prevention of phagocytosis Resistance to complement lysis	Brogden et al. 1989; Czuprynski et al. 1989; Chae et al. 1990
Fimbriae/adhesin	Attachment and colonization	Lo, unpublished
Superoxide dismutase	Survival from oxidative burst	Lainson 1996

Lkt is considered to be the single most important virulence factor in the pathogenesis of shipping fever pneumonia. An *M. haemolytica* A1 *lkt* mutant was unable to lyse bovine lymphoma cells or sheep erythrocytes compared to the weak hemolytic activity of the parent strain (Chidambaram et al. 1995; Murphy et al. 1995). Further, calves challenged with an *lkt* mutant showed significantly less mortality and lung pathology than calves challenged with the parent strain (Petras et al. 1995). Conversely, calves vaccinated with a crude culture supernatant vaccine enriched with rLkt showed reduced mortality and lung pathology in a vaccine and challenge experiment (Conlon et al. 1991). These results are consistent with a crucial role for Lkt in the virulence of *M. haemolytica* A1 and in the induction of a protective immune response.

Sialoglycoprotease (Gcp)

M. haemolytica A1 secretes a metallo-endopeptidase into the culture supernatant, which is specific for *O*-sialoglycoproteins (Abdullah et al. 1992). The enzyme is specific for proteins that have *O*-sialoglycans attached to serine or threonine residues (Abdullah et al. 1992). Convalescent sera from cattle exhibits Gcp neutralizing activity, suggesting that Gcp is immunogenic in the animals (Lee et al. 1994). The targets for the Gcp have not been identified, but are likely sialoglycoproteins on the surface of mucosal epithelial cells, alveolar macrophages, alveolar neutrophils, or other leukocytes. Even though the role of Gcp in pathogenesis has not been demonstrated, it has been suggested that the activity of Gcp interferes with neutrophil adhesion by the cleavage of *O*-sialoglycoprotein from cellular adhesion molecules. Alternatively, Gcp may interfere with the host immune response by cleavage of serum IgG molecules or may aid in bacterial colonization (Mellors and Lo 1995). Recently, vaccination with rGcp induced protection in cattle against pneumonic challenge with *M. haemolytica* A1 (Shewen et al. 2003).

Neuraminidase

The enzyme neuraminidase is produced by 15 of the 16 classical *P. haemolytica* serotypes (Straus et al. 1993b). Neuraminidase is produced by *P. haemolytica in vivo* since antineuraminidase antibodies can be found in cattle serum after transthoracic infection with the bacterium (Straus and Purdy 1994). Although the role of neuraminidase in bovine pneumonia is unknown, this enzyme has been implicated as a virulence factor in several mucosal pathogens. It has been reported that *M. haemolytica* A1 adheres to bovine nasal mucus, which can be altered by enzymatic degradation of protein and carbohydrate components of the mucus (Uhlich et al. 1993). The protective activity of salivary secretion is reduced when sialic acid residues are removed from the salivary glycoproteins. The *M. haemolytica* neuraminidase may exert a pathogenic effect by removing sialic acid residues from host glycoproteins, thereby reducing the protective effects of mucus while enhancing adherence to mucosal epithelium (Confer et al. 1995). Further, the production of neuraminidase by *M. haemolytica* A1 parallels bacterial growth *in vitro* and is maximal in the stationary phase (Straus et al. 1993a), suggesting a possible role during an infection.

Iron-Regulated Outer Membrane Proteins (IROMP)

It has been shown that *M. haemolytica* produces a number of outer membrane proteins in respond to iron depletion (Donachie and Gilmour 1988). These IROMPs are recognized by convalescent bovine (and ovine) serum and demonstrated that they are antigenic and expressed *in vivo* (Deneer and Potter 1989). Three IROMPs of 71, 77, and 100 kDa expressed by *M. haemolytica* A1 grown under iron restriction *in vitro* have been characterized (Deneer and Potter 1989; Morck et al. 1991; Confer et al. 1995). The 71- and 100-kDa IROMPs have been identified as the bovine-specific transferrin-binding proteins (Tbps), which are responsible for iron acquisition (Ogunnariwo and Schryvers 1990). The genes coding for TbpA and TbpB have been cloned and characterized (Ogunnariwo et al. 1997). *tbpA* codes for the 100-kDa TbpA protein and *tbpB* codes for the 71-kDa TbpB lipoprotein. Genetic manipulation of the *tbpA/tbpB* genes to produce recombinant Tbps in *Escherichia* have subsequently shown Tbps to be effective vaccine candidates in a vaccine trial and challenge study (Potter et al. 1999).

Lipopolysaccharide (LPS)

The LPS of *M. haemolytica* A1 is structurally similar to that of typical gram-negative bacteria. The O antigen structure is composed of repeating units of -3)- *p* -D-galactose-*p*-(1,3)-β-D-galactosamine-(1,4)-β-D-galactose-*p*-(1- (Lacroix et al. 1993). This O-antigen structure was found to be accessible to the host's immune system since it was found on the surface of encapsulated *M. haemolytica* A1 cells (Wilson et al. 1992). The LPS of *M. haemolytica*

exhibits activities typical of gram-negative endotoxin including: pyrogenicity, macrophage activation, induction of tumor necrosis factor, activation of the coagulation cascade, platelet aggregation, and induction of hypotensive shock (Paulsen et al. 1989). Intrabronchial administration of purified *M. haemolytica* LPS causes neutrophil and fibrin exudation, pulmonary edema, and platelet aggregation in capillaries (Paulsen et al. 1990), implicating LPS as a major cause of microvascular necrosis and thrombosis (Whiteley et al. 1992).

The involvement of the *M. haemolytica* LPS in pathogenesis could be the result of modulating bovine leukocyte functions and stimulation of the host inflammatory response, thereby exacerbating host tissue damage. The LPS mediates the release of proinflammatory cytokines, lipid mediators, procoagulants, oxygen radicals, and proteases from bovine monocytes and macrophages (Whiteley et al. 1992). Acute lung injury results when increasing numbers of recruited neutrophils adhere to endothelial cells and degranulation occurs (Paulsen et al. 1989). Recently, it has been suggested that Lkt is complexed with LPS, accounting for some of the proinflammatory effects of sublethal concentrations of Lkt (Li and Clinkenbeard 1999).

Capsular Polysaccharide (CPS)

The serotype-specific CPS of *M. haemolytica* A1 consist of a polysaccharide structure with the repeating unit of: -3)-O-ManNAcA-(β-1-4)-ManNAc-β1- (Adlam et al. 1984; Adlam, 1989). CPS can be detected in lungs, airways, alveoli, and alveolar macrophages of animals infected by *M. haemolytica* (Whiteley et al. 1990). The production of CPS *in vitro* has been shown to be growth-phase dependent, with the greatest encapsulation observed in exponential phase cells (Corstvet et al. 1982). The involvement of CPS in pathogenesis is likely indirect since it does not appear to mediate any tissue damage. CPS may be involved in adherence of the bacteria to alveolar walls (Brogden et al. 1989), the attraction of neutrophils to infection sites, and protection of the bacteria from complement-mediated serum killing (Czuprynski et al. 1989; Confer et al. 1990).

The genetic cluster involved in the biosynthesis of CPS in *M. haemolytica* A1 has been characterized (Lo et al. 2001). The cluster contains 12 genes and is organized in a manner typical of the group II CPS biosynthetic clusters of gram-negative bacterium. Further, an acapsular mutant of *M. haemolytica* A1 has been constructed by the interruption of two genes within the cluster (McKerral and Lo 2002). Animal challenge studies using this acapsular mutant are being carried out to define the role of CPS in pathogenesis.

Fimbriae and Adhesins

Structures resembling fimbriae (5 and 12 nm in diameter) have been observed by electron microscopy on some *M. haemolytica* A1 isolates from lung washings of experimentally infected animals (Morck et al. 1987, 1989). However, these structures have not been characterized in detail and their presence does not appear to be a universal feature of *M. haemolytica* isolates (van Alphan 1995). Therefore, there is no evidence that *M. haemolytica* produces fimbriae that play any role in pathogenesis.

Recently, a high molecular weight (Hmw) adhesin protein (molecular mass of >250 kDa) has been identified in *M. haemolytica* A1 (Lo, manuscript in preparation). The Hmw adhesin protein of *M. haemolytica* A1 is similar to the high molecular weight adhesins characterized in nonpiliated, nontypeable strains of *Haemophilus influenzae* (NTHi). The prototype of these high molecular weight adhesins is a highly immunogenic 115-kDa protein (Hia), which mediates adhesion of NTHi to conjunctival epithelium (Barenkamp and St. Geme III 1996). Other members of these Hmw adhesins include HMW1 and HMW2 of NTHi, the LspA1 and LspA2 proteins of *H. ducreyi,* and FHA of *Bordetella pertussis* (Barenkamp and Bodor 1990; Barenkamp and Leininger 1992; Ward et al. 1998). In most NTHi strains that lack fimbriae and Hia, the HMW adhesions mediate attachment to human epithelial cells and mononuclear cells (Noel et al. 1994). It has also been demonstrated that purified HMW adhesion proteins from NTHi can be used to immunize animals to stimulate a protective immune response (Barenkamp 1996). The *M. haemolytica* A1 Hmw adhesin exhibits domains similar to those in HMW and Hia from NTHi and can be detected by immunoblot using antibodies against the HMW proteins (manuscript in preparation). It is likely that this Hmw adhesin is involved in bacteria–epithelial cell interactions during an infection. Research into the role of this Hmw adhesin in the attachment of *M. haemolytica* A1 to target cells is currently in progress.

Superoxide Dismutase (SOD)

When neutrophils are damaged by the *M. haemolytica* leukotoxin during an infection, they generate oxidative burst and produce superoxide radical

anions (Czuprynski 1995). The superoxide radicals promote formation of toxic hydroxide radicals, which can depolymerize polysaccharides, cause DNA lesions, inactivate enzymes, and the destruction of membranes (Weinberg 1999). Although these activities can result in extensive host tissue damage, they are also detrimental to the bacteria. SOD is a metallo-enzyme that catalyzes the detoxification of oxygen free radicals to form hydrogen peroxide and molecular oxygen (Braun 1997). It has been reported that *M. haemolytica* A2 and A7 produce a periplasmic SOD, which dismutate exogenously generated superoxide and may enable bacterial survival from the oxidative burst of the host neutrophils during an infection (Lainson 1996).

Pathogenesis

Overview

Bovine respiratory disease is an economically significant disease of cattle, accounting for approximately 30% of the total cattle deaths globally, and is associated with an annual economic loss of over one billion dollars in North America alone (Griffin 1997). The major cause of death is pneumonic pasteurellosis, an acute fibrinous pneumonia that often occurs within days of cattle transport. *M. haemolytica* A1 is the predominant serotype recovered from pneumonic lesions in naturally occurring cases of shipping fever, though serotypes 2, 5, 6 are sometimes recovered. Shipping fever is characterized by fever, cough, respiratory distress, and nasal discharge. Morbidity is high due to substantial weight loss, obstruction of bronchioles due to fibrinous exudate and deposition of necrotic debris, accumulation of macrophages and fibrin in the alveoli, and subsequent thrombosis and lymphatic vessel distention (Friend et al. 1977b). At necropsy, the disease is typified by an acute fibrinous bronchopneumonia of the lung tissue. The disease can be rapidly fatal, making expeditious detection and treatment important. Prevention of disease by vaccination is the focus of many research efforts.

Interactions Between the Bacterium and Host Defenses

The pathogenesis of pneumonic pasteurellosis is complex and poorly understood. *M. haemolytica* A1 is a normal commensal of the nasopharyngeal tract of healthy cattle and sheep (Frank et al. 1986). Small numbers of the microorganism, which are routinely inhaled in aerosolized droplets, are removed by the clearing mechanisms in the alveolar (Lillie and Thomson 1972). However, when the animals are under stress, such as during transport, clearance of the bacteria does not occur efficiently and the animals rapidly succumb to the disease (Lopez et al. 1976). In addition, concurrent infection with other bacteria and respiratory viruses further impair the clearing mechanism, resulting in proliferation and colonization of *M. haemolytica* in the lungs (Frank et al. 1986; Jericho 1989). The characteristics of bovine pneumonic pasteurellosis is extensive influx and death of polymorphonuclear neutrophils and macrophages within the pulmonary alveoli (Friend et al. 1977b; Rehmtulla and Thomson 1981).

The activity of Lkt on leukocytes prevents the host cells from phagocytosing invading bacteria and increases the proinflammatory response (Clinkenbeard et al. 1989). At sublethal doses, Lkt stimulates bovine neutrophils and mononuclear phagocytes to produce reactive oxygen intermediates, to degranulate, and to secrete proinflammatory sicosanoids and cytokines (Czuprynski and Ortiz-Carranza 1992; Czuprynski 1995). These inflammatory mediators act synergistically to increase neutrophil influx and potentiate the magnitude of tissue necrosis, contributing to tissue damage of the lung (Confer et al. 1995). It has also been reported that sublethal doses of Lkt may also cause bovine leukocytes to undergo apoptosis (Stevens and Czuprynski 1996; Leite et al. 1999). The net result of these Lkt activities is to interfere with normal host immune function and to cause severe inflammation of the lung resulting in fibrinous bronchopneumonia.

Influence of Environmental Factors

When cattle are stressed by environmental factors such as crowding, change of climate, change of diet, or movement in a truck or train associated with shipping of the animals from the farm to feedlots, the pulmonary clearance activity within the lungs is impaired. A concurrent viral infection associated with mixing and transporting animals from different sources further reduces the immune capability of the animal (Jacab and Green 1976). This facilitates colonization and establishment of the disease (Thomson et al. 1969).

The process by which the lung is colonized by *M. haemolytica* A1 is not clearly understood, but it has been suggested that an increase in the number of bacteria in the nasal cavity results in entry of the bacterium into the lungs resulting in the development of pneumonia (Jericho et al. 1986). The

healthy animal is able to remove the invading bacterium (Lillie and Thompson 1972), but the pulmonary clearance may be impaired in animals for reasons described above. Gilka et al. (1974) showed the importance of alveolar macrophages as the primary phagocytic cell that can engulf *M. haemolytica* after aerosol exposure. It was shown that healthy animals were able to clear 75% of aerosolized *M. haemolytica* A1 within 2 h, 95% within 4 h, and 92% within 8 h of exposure. The ability of the lung macrophages to engulf invading *M. haemolytica* A1 has been demonstrated to be increased in the presence of opsonizing antibodies *in vitro* (Maheswaran et al. 1980; Markham and Wilkie 1980). The similar activity was demonstrated for neutrophils that can phagocytose oposonized bacterium more efficiently (Czuprynski et al. 1987). Once ingested, the bacterium are killed within 1 to 4 h. Impairment of the phagocytic capability of the macrophages and neutrophils therefore predisposes the lung to colonization by *M. haemolytica* through reduction in bacterial clearance and/or immunosuppression (Frank et al. 1987).

Lesions

Clinically, pneumonic pasteurellosis in cattle is characterized by depression, anorexia, fever, nasal discharge, and bronchial/pleuritic sounds over the anterior-ventral lung lobes. Pathological features include bronchiolitis, with the infiltration of neutrophils and the accumulation of both macrophages and fibrin in the alveolar spaces. In addition, there is distension of the lymphatic vessels by both neutrophils and fibrin, with small areas of coagulation necrosis, which might develop into liquefaction necrosis (Friend et al. 1977a; Rehmtulla and Thomson 1981).

IMMUNITY

The most common approach to examine immune response in animals against *M. haemolytica* is to correlate antibody titers to a particular antigen and resistance to infection by the bacterium, or severity of the pneumonia. Numerous secreted and surface molecules of *M. haemolytica* A1 have been characterized and examined for their roles in protection and immunity against the bacterial infection (Confer 1993; McBride et al. 1996). Despite the severe clinical effects caused by LPS, serum antibody titers to *M. haemolytica* A1 LPS do not correlate to protection (Confer and Simons 1986). Similarly, the use of *M. haemolytica* A1 CPS preparations in a vaccine does not provide adequate immunity and protection against pneumonia (Confer et al. 1989; Conlon and Shewen 1993). On the other hand, the leukotoxin has been shown to be highly immunogenic, and animals with high Lkt-neutralizing antibodies have a higher resistance to pneumonia both in the field and in experimental studies (Cho et al. 1984; Gentry et al. 1985). However, the high titer of Lkt-neutralizing antibodies alone is not sufficient to provide adequate protection against *M. haemolytica* A1 infection (Shewen and Wilkie 1988; Conlon et al. 1991). It has been shown that animals vaccinated with rGcp have a lower degree of pneumonic lung tissue compared to control animals, indicating Gcp as a potentially protective immunogen (Shewen et al. 2003). As mentioned above, the IROMPs, in particular the transferrin-binding proteins, have been shown to be an important immunogen in experimental studies (Gilmour et al. 1991; Potter et al. 1999). In addition, Moiser et al. (1989) identified 13 saline-extractable surface antigens that were recognized by convalescent serum and were correlated to resistance in experimental challenge. Weldon et al. (1994) reported that a high antibody titer against a 6-kDa antigen was correlated to resistance to pneumonia. It turned out that the 6-kDa antigen is a partial fragment of a larger protein. The gene that codes for the entire 60-kDa antigen has been cloned and characterized (Lo and Mellors 1996). Other reports of antigens from *M. haemolytica* A1 that are correlated to resistance include: the lipoproteins PlpE (Pandher et al. 1998); surface or membrane associated proteins (Cooney and Lo 1993; Frank et al. 1994; Confer et al. 1985a; Pandher et al. 1999), and capsular polysaccharide (McVey et al. 1990). Therefore, there is a collection of potential antigens that could elicit a high antibody response in the animals and could be developed as components of an efficacious vaccine (McVey et al. 1989).

A number of commercial vaccines have been developed for the prevention of pneumonic pasteurellosis. Formalinized whole-cell preparations (bacterins) have been in use for almost 70 years. However, field trials and experimental studies have shown that the bacterin vaccine is not effective. In fact, it may cause an adverse effect resulting in a higher incidence of pneumonia and more severe pulmonary lesions in the vaccinated animals compared to unvaccinated controls (Confer et al. 1987; Mosier et al. 1989). This is due to the inability of the bacterin to induce an antileukotoxin response in the animals. In naturally infected animals, immunity to pneumonic pasteurellosis has been correlated with a

high serum antibody titer to both surface antigens and antileukotoxic activity (Shewen and Wilkie 1983). Recently, vaccines based on extractable surface antigens of *M. haemolytica* A1 have been shown to be more effective in protection (Confer et al. 1985a; 1989; Durham et al. 1986). Vaccine based on live *M. haemolytica* A1 has also been shown effective in experimental challenge studies (Confer et al. 1985b, 1986; Mosier et al. 1989). A vaccine based on cell-free culture supernatant containing soluble antigens harvested during logarithmic growth phase has been shown to be 60–70% effective in both experimental studies and field trials (Jim et al. 1988; Shewen et al. 1988; Shewen and Wilkie 1988). The efficacy of this vaccine can be enhanced by supplementation with recombinant leukotoxin produced from *Escherichia coli* (Conlon et al. 1991).

Conclusion

Within the last two decades, research into the pathogenesis of *M. haemolytica* and shipping fever has been greatly advanced with the use of molecular techniques. The nucleotide sequence of the entire genome of the bacterium has now been determined and is undergoing annotation to identify all of the encoded functions. This identification of all of the genes and their functions should provide researchers with a framework to focus on genes/functions that play important roles in pathogenesis. In addition, DNA microarrays that contain all of the encoded genes are being designed. The use of microarray technology will be important in the analysis of gene expression under various conditions that affect different stages of infection and disease.

The genetic analysis of *M. haemolytica* has been hampered by the lack of tools to construct specific mutations in the bacterium. Without defined mutants, it is not possible to carry out experiments on each virulence factor to the satisfaction of molecular Koch's postulates (Falkow 1988). Procedures have been developed for the isolation of specific knockout mutations in *M. haemolytica* by allelic replacement (Murphy and Whitworth 1994) and using a plasmid-based selection (Fedorova and Highlander 1997). Following this procedure, mutations in the *lkt* operon (Fedorova and Highlander 1997) and in the CPS biosynthetic operon (McKerral and Lo 2002) have been created. A chloramphenicol cassette with steady expression of Cm^R has been designed for stable selection of this antibiotic resistance as a selectable marker in *M. haemolytica* (McKerral and Lo 2002). This development of a genetic approach for the construction of defined mutations also will greatly facilitate continuing studies of the bacterium.

Finally, novel approaches to develop an effective vaccine against bovine pneumonic pasteurellosis are on the way. Current available vaccines are based primarily on soluble or extractable surface antigens of *M. haemolytica*. These vaccines have a field efficacy of approximately 75%. The animals are immunized by injection, which is labor intensive. Alternatives to needle injection for immunization are being considered. An *aroA* mutant that has impaired growth in animals has been created as the basis of a live vaccine (Homchampa et al. 1994; Tatum et al. 1994). Another mutant that has a deletion within the *lkt* genes is also being considered for use a live vaccine. The expression of *M. haemolytica* antigens in transgenic forage crops to be used as an edible vaccine is also being developed (Lee et al. 2001).

There have been great advances in the understanding of the basic biology of *M. haemolytica*, its virulence factors, and the pathogenesis of the disease it causes. It is anticipated that there will be continuing development and accumulation of knowledge, and perhaps an effective control of this bacterium in the next decades to come.

REFERENCES

Aalbaek, B., L. Eriksen, R. B. Rimler, P. S. Leifsson, A. Basse, T. Christiansen, and E. Eriksen. 1999. Typing of *Pasteurella multocida* from haemorrhagic septicaemia in Danish fallow deer (*Dama dama*). APMIS 107:913–920.

Abdullah, K. M., E. A. Udoh, P. E. Shewen, and A. Mellors. 1992. A neutral lycoprotease of *Pasteurella haemolytica* A1 specifically cleaves *O*-sialoglycoproteins. Infect. Immun. 60:56–62.

Adlam, C. 1989. The structure, function and properties of cellular and extracellular components of *Pasteurella haemolytica*. In *Pasteurella* and *Pasteurellosis.* C. Adlam and J. M. Rutter (eds.). Toronto: Academic Press, pp.75–92.

Adlam, C., J. M. Knights, J. C. Mugridge, J. C. Lindon, P. R. W. Baker, J. E. Beesley, B. Spacey, G. R. Craig, and L. K. Nagy. 1984. Purification, characterization and immunological properties of the serotype-specific capsular polysaccharide of *Pasteurella haemolytica* (serotype 1) organisms. J. Gen. Microbiol. 130:2415–2426.

Al-Haddawi, M. H., S. Jasni, M. Zamri-Saad, A. R. Mutalib, I. Zulkifli, R. Son, and A. R. Sheikh-Omar. 2000. In vitro study of *Pasteurella multocida*

adhesion to trachea, lung and aorta of rabbits. Vet. J. 159:274–281.

Amonsin, A., J. F. X. Wellehan, L. Li, J. Laber, and V. Kapur. 2002. DNA fingerprinting of *Pasteurella multocida* recovered from avian sources. J. Clin. Microbiol. 40:3025–3031.

Angen, O., M. Quirie, W. Donachie, and M. Bisgaard. 1999a. Investigations on the species specificity of *Mannheimia* (*Pasteurella*) *haemolytica* serotyping. Vet. Microbiol. 65:283–290.

Angen, O., R. Mutters, D. A. Caugert, J. E. Olsen, and M. Bisgaard. 1999b. Taxonomic relationships of the [*Pasteurella*] *haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov.. Int. J. Syst. Bacteriol. 49:67–86.

Baba, T. 1984. Cell-mediated immune protection in chickens against *Pasteurella multocida*. Res. Vet. Sci. 36:225–30.

Barenkamp, S. J. 1996. Immunization with high-molecular-weight adhesin proteins of nontypeable *Haemophilus influenzae* modifies experimental otitis media in chinchillas. Infect. Immun. 64:1246–1251.

Barenkamp, S. J., and E. Leininger. 1992. Cloning, expression and DNA sequence analysis of genes encoding nontypeable *Haemophilus influenzae* high-molecular-weight surface-exposed proteins related to filamentous hemagglutinin of *Bordetella pertussis*. Infect. Immun. 60:1302–1313.

Barenkamp, S. J., and F. F. Bodor. 1990. Development of serum bacteriocidal activity following nontypeable *Haemophilus influenzae* acute otitis media. Pediatr. Infect. Dis. 9:333–339.

Barenkamp, S. J., and J. W. St. Geme III. 1996. Identification of a second family of high-molecular-weight adhesion proteins expressed by nontypeable *Haemophilus influenzae*. Mol. Microbiol. 19:1215–1223.

Biberstein, E. L. 1978. Biotyping and serotyping of *Pasteurella haemolytica*. In Methods in microbiology. T. Bergan and J. R. Norris (eds.). London: Academic Press, pp. 253–269.

Bingham, D. P., R. Moore, and A. B. Richards. 1990. Comparison of DNA:DNA homology and enzymatic activity between *Pasteurella haemolytica* and related species. Am. J. Vet. Res. 51:1161–1166.

Birkbeck, T. H., L. A. Laidler, A. N. Grant, and D. I. Cox. 2002. *Pasteurella skyensis* sp nov., isolated from Atlantic salmon (*Salmo salar* L.). Int. J. Syst. Evol. Microbiol. 52:699–704.

Blackall, P. J., and J. K. Miflin. 2000. Identification and typing of *Pasteurella multocida*: a review. Avian Path. 29:271–287.

Blackall, P. J., N. Fegan, J. L. Pahoff, G. J. Storie, G. B. McIntosh, R. D. A. Cameron, D. O'Boyle, A. J. Frost, M. R. Bara, G. Marr, and J. Holder. 2000. The molecular epidemiology of four outbreaks of porcine pasteurellosis. Vet. Microbiol. 72:111–120.

Bosch, M., E. Garrido, M. Llagostera, A. M. Perez de Rozas, I. Badiola, and J. Barbe. 2002. *Pasteurella multocida exbB*, *exbD* and *tonB* genes are physically linked but independently transcribed. FEMS Microbiol. Lett. 210:201–208.

Boyce, J. D., and B. Adler. 2000. The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B:2). Infect. Immun. 68:3463–3468.

———. 2001. Acapsular *Pasteurella multocida* B:2 can stimulate protective immunity against pasteurellosis. Infect. Immun. 69:1943–1946.

Boyce, J. D., I. Wilkie, M. Harper, M. L. Paustian, V. Kapur, and B. Adler. 2002. Genomic-scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. Infect. Immun. 70:6871–6879.

Braun, V. 1997. Surface signaling: novel transcriptional initiation mechanism starting from the cell surface. Arch. Microbiol. 167:325–331.

Bredy, J. P., and R. G. Botzler. 1989. The effects of six environmental variables on *Pasteurella multocida* populations in water. J. Wild. Dis. 25:232–239.

Brogden, K. A., C. Adlam, H. D. Lehmkuhl, R. C. Cutlip, J. M. Knights, and R. L. Engen. 1989. Effect of *Pasteurella haemolytica* (A1) capsular polysaccharide on sheep lung *in vivo* and on pulmonary surfactant *in vitro*. Am. J. Vet. Res. 50:555–559.

Burrows, L. L. 1993. Molecular characterization of the RTX cytolysin determinants from Gram-negative pathogens of veterinary importance. Ph.D. Thesis. The Faculty of Graduate Studies, University of Guelph, Guelph.

Burrows, L. L., E. Olah-Winfield, and R. Y. C. Lo. 1993. Molecular analysis of the leukotoxin determinants from *Pasteurella haemolytica* serotypes 1 to 16. Infect. Immun. 61:5001–5007.

Carpenter, T. E., K. P. Snipes, R. W. Kasten, D. W. Hird, and D. C. Hirsh. 1991. Molecular epidemiology of *Pasteurella multocida* in turkeys. Am. J. Vet. Res. 52:1345–1349.

Carter, G. R. 1967. Pasteurellosis: *Pasteurella multocida* and *Pasteurella hemolytica*. Adv. Vet. Sci. 11:321–379.

Chae, C. H., M. J. Gentry, A. W. Confer, and G. A. Anderson. 1990. Resistance to host immune

defense mechanisms afforded by capsular material of *Pasteurella haemolytica*, serotype 1. Vet. Microbiol. 25:241–251.

Chanter, N. 1990. Molecular aspects of the virulence of Pasteurella multocida. Can. J. Vet. Res. 54 Suppl: S45–7.

Chanter, N., and J. M. Rutter. 1990. Colonisation by *Pasteurella multocida* in atrophic rhinitis of pigs and immunity to the osteolytic toxin. Vet. Microbiol. 25:253–265.

Chidambaram, M., B. Sharma, S. Petras, C. Reese, S. Froshauer, and G. Weinstock. 1995. Isolation of *Pasteurella haemolytica* leukotoxin mutants. Infect. Immun 63:1027–1032.

Cho, H. J., J. G. Bohac, W. D. G. Yates, and H. B. Ohman. 1984. Anticytotoxin activity of bovine sera and body fluids against *Pasteurella haemolytica* A1 cytotoxin. Can. J. Comp. Med. 48:151–155.

Chrisp, C. E., and N. T. Foged. 1991. Induction of pneumonia in rabbits by use of a purified protein toxin from *Pasteurella multocida*. Am. J. Vet. Res. 52:56–61.

Christensen, J. P., H. H. Dietz, and M. Bisgaard. 1998. Phenotypic and genotypic characters of isolates of *Pasteurella multocida* Obtained from backyard poultry and from two outbreaks of avian cholera in avifauna in Denmark. Avian Path. 27:373–381.

Chung, J. Y., I. Wilkie, J. D. Boyce, K. M. Townsend, A. J. Frost, M. Ghoddusi, and B. Adler. 2001. Role of capsule in pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. Infect. Immun. 69:2487–2492.

Clinkenbeard, K. D., D. A. Mosier, and A. W. Confer. 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. Infect. Immun. 57:420–425.

Collins, F. M., C. J. Niederbuhl, and S. G. Campbell. 1983. Bactericidal activity of alveolar and peritoneal macrophages exposed in vitro to three strains of *Pasteurella multocida*. Infect. Immun. 39:779–784.

Confer, A., R. Panciera, K. Clinkenbeard, and D. Mosier. 1990. Molecular aspects of virulence of *Pasteurella haemolytica*. Can. J. Vet. Res. 54 Suppl:S48–52.

Confer, A. W. 1993. Immunogens of *Pasteurella*. Vet. Microbiol. 37:353–368.

Confer, A. W., and K. R. Simons. 1986. Effects of *Pasteurella haemolytica* lipopolysaccharide on selected functions of bovine leukocytes. Am. J. Vet. Res. 47:154–157.

Confer, A. W., B. A. Lessley, R. J. Panciera, R. W. Fulton, and J. A. Kreps. 1985a. Serum antibodies to antigens associated with a saline extract of *Pasteurella haemolytica*: correlation with resistance to experimental bovine pneumonic pasteurellosis. Vet. Immunol. Immunopathol. 10:265–278.

Confer, A. W., K. D. Clinkenbeard, and G. L. Murphy. 1995. Pathogenesis and virulence of *Pasteurella haemolytica* in cattle: an analysis of current knowledge and future approaches. In W. Donachie, F. A. Lainson, and J.C. Hodgson (eds.). *Haemophilus, Actinobacillus* and *Pasteurella*. New York: Plenum Press, pp.51–62.

Confer, A. W., K. R. Simons, R. J. Panciera, A. J. Mortand, and D. A. Mosier. 1989. Serum antibody response to carbohydrate antigens of *Pasteurella haemolytica* serotype 1: Relation to experimentally induced bovine pneumonic pasteurellosis. Am. J. Vet. Res. 50:98–105.

Confer, A. W., R. J. Panciera, M. J. Gentry, and R. W. Fulton. 1986. Immunologic response and resistance to experimentally induced pneumonic pasteurellosis immunized with various dosages of lyophilized *Pasteurella haemolytica*. Am. J. Vet. Res. 47:1853–1857.

———. 1987. Immunologic response to *Pasteurella haemolytica* and resistance to experimental bovine pneumonic pasteurellosis induced by bacterins in oil adjuvants. Am. J. Vet. Res. 48:163–168.

Confer, A. W., R. J. Panciera, R. W. Fulton, M. J. Gentry, and J. A. Rummage. 1985b. Effect of vaccination with live or killed *Pasteurella haemolytica* on resistance to experimental bovine pneumonic pasteurellosis. Am. J. Vet. Res. 46:342–346.

Conlon, J. A., and P. E. Shewen. 1993. Clinical and serological evaluation of a *Pasteurella haemolytica* A1 capsular polysaccharide vaccine. Vaccine 11:767–772.

Conlon, J. A., P. E. Shewen, and R. Y. C. Lo. 1991. Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. Infect. Immun. 59:587–591.

Cooney, B. J., and R. Y. C. Lo. 1993. Three contiguous lipoprotein genes in *Pasteurella haemolytica* A1 which are homologous to a lipoprotein gene in *Haemophilus influenzae* type b. Infect. Immun. 61:4682–4688.

Corstvet, R. E., M. J. Gentry, P. R. Newman, J. A. Rummage, and A. W. Confer. 1982. Demonstration of age-dependent capsular material on *Pasteurella haemolytica* serotype 1. J. Clin. Microbiol. 16:1123–1126.

Czuprynski, C. J. 1995. Modulation of leukocytes by exotoxins produced by HAP organisms. In *Haemophilus, Actinobacillus* and *Pasteurella*. W. Donachie, F. A. Lainson, and J. C. Hodgson (eds.). New York: Plenum Press, pp. 143–152.

Czuprynski, C. J., and O. Ortiz-Carranza. 1992. *Pasteurella haemolytica* leukotoxin inhibits mitogen-induced bovine peripheral blood mononuclear cell proliferation *in vitro*. Microb. Pathog. 12:459–463.

Czuprynski, C. J., E. J. Noel, and C. Adlam. 1989. Modulation of bovine neutrophil antibacterial activities by *Pasteurella haemolytica* A1 purified capsular polysaccharide. Microb. Pathog. 6:133–141.

Czuprynski, C. J., H. L. Hamilton, and E. F. Noel. 1987. Ingestion and killing of *Pasteurella haemolytica* A1 by bovine neutrophils *in vitro*. Vet. Microbiol. 14:61–74.

de Alwis, M. C. L. 1992. The epidemiology of haemorrhagic septicaemia in Sri Lanka. The International Workshop on Pasteurellosis in Production Animals, Bali, Indonesia, Australian Centre for International and Agricultural Research.

DeAngelis, P. L., N. S. Gunay, T. Toida, W-j. Mao, and R. J. Linhardt. 2002. Identification of the capsular polysaccharides of Type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively. Carbohydr. Res. 337:1547–1552.

Deneer, H., and A. Potter. 1989. Iron-repressible outer-membrane proteins of *Pasteurella haemolytica*. J. Gen. Microbiol. 135:435–443.

Dewhirst, F. E., B. J. Paster, I. Olsen, and G. J. Fraser. 1993. Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. Int. J. Med. Microbiol. 279:35–44.

Diallo, I. S., and A. J. Frost. 2000. Survival of avian strains of Pasteurella multocida in chicken serum. Vet. Microbiol. 72:153–161.

Donachie, W., and N. J. L. Gilmour. 1988. Sheep antibody responses to cell wall antigens expressed in vivo by *Pasteurella haemolytica* serotype A2. FEMS Microbiol. Letts. 56:271–276.

Durham, J. A., A. W. Confer, D. A. Mosier, and B. A. Lessley. 1986. Comparison of antigens associated with saline solution, potassium thiocynate and sodium salicylate extract of *Pasteurella haemolytica* serotype 1. Am. J. Vet. Res. 47:1946–1951.

Falkow, S. 1988. Molecular Koch's postulates applied to microbial pathogenicity. Rev. Infect. Dis. 10, suppl.2:S274–S276.

Fedorova, N. D., and S. K. Highlander. 1997. Generation of targeted nonpolar gene insertions and operon fusions in *Pasteurella haemolytica* and creation of a strain that produces and secretes inactive leukotoxin. Infect. Immun. 65:2593–2598.

Foreyt, W. J. 1989. Fatal *Pasteurella haemolytica* pneumonia in bighorn sheep after direct contact with clinically normal domestic sheep. Am. J. Vet. Res. 50:341–344.

Frank, G. H., R. E. Briggs, and K. G. Gillette. 1986. Colonization of the nasal passages of calves with *Pasteurella haemolytica* serotype 1 and regeneration of colonization after experimentally induced viral infection of the respiratory tract. Am. J. Vet. Res. 47:1704–1707.

———. 1987. *Pasteurella haemolytica* serotype 1 colonization of the nasal passages of virus-infected calves. Am. J. Vet. Res. 48:1674–1677.

Frank, G. H., R. E. Briggs, R. W. Loan, C. W. Purdy, and E. S. Zehr. 1994. Serotype-specific inhibition of colonization of the tonsils and nasopharynx of calves after *Pasteurella haemolytica* serotype A1 after vaccination with the organism. Am. J. Vet. Res. 55:1107–1110.

Friend, S. C., R. G. Thomson, and B. N. Wilkie. 1977a. Bovine pneumonic pasteurellosis: experimental induction in vaccinated and nonvaccinated calves. Can. J. Comp. Med. 41:77–83.

———. 1977b. Pulmonary lesions induced by *Pasteurella haemolytica* in cattle. Can. J. Comp. Med. 41:219–223.

Fuller, T. E., M. J. Kennedy, and D. E. Lowery. 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. Microb. Pathog. 29:25–38.

Gentry, M. J., A. W. Confer, and R. J. Panciera. 1985. Serum neutralization of cytotoxin from *Pasteurella haemolytica* serotype 1 and resistance to experimental bovine pneumonic pasteurellosis. Vet. Immunol. Immunopathol. 9:239–250.

Gilka, F., R. G. Thomson, and M. Savan. 1974. The effect of edema, hydrocorisone acetate, concurrent viral infection and immunization on the clearance of *Pasteurella haemolytica* from bovine ling. Can. J. Comp. Med. 38:251–259.

Gilmour, N. J. L., and J. S. Gilmour. 1989. Pasteurellosis of sheep. In *Pasteurella* and pasteurellosis. C. Adlam and J. M. Rutter (eds.). London: Acad. Press, pp. 223–262.

Gilmour, N. J. L., W. Donachie, A. Sutherland, J. S. Gilmour, G. Jones, and M. Quirie. 1991. Vaccine containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. Vaccine 9:137–140.

Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullmann, and A. Danchin. 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. Mol. Microbiol. 2:19–30.

Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. In Veterinary clinics of North America: food animal practice. J.

Vestweber and G. St. Jean (eds.). Philadelphia: W. B. Saunders, pp. 367–377.

Hansen, L. M., and D. C. Hirsh. 1989. Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. Vet. Microbiol. 21:177–184.

Harmon, B. G., J. R. Glisson, K. S. Latimer, W. L. Steffens, and J. C. Nunnally. 1991. Resistance of *Pasteurella multocida* A:3,4 to phagocytosis by turkey macrophages and heterophils. Am. J. Vet. Res. 52:1507–1511.

Hassanin, H. H., T. E. Toth, M. Eldimerdash, and P. B. Siegel. 1995. Stimulation of avian respiratory phagocytes by *Pasteurella multocida*—effects of the route of exposure, bacterial dosage and strain, and the age of chickens. Vet. Microbiol. 46:401–413.

Heddleston, K. L., and P. A. Rebers. 1972. Fowl cholera: cross-immunity induced in turkeys with formalin-killed in-vivo-propagated *Pasteurella*. Avian Dis. 16:578–586.

Heddleston, K. L., P. A. Rebers, and G. Wessman. 1975. Fowl cholera: immunologic and serologic response in turkeys to live *Pasteurella multocida* vaccine administered in the drinking water. Poult. Sci. 54:217–221.

Homchampa, P., R. A. Strugnell, and B. Adler. 1994. Construction and vaccine potential of an *aroA* mutant of *Pasteurella haemolytica*. Vet. Microbiol. 42:35–44.

———. 1997. Cross protective immunity conferred by a marker-free *aroA* mutant of *Pasteurella multocida*. Vaccine 15:203–208.

Hopkins, B. A., and L. D. Olson. 1997. Comparison of live avirulent Pm-1 and Cu fowl cholera vaccines in turkeys. Avian Dis. 41:317–325.

Hopkins, B. A., T. H. M. Huang, and L. D. Olson. 1998. Differentiating turkey postvaccination isolants of *Pasteurella multocida* using arbitrarily primed polymerase chain reaction. Avian Dis. 42:265–274.

Horadagoda, N. U., J. C. Hodgson, G. M. Moon, T. G. Wijewardana, and P. D. Eckersall. 2002. Development of a clinical syndrome resembling haemorrhagic septicaemia in the buffalo following intravenous inoculation of *Pasteurella multocida* serotype B:2 endotoxin and the role of tumour necrosis factor-alpha. Res. Vet. Sci. 72:194–200.

Hunt, M. L., C. G. Ruffolo, K. Rajakumar, and B. Adler. 1998. Physical and genetic map of the *Pasteurella multocida* A:1 chromosome. J. Bacteriol. 180:6054–6058.

Hunt, M. L., D. J. Boucher, J. D. Boyce, and B. Adler. 2001. In vivo expressed genes of *Pasteurella multocida*. Infect. Immun. 69:3004–3012.

Ibrahim, R. S., T. Sawada, M. Shahata, and A. Ibrahim. 2000. Cross-protection and antigen expression by chicken embryo-grown *Pasteurella multocida* in chickens. J. Comp. Pathol. 123:278–284.

Jacab, G. J., and G. M. Green. 1976. Defect in intracellular killing of *Staphylococcus aureus* within the alveolar macrophages in Sendi virus infected murin lungs. J. Clin. Invest. 57:1533–1539.

Jacques, M., M. Kobisch, M. Belanger, and F. Dugal. 1993. Virulence of capsulated and noncapsulated isolates of *Pasteurella multocida* and their adherence to porcine respiratory tract cells and mucus. Infect. Immun. 61:4785–4792.

Jericho, K. W., A. Lejune, and G. N. Tiffin. 1986. Bovine perpesvirus-1 and *Pasteurella haemolytica* aerobiology in experimental infection. AM. J. Vet. Res. 47:205–209.

Jericho, K. W. F. 1989. Histological changes in lungs of calves exposed to an aerosol of *Pasteurella haemolytica*. J. Comp. Path. 101:87–99.

Jim, K., T. Guichon, and G. Shaw. 1988. Protecting feedlot calves from pneumonic pasteurellosis. Vet. Med. 83:1984–1087.

Kolodrubetz, D., T. Dailey, J. Ebersole, and E. Kraig. 1989. Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*. Infect. Immun. 57:1465–1469.

Koronakis, V., M. Cross, B. Senior, E. Koronakis, and C. Hughes. 1987. The secreted hemolysins of *Proteus mirabilis*, *Proteus vulgaris* and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. J. Bacteriol. 169:1509–1515.

Lacroix, R. P., J. R. Duncan, R. P. Jenkins, R. A. Leitch, J. A. Perry, and J. C. Richards. 1993. Structural and serological specificites of *Pasteurella haemolytica* lipopolysaccharides. Infect. Immun. 61:170–181.

Lainson, F. A., N. Thomson, H. A. Rowe, P. R. Langford, K. D. Aitchison, W. Donachie, and J. S. Kroll. 1996. Occurrence of [copper, zinc]-cofactored superoxide dismutase in *Pasteurella haemolytica* and its serotype distribution. FEMS Microbiol. Lett. 142:11–17.

Lax, A. J., and A. E. Grigoriadis. 2001. *Pasteurella multocida* toxin: the mitogenic toxin that stimulates signalling cascades to regulate growth and differentiation. Int. J. Med. Microbiol. 291:261–268.

Lee, C. W., P. E. Shewen, W. M. Cladman, J. A. R. Conlon, A. Mellors, and R. Y. C. Lo. 1994. Sialoglycoprotease of *Pasteurella haemolytica* A1: Detection of anti-sialoglycoprotease antibodies in sera of calves. Can. J. Vet. Res. 58:93–98.

Lee, M. D., R. E. Wooley, and J. R. Glisson. 1994. Invasion of epithelial cell monolayers by turkey

strains of *Pasteurella multocida*. Avian Dis. 38:72–77.

Lee, R. W. H., J. N. Strommer, D. Hodgins, P. E. Shewen, Y. Niu, and R. Y. C. Lo. 2001 Towards development of an edible vaccine against bovine pneumonic pasteurellosis using trangenic white clover expressing a *Mannheimia haemolytica* A1 Lkt50 fusion protein. Infect. Immun. 69:5786–5793.

Lei, W. B., E. M. Talley, and D. A. Bayliss. 2001. Receptor-mediated inhibition of G protein-coupled inwardly rectifying potassium channels involves G alpha(q) family subunits, phospholipase C, and a readily diffusible messenger. J. Biol. Chem. 276:16720–16730.

Leite, F., C. Malazdrewich, H. S. Yoo, S. K. Maheswaran, and C. J. Czuprynski. 1999. Use of TUNEL staining to detect apoptotic cells in the lungs of cattle experimentally infected with *Pasteurella haemolytica*. Microb. Pathog. 27:179–185.

Li, J., and K. D. Clinkenbeard. 1999. Lipopolysaccharide complexes with *Pasteurella haemolytica* leukotoxin. Infect. Immun. 67:2920–2927.

Li, J., K. D. Clinkenbeard, and J. W. Ritchey. 1999. Bovine CD18 identified as a species specific receptor for *Pasteurella haemolytica* leukotoxin. Vet. Microbiol. 67:91–97.

Lillie, L. E., and R. G. Thomson. 1972. The pulmonary clearance of bacteria by calves and mice. Can. J. Comp. Med. 36:129–137.

Lo, R. Y. C., and A. Mellors. 1996. The isolation of recombinant plasmids expressing secreted antigens of *Pasteurella haemolytica* A1 and the characterization of an immunogenic 60 kDa antigen. Vet. Microbiol. 51:381–391.

Lo, R. Y. C., and P. E. Shewen. 1992. The genus *Pasteurella*. In The prokaryotes, 2nd ed. A. Barlows, H. G. Truper, M. Dworkin, W. Hardr, and K.-H Schleifer (eds.). Toronto: Springer-Verlag, pp. 3331–3338.

Lo, R. Y. C., C. A. Strathdee, and P. E. Shewen. 1987. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. Infect. Immun. 55:1987–1996.

Lo, R. Y. C., L. J. McKerral, T. L. Hills, and M. Kostrzynska. 2001. Analysis of the capsule biosynthetic locus of *Mannheimia (Pasteurella) haemolytica* A1 and proposal of a nomenclature system. Infect. Immun. 69:4458–4464.

Lo, R. Y. C., P. E. Shewen, C. A. Strathdee, and C. N. Greer. 1985. Cloning and expression of the leukotoxin gene of *Pasteurella haemolytica* A1 in *Escherichia coli*. K-12. Infect. Immun. 50:667–671.

Lopez, A., R. G. Thomson, and M. Savan. 1976. The pulmonary clearance of *Pasteurella haemolytica* in calves infected with bovine parainfluenza-3 virus. Can. J. Comp. Med. 40:385–391.

Luo, Y. G., Q. D. Zeng, J. R. Glisson, M. W. Jackwood, I. H. N. Cheng, and C. L. Wang. 1999. Sequence analysis of *Pasteurella multocida* major outer membrane protein (OmpH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. Vaccine 17:821–831.

Magyar, T., V. L. King, and F. Kovacs. 2002. Evaluation of vaccines for atrophic rhinitis—A comparison of three challenge models. Vaccine 20:1797–1802.

Maheswaran, S. K., K. A. Berggren, R. R. Simonson, G. E. Ward, and C. C. Muscoplat. 1980. Kinetics of interaction and fate of *Pasteurella haemolytica* in bovine alveolar macrophage. Infect. Immun. 30:245–262.

Markham, R. J. F., and B. N. Wilkie. 1980. Interaction between *Pasteurella haemolytica* and bovine alveolar macrophages: cytotoxic effect on macrophages and impaired phagocytosis. Am. J. Vet. Res. 41:18–22.

Matsumoto, M., and J. G. Strain. 1993. Pathogenicity of *Pasteurella multocida*—its variable nature demonstrated by *in vivo* passages. Avian Dis. 37:781–785.

May, B. J., Q. Zhang, L. L. Li, M. L. Paustian, T. S. Whittam, and V. Kapur. 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. Proc. Natl. Acad. Sci. USA 98:3460–3465.

McBride, J. W., R. E. Corstvet, D. B. Paulsen, J. R. McClure, and F. M. Enright. 1996. Characterization of bovine pulmonary and serum antibody responses after parenteral or intrapulmonary vaccination with live *Pasteurella haemolytica*. Comp. Immun. Microbiol. Infect. Dis. 19:99–115.

McKerral, L. J., and R. Y. C. Lo. 2002. Construction and characterization of an acapsular mutant of *Mannheimia haemolytica* A1. Infect. Immun. 70:2622–2629.

McVey, D. S., R. W. Loan, C. W. Purdy, and A. E. Richards. 1989. Antibodies to *Pasteurella haemolytica* somatic antigens in two models of the bovine respiratory disease complex. Am. J. Vet. Res. 50:443–447.

McVey, D. S., R. W. Loan, C. W. Purdy, and W. J. Shuman. 1990. Specificity of bovine serum antibody to capsular carbohydrate antigens from *Pasteurella haemolytica*. J. Clin. Microbiol. 28:1151–1158.

Mellors, A., and R. Y. C. Lo. 1995. *O*-sialoglycoprotease from *Pasteurella haemolytica*. In Proteolytic enzymes: aspartic and metallopeptidases. A. J.

Barrett (ed.). San Diego: Academic Press, pp. 728–740.

Morck, D., B. Ellis, P. Domingue, M. Olson, and J. Costerton. 1991. *In vivo* expression of iron regulated outer-membrane proteins in *Pasteurella haemolytica*-A1. Microb. Pathog. 11:373–378.

Morck, D. W., M. E. Olson, S. D. Acres, P-Y. Daost, and J. W. Costerton. 1989. Presence of bacterial glycocalyx and fimbriae on *Pasteurella haemolytica* in feedlot cattle with pneumonic pasteurellosis. Can. J. Vet. Res. 53:167–171.

Morck, D. W., T. J. G. Raybould, S. D. Acres, L. A. Babiuk, J. Nelligan, and J. W. Costerton. 1987. Electron microscopic description of glycocalyx and fimbriae on the surface of *Pasteurella haemolytica*-A1. Can. J. Vet. Res. 51:83–88.

Mosier, D. A., K. R. Simons, A. W. Confer, R. J. Panciera, and K. D. Clinkenbeard. 1989. *Pasteurella haemolytica* antigens associated with resistance to pneumonic pasteurellosis. Infect. Immun. 57:711–716.

Muhairwa, A. P., J. P. Christensen, and M. Bisgaard. 2000. Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. Avian Path. 29:133–142.

Muhairwa, A. P., M. M. A. Mtambo, J. P. Christensen, and M. Bisgaard. 2001. Occurrence of *Pasteurella multocida* and related species in village free ranging chickens and their animal contacts in Tanzania. Vet. Microbiol. 78:139–153.

Mullan, P. B., and A. J. Lax. 1998. *Pasteurella multocida* toxin stimulates bone resorption by osteoclasts via interaction with osteoblasts. Calcif. Tissue Int. 63:340–345.

Murphy, G. L., and L. C. Whitworth. 1994. Construction of isogenic mutants of *Pasteurella haemolytica* by allelic replacement. Gene 148:101–105.

Murphy, G. L., L. C. Whitworth, K. D. Clinkenbeard, and P. A. Clinkenbeard. 1995. Hemolytic activity of the *Pasteurella haemolytica* leukotoxin. Infect. Immun. 63:209–3212.

Mutters, R., H. Christensen, and M. Bisgaard. 2003. Genus Pasteurella. In Bergey's manual of systematic bacteriology, vol. II. The proteobacteria. G. Garrity (ed.). Heidelberg: Springer. (In press)

Myint, A., and G. R. Carter. 1989. Prevention of haemorrhagic septicaemia in buffaloes and cattle with a live vaccine. Vet. Rec. 124:508–509.

Myint, A., G. R. Carter, and T. O. Jones. 1987. Prevention of experimental haemorrhagic septicaemia with a live vaccine. Vet. Rec. 120:500–501.

Noel, G. J., D. C. Love, and D. M. Mosser. 1994. High-molecular-weight proteins of nontypeable *Haemophilus influenzae* mediate bacterial adherence to cellular proteoglycans. Infect. Immun. 62:4028–4033.

Ogunnariwo, J. A., and A. B. Schryvers. 1990. Iron acquisition in *Pasteurella haemolytica*: expression and identification of a bovine-specific transferrin receptor. Infect. Immun. 58:2091–2097.

Ogunnariwo, J. A., T. K. W. Woo, R. Y. C. Lo, G. C. Gonzalez, and A. B. Schryvers. 1997. Characterization of the *Pasteurella haemolytica* transferrin receptor genes and the recombinant receptor proteins. Microb. Pathog. 23:273–284.

Ohnishi, T., Y. Horiguchi, M. Masuda, N. Sugimoto, and M. Matsuda. 1998. *Pasteurella multocida* toxin and *Bordetella bronchiseptica* dermonecrotizing toxin elicit similar effects on cultured cells by different mechanisms. J. Vet. Med. Sci. 60:301–305.

Pabs-Garnon, L. F., and M. A. Soltys. 1971a. Methods of transmission of fowl cholera in turkeys. Am. J. Vet. Res. 32:1119–20.

———. 1971b. Multiplication of *Pasteurella multocida* in the spleen, liver and blood of turkeys inoculated intravenously. Can. J. Comp. Med. 35:147–149.

Pandher, K., A. W. Confer, and G. L. Murphy. 1998. Genetic and immunologic analysis of PlpE, a lipoprotein important in complement-mediated killing of *Pasteurella haemolytica* serotype 1. Infect. Immun. 66:5613–5619.

Pandher, K., G. L. Murphy, and A. W. Confer. 1999. Identification of immunogenic, surface-exposed outer membrane proteins of *Pasteurella haemolytica* serotype 1. Vet. Microbiol. 65:215–226.

Paulsen, D. B., A. W. Confer, K. D. Clinkenbeard, and D. A. Mosier. 1990. *Pasteurella haemolytica* lipopolysaccharide-induced arachidonic acid release from and neutrophil adherence to bovine pulmonary artery endothelial cells. Am. J. Vet. Res. 51:1635–1639.

Paulsen, D. B., D. A. Mosier, K. D. Clinkenbeard, and A. W. Confer. 1989. Direct effects of *Pasteurella haemolytica* lipopolysaccharide on bovine pulmonary endothelial cells *in vitro*. Am. J. Vet. Res. 50:1633–1637.

Paustian, M. L., B. J. May, and V. Kapur. 2001. *Pasteurella multocida* gene expression in response to iron limitation. Infect. Immun. 69:4109–4115.

———. 2002. Transcriptional response of *Pasteurella multocida* to nutrient limitation. J. Bacteriol. 184:3734–3739.

Petersen, K. D., J. P. Christensen, A. Permin, and M. Bisgaard. 2001. Virulence of *Pasteurella multocida* subsp *multocida* isolated from outbreaks of fowl cholera in wild birds for domestic poultry and game birds. Avian Path. 30:27–31.

Petras, S., M. Chidambaram, E. Illyes, S. Froshauer, G. Weinstock, and C. Reese. 1995. Antigenic and

virulence properties of *Pasteurella haemolytica* leukotoxin mutants. Infect. Immun. 63:1033–1039.

Pohl, S. 1981. DNA relatedness among members of *Haemophilus, Pasteurella* and *Actinobacillus*. In *Haemophilus, Pasteurella* and *Actinobacillus*. M. Kilian, W. Frederikson, and E. L. Biberstein (eds.). London: Acad. Press, pp. 243–253.

Potter, A. A., A. B. Schryvers, J. A. Ogunnariwo, W. A. Hutchins, R. Y. C. Lo, and T. Watts. 1999. Protective capacity of the *Pasteurella haemolytica* transferrin-binding proteins TbpA and TbpB in cattle. Microb. Pathog. 27:197–206.

Pruimboom, I. M., R. B. Rimler, and M. R. Ackermann. 1999. Enhanced adhesion of *Pasteurella multocida* to cultured turkey peripheral blood monocytes. Infect. Immun. 67:1292–1296.

Pruimboom, I. M., R. B. Rimler, M. R. Ackermann, and K. A. Brogden. 1996. Capsular hyaluronic acid-mediated adhesion of *Pasteurella multocida* to turkey air sac macrophages. Avian Dis. 40:887–893.

Rabier, M. J., N. K. Tyler, N. J. Walker, L. M. Hansen, D. C. Hirsh, and F. Tablin. 1997. *Pasteurella multocida* enters polarized epithelial cells by interacting with host F-Actin. Vet. Microbiol. 54:343–355.

Rebers, P. A., A. E. Jensen, and G. A. Laird. 1988. Expression of pili and capsule by the avian strain P-1059 of *Pasteurella multocida*. Avian Dis. 32:313–318.

Rehmtulla, A. J., and R. G. Thomson. 1981. A review of the lesions in shipping fever of cattle. Can. Vet. J. 22:1–8.

Rhoades, K. R., and R. B. Rimler. 1987. Effects of *Pasteurella multocida* endotoxins on turkey poults. Avian Dis. 31:523–526.

Rimler, R. B. 2001. Purification of a cross-protective antigen from *Pasteurella multocida* grown in vitro and in vivo. Avian Dis. 45:572–580.

Rimler, R. B., and K. A. Brogden. 1986. *Pasteurella multocida* isolated from rabbits and swine: serologic types and toxin production. Am. J. Vet. Res. 47:730–737.

Rimler, R. B., and K. R. Rhoades. 1987. Serogroup F, a new capsule serogroup of *Pasteurella multocida*. J. Clin. Microbiol. 25:615–618.

Rimler, R. B., P. A. Rebers, and K. R. Rhoades. 1979. Fowl cholera: cross-protection induced by *Pasteurella multocida* separated from infected turkey blood. Avian Dis. 23:730–741.

Rose, D. M., S. N. Giri, J. G. Zinkl, K. P. Snipes, J. N. Davidson, R. H. McCapes, and G. M. Conzelman. 1988. Changes in circulating prostaglandin E and F2 alpha levels, cloacal temperature, and leukocyte counts in turkeys inoculated with *Pasteurella multocida*. Avian Dis. 32:204–208.

Ruffolo, C. G., J. M. Tennent, W. P. Michalski, and B. Adler. 1997. Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. Infect. Immun. 65:339–343.

Saharee, A. A., N. B. Salin, A. Rasedee, and M. R. Jainudeen. 1992. Haemorrhagic septicaemia carriers among cattle and buffalo in Malaysia. The International Workshop on Pasteurellosis in Production Animals, Bali, Indonesia, Australian Centre for International and Agricultural Research.

Scott, P. V., J. F. Markham, and K. G. Whithear. 1999. Safety and efficacy of two live *Pasteurella multocida aroA* mutant vaccines in chickens. Avian Dis. 43:83–88.

Shewen, P. E., A. Sharpe, and B. N. Wilkie. 1988. Efficacy testing of a *Pasteurella haemolytica* extract vaccine. Vet. Med. 10:1078–1083.

Shewen, P. E., and B. N. Wilkie. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infect. Immun. 35:91–94.

———. 1983. *Pasteurella haemolytica* cytotoxin: production by recognized serotypes and neutralization by type specific rabbit antisera. Am. J. Vet. Res. 44:715–719.

———. 1985. Evidence for *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res. 45:1212–1214.

———. 1988. Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. Can. J. Vet. Res. 52:30–36.

Shewen, P. E., C. W. Lee, A. Perets, D. C. Hodgins, K. Baldwin, and R. Y. C. Lo. 2003. Efficacy of recombinant sialoglycoprotease in protection of cattle against pneumonic challenge with *Mannheimia (Pasteurella) haemolytica* A1. Vaccine 3679:1–6.

Simensen, E., and L. D. Olson. 1980. Aerosol transmission of *Pasteurella multocida* in turkeys. Avian Dis. 24:1007–1010.

Smith, G. R. 1961. The characteristics of two types of *Pasteurella haemolytica* associated with different pathological conditions of sheep. J. Path. Bact. 81:431–440.

Sneath, P. H. A., and M. Stevens. 1990. *Actinobacillus seminis* sp. nov., nom. rev., *Pasteurella bettii* sp. nov., *Pasteurella lymphangitidis* sp. nov., *Pasteurella mairi* sp. nov., and *Pasteurella trehalosi* sp. nov. Int. J. Syst. Bacteriol. 40:148–153.

Snipes, K. P., G. Y. Ghazikhanian, and D. C. Hirsh. 1986. Fate of *Pasteurella multocida* in the blood

vascular system of turkeys following intravenous inoculation: comparison of an encapsulated, virulent strain with its avirulent, acapsular variant. Avian Dis. 31:254–259.

Stevens, P., and C. Czuprynski. 1996. *Pasteurella haemolytica* leukotoxin induces bovine leukocytes to undergo morphologic changes consistent with apoptosis *in vitro.* Infect. Immun. 64:2687–2694.

Strathdee, C. A., and R. Y. C. Lo. 1987. Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli*. Infect. Immun. 55:3233–3236.

———. 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* leukotoxin determinant. J. Bacteriol. 171:916–928.

Straus, D. C., and C. W. Purdy. 1994. *In vivo* production of neuraminidase by *Pasteurella haemolytica* A1 in goats after transthoracic challenge. Infect. Immun. 62:4675–4678.

Straus, D. C., P. J. Unbehagen, and C. W. Purdy. 1993a. Neuraminidase production by *Pasteurella haemolytica* A1 strain associated with bovine pneumonia. Infect. Immun. 61:253–259.

Straus, D. C., W. L. Jolley, and C. W. Purdy. 1993b. Characterization of neuraminidases produced by various serotypes of *Pasteurella haemolytica*. Infect. Immun. 61:669–4674.

Tatum, F. M., R. E. Briggs, and S. M. Halling. 1994. Molecular gene cloning and nucleotide sequencing and construction of an *aroA* mutant of *Pasteurella haemolytica* serotype A1. Appl. Environ. Microbiol. 60:2011–2016.

Thompson, S., L. Wang, A. West, and P. Sparling. 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J. Bacteriol. 175:811–818.

Thomson, R. G., M. Benson, and M. Savan. 1969. Pneumonic pasteurellosis of cattle: microbiology and immunology. Can. J. Comp. Med. 33:194–206.

Townsend, K. T., J. D. Boyce, J. Y. Chung, A. J. Frost, and B. Adler. 2001. Genetic organization of *Pasteurella multocida cap* loci and the development of a multiplex capsular PCR typing system. J. Clin. Microbiol. 39:924–929. Erratum in: J. Clin. Microbiol. 39:2378.

Tsuji, M., and M. Matsumoto. 1989. Pathogenesis of fowl cholera: influence of encapsulation on the fate of *Pasteurella multocida* after intravenous inoculation into turkeys. Avian Dis. 33:238–247.

———. 1990. Immune defence mechanism against blood-borne *Pasteurella multocida* in turkeys. Res. Vet. Sci. 48:344–349.

Uhlich, G. A., D. A. Mosier, and K. R. Simons. 1993. An enzyme-linked immunosorbent assay for detection of adhesion of *Pasteurella haemolytica* to bovine nasal mucus. Proceedings of the 74th Annual Meeting of the Conference Workers in Animal Diseases (CRWAD). Chicago, IL. Abstract#P-24.

van Alphen, L. 1995. Adhesin-receptor interaction of *Haemophilus influenzae* and other bacteria from the HAP group. In *Haemophilus, Actinobacillus* and *Pasteurella.* W. Donachie, F. A. Lainson, and J. C. Hodgson (eds.). New York: Plenum Press, pp. 89–100.

Verma, R., and T. N. Jaiswal. 1997. Protection, humoral and cell-mediated immune responses in calves immunized with multiple emulsion haemorrhagic septicaemia vaccine. Vaccine 15:1254–1260.

———. 1998. Haemorrhagic septicaemia vaccines. Vaccine 16:1184–1192.

Ward, C. K., S. R. Lumbley, J. L. Latimer, L. D. Cope, and E. J. Hansen. 1998. *Haemophilus ducreyi* secretes a filamentous hemagglutinin-like protein. J. Bacteriol. 180:6013–6022.

Wassenaar, T. M., and W. Gaastra. 2001. Bacterial virulence: can we draw the line? FEMS Microbiol. Lett. 201:1–7.

Weinberg, E. D. 1999. Iron loading and disease surveillance. Emerg. Infect. Dis. 5:346–352.

Welch, R. A., M. E. Bauer, A. D. Kent, J. A. Ledds, M. Moayeri, L. B. Regassa, and D. L. Swenson. 1995. Battling against host phagocytes: the wherefore of the RTX family of toxins. Infect. Agents Dis. 4:254–272.

Weldon, S. K., D. A. Moiser, K. R. Simons, R. C. Craven, and A. W. Confer. 1994. Identification of a potentially important antigen of *Pasteurella haemolytica*. Vet. Microbiol. 40:283–291.

Whiteley, L. O., S. K. Maheswaran, D. J. Weiss, and T. R. Ames. 1990. Immunohistochemical localization of *Pasteurella haemolytica* A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine pasteurella pneumonia. Vet. Pathol. 27:150–161.

Whiteley, L. O., S. K. Maheswaran, D. J. Weiss, T. R. Ames, and M. S. Kannan. 1992. *Pasteurella haemolytica* A1 and bovine respiratory disease: pathogenesis. J. Vet. Intern. Med. 6:11–22.

Wilson, B. A., L. R. Aminova, V. G. Ponferrada, and M. F. Ho. 2000. Differential modulation and subsequent blockade of mitogenic signaling and cell cycle progression by *Pasteurella multocida* toxin. Infect. Immun. 68:4531–4538.

Wilson, C. F., A. D. Sutherland, L. Inglis, and W. Donachie. 1992. Characterization and biological

activity of monoclonal antibodies specific for *Pasteurella haemolytica* A1 capsule and lipopolysaccharide. Vet. Microbiol. 31:161–168.

Younan, M., and L. Fodar. 1995. Characterisation of a new *Pasteurella haemolytica* serotype (A17). Res. Vet. Sci. 58:98.

Zywietz, A., A. Gohla, M. Schmelz, G. Schultz, and S. Offermanns. 2001. Pleiotropic effects of *Pasteurella multocida* toxin are mediated by G(q)-dependent and -independent mechanisms—involvement of G(q) but not G(11). J. Biol. Chem. 276:3840–3845.

21
Yersinia

J. Mecsas and R. Chafel

Yersinia species cause a variety of diseases in mammals, birds, and fish. *Yersinia* are named for Alexandre Yersin, who first identified *Y. pestis*, the causative agent of bubonic and pneumonic plague, in 1894. Originally, the bacteria were classified as *Pasteurella*; however, they were given a separate name as their relatedness to the Enterobacteriaceae became apparent. Like other Enterobacteriaceae, *Yersinae* are facultative, anaerobic, oxidase-negative, gram-negative rods or coccobacilli capable of fermenting glucose. They often have bipolar staining in tissue smears. In the laboratory, they are grown at room temperature, and it takes 2–3 days for formation of single colonies.

Of the 11 species of *Yersinia*, four—*Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica,* and *Y. ruckeri*—cause disease. *Yersinia 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 contaminated fecal matter (fig. 21.1). 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 and septicemia, is generally transmitted from animal to animal via the bite of an infected flea (fig. 21.1). Occasionally transmission occurs via aerosolization from an animal or human with pneumonic plague, resulting in an often-fatal pneumonia. *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 rainbow trout, until stresses precipitate disease outbreak. Very little is known about the genes required for virulence in *Y. ruckeri*; therefore, only its disease pathogenesis will be discussed in this chapter.

CLASSIFICATION OF *YERSINIA* SPECIES

Y. pseudotuberculosis is divided into 14 serotypes based on the immunoreactivity of 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 not associated with any 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, so named for the three *Y. pestis* pandemics and the strains thought to have caused these three pandemics.

Although both *Y. pseudotuberculosis* and *Y. enterocolitica* are enteric pathogens, they are much less closely related at a DNA level than *Y. pestis* is with *Y. pseudotuberculosis*. In fact, *Y. pestis* is thought to have evolved from *Y. pseudotuberculosis* serotype IB within the last 20,000 years and possibly as recently as 1,500 years ago (Achtman et al. 1999). In contrast, *Y. enterocolitica* and *Y. pseudotuberculosis* are thought to have diverged from a common ancestor about 50 million years ago. Because of the high degree of DNA homology between *Y. pestis*

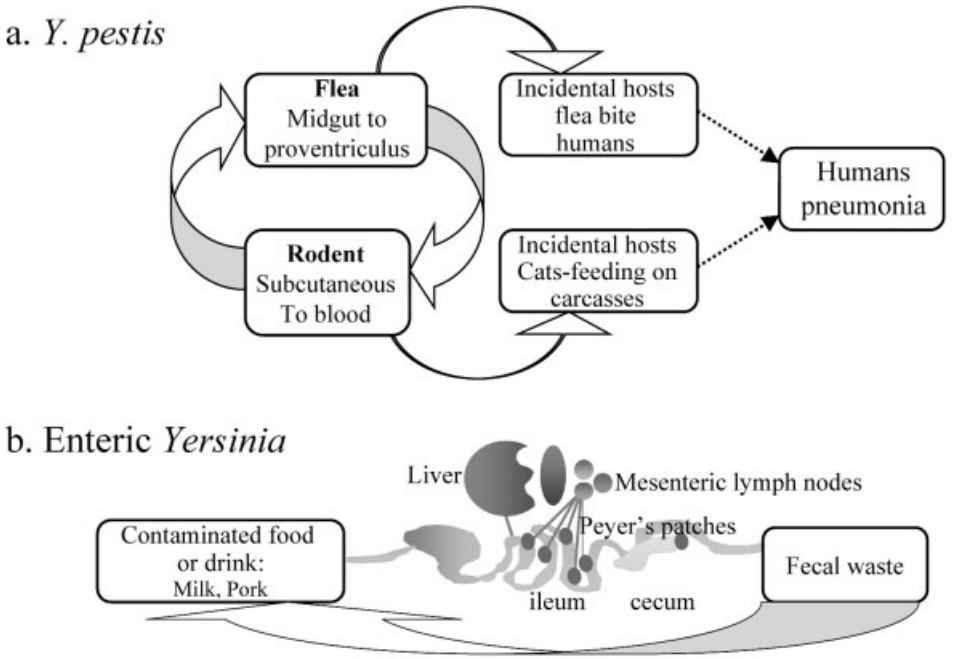


Figure 21.1. A comparison of transmission cycles of (*A*) *Y. pestis* and (*B*) the enteric *Yersinia* spp. *Y. pestis* life cycle involves transmission between fleas and rodents with the bacteria inoculated subcutaneously into rodents, eventually spreading to blood, followed by ingestion by a flea. *Y. enterocolitica* and *Y. pseudotuberculosis* are transmitted by ingestion of contaminated foods.

and *Y. pseudotuberculosis*, *Y. pestis* could be considered a subspecies of *Y. pseudotuberculosis*. However, the designation *Y. pestis* is still commonly used because there are distinct and drastic differences between *Y. pestis* and *Y. pseudotuberculosis* in regard to their ability to survive in the environment, the spectrum and severity of disease, their mode of transmission, and some of their virulence factors.

Recently, the DNA sequences of two strains of *Y. pestis* have been completed and annotated, and the sequence of one strain of *Y. pseudotuberculosis* is nearing completion and available for perusal. A comparison between the two *Y. pestis* genomes and the *Y. pseudotuberculosis* genome indicates that the *Y. pestis* genome is likely in flux, rapidly acquiring and losing genetic elements and undergoing genomic rearrangements. For instance, *Y. pestis* has acquired two additional plasmids, pFra and pPCP1, which are absent from *Y. pseudotuberculosis* strains. As will be discussed below, 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 site. In addition, there are many insertion elements in the *Y. pestis* genome that inactivate genes that are functional in *Y. pseudotuberculosis*. Some of these genes are important for the virulence and/or transmission of enteric *Yersinia* spp. and others are likely needed by the enterics while growing in the environment. Plasmids, pathogenicity islands, and proteins expressed in various *Yersinia* spp. are shown in table 21.1.

SOURCES OF *YERSINIA* SPECIES

The enteric *Yersinia* pathogens are usually acquired through ingestion of contaminated food or water (fig. 21.1). 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. Frequently, animals acquire yersiniosis from contaminated water supplies. Birds and rodents are also reservoirs of infection for *Y. pseudotuberculosis* and may contaminate animal feeds. Swine are a significant source of *Y. entercolitica* and may infect ruminants if the two species share adjacent enclosures. The enteric *Yersinia* spp. replicate at temperatures as low as

Table 21.1. Plasmids, Pathogenicity Islands, and Proteins Expressed in Various *Yersinia* spp.

	pYV	pPCP	pFra	HPI	HMS	Ysa	Inv[3]	YadA[3]
Y. pestis								
Antiqua	+	+	+	+	+	+	-	-
Medievals	+	+	+	+	+	+	-	-
Orientalis	+	+	+	+	+	+	-	-
Y. pseudotuberculosis								
I	+	-	-	+	+	+	+	+
II	+	-	-	-	+	?	+	+
III	+	-	-	(+)[1]	+	+	+	+
IV	+	-	-	-	+	?	+	+
V	+	-	-	-	+	?	+	+
Y. enterocolitica								
1a	-	-	-	-	-	-	?	-
1b	+	-	-	+	-	+[2]	+	+
2	+	-	-	-	-	-	+	+
3	+	-	-	-	-	+[2]	+	+
4	+	-	-	-	-	-	+	+
5	+	-	-	-	-	?		+
Y. ruckeri	-	-	-	-	-	?		-
Y. intermedia	-	-	-	-		-		-
Y. frederiksenii	-	-	-	-		-		-
Y. kristensenii	-	-	-	-		-		-
Y. aldovae	-	-	-	-		-		-
Y. rhodei	-	-	-	-		?		-
Y. mollaretti	-	-	-	-		-		-
Y. bercoviere	-	-	-	-		-		-

[1] The HPI contains a 9-kb truncation in *Y. pseudotuberculosis* serotype III.

[2] *Y. enterocolitica* contains a different type III secretion system on the chromosome than the systems found in *Y. pestis* and *Y. pseudotuberculosis.*

[3] 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.

4°C, so refrigeration does not make contaminated foods safe. Since the enteric *Yersinia* can spend significant portions of their life in outside-of-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 (fig. 21.1). 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 of 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 subcutaneously 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 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, such as carcasses, but is otherwise sensitive to desiccation and temperatures above 40°C. Colder temperatures or freezing may prolong the viability of the organism.

VIRULENCE FACTORS

Factors Necessary for *Y. 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* at late stages of septicemia, hypotension and vascular collapse occur. However, lethality of *Y. pestis* in mice is not dependent on Ymt, and Ymt is expressed at higher levels at 26°C compared to 37°C, observations that prompted a recent study of Ymt in fleas. These recent findings indicate that *Y. pestis* strains that lack Ymt are rapidly killed in the midgut of fleas. Ymt is a phospholipase D that remains within the bacteria unless the bacteria lyse (Hinnebusch et al. 2000, 2002a). How PLD protects *Y. pestis* from bactericidal components in the flea midgut and what in the midgut is bactericidal to *Y. pestis* are not yet 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, *hms*, is necessary for efficient transmission of *Y. pestis* from the flea to subcutaneous 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 (hemin storage locus). 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). The presence of the HPI correlates with the level of pathogenicity of enteric *Yersinia* spp., which supports the notion that this segment of DNA is required for high pathogenicity. Similar DNA sequences have also been identified in some *E. coli* strains isolated from patients with diarrhea and in some strains of *Salmonella, Citrobacter,* and *Klebsiella*. The HPI was likely acquired by *Yersinia* spp. from another organism, as its GC base composition differs from the rest of the genomic sequence, it is flanked by insertion sequence elements, IS100, and the element encodes a putative integrase. These are all hallmarks of horizontally transmitted elements. Furthermore, different *Yersinia* spp. 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 appealing 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 Fe3+ with a higher affinity than Fe2+. In general, siderophores scavenge Fe3+ bound to host proteins and transport it back to bacteria. Once within the cytoplasm, the Fe3+ can be used for 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, 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.

Mutations in this locus render *Y. pestis* significantly less virulent after subcutaneous inoculation. In fact, strain EV7651, which lacked both the HPI and the *hms* locus, has been used as a vaccine strain at various times over the past 60 years (Russell et al. 1995). This vaccine strain was given to people in Vietnam from 1967 to 1969, but because of the ongoing war, no studies were possible to test its efficacy in preventing plague.

Y. pestis has additional iron-acquisition mechanisms, including the siderophore-independent processes involving ferric uptake controlled by Yfu and Yfe (Bearden and Perry 1999; Gong et al. 2001; Perry et al. 2003).

Fraction 1 Antigen

Fraction 1 (F1) antigen is associated with the capsule and is encoded on the p60 or pFra plasmid found only in *Y. pestis* (Drozdov et al. 1995). F1 can prevent opsonization by complement and thus F1 production is one of several mechanisms utilized by *Y. pestis* for evading phagocytosis by neutrophils and macrophages. An immune response to F1 provides protective immunity, and F1 is one of two components of an acellular vaccine under active development; however, F1 is not an essential virulence factor in mice. Thus, if strains evolve or are developed that lack FI, the vaccine would become significantly less useful.

Pla

Pla is encoded on the pPCP1 plasmid found in *Y. pestis*. Pla is required for *Y. pestis* to disseminate from a subcutaneous site of inoculation to surrounding lymph tissues or the bloodstream (Sodeinde et al. 1992). Pla is an outer membrane protein that has proteolytic activity and activates plasminogen. 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 that establishes a site of infection in the GI tract to one that establishes infection after subcutaneous 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 subcutaneous location to lymph nodes or other organs.

Adherence and Invasion Factors

A number of adherence factors have been identified in *Yersinia* spp.; but curiously, several of the adherence factors found in the enteric *Yersinia* spp. have been inactivated in *Y. pestis* by insertion of IS elements or by frame shift 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), 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* spp.; pH 6 antigen is found in both *Y. pestis* and *Y. pseudotuberculosis;* and Pla is found in *Y. pestis*. Analysis of the *Y. pestis* genome indicates that several putative genes have homology to Invasin or other adherence factors; however, no studies have been done that 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* spp. It binds avidly to $\alpha\beta1$ integrins found on the surface of many types of mammalian cells. This tight binding triggers normally, nonphagocytic cells to internalize *Yersinia* (Bliska et al. 1993), if the Yops are not being expressed (see below). A primary role of Invasin in enteric *Yersinia* pathogenesis appears to be to allow the pathogen to survive in the gastrointestinal tract (Mecsas et al. 2001). *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* spp. 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 a mouse model system of infection, *yadA* mutants fail to persist in lymph tissues, a finding that demonstrates the importance of YadA for pathogenesis of *Y. enterocolitica* (Pepe et al. 1995).

Type III Secretion System Apparatus

One of the key virulence features of *Yersinia*, a type III secretion system (fig. 21.2), 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 (reviewed in Cornelis et al. 1998; Cornelis 2002). Once within host cells, these proteins alter, neutralize, or destroy normal host-cell functions. A number of other gram-negative plant and animal pathogens, including *Salmonella enteriditis, Bordetella*, and *Pseudomonas*, contain type III secretion systems (Hueck 1998); however, the *Yersinia* pYV-encoded system was the first identified and remains the best characterized.

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 make up the structural components of the type III secretion system, which extends from the cytoplasm to the outer membrane and includes a needlelike structure that goes beyond the outer membrane, through which exported proteins presumably travel. In

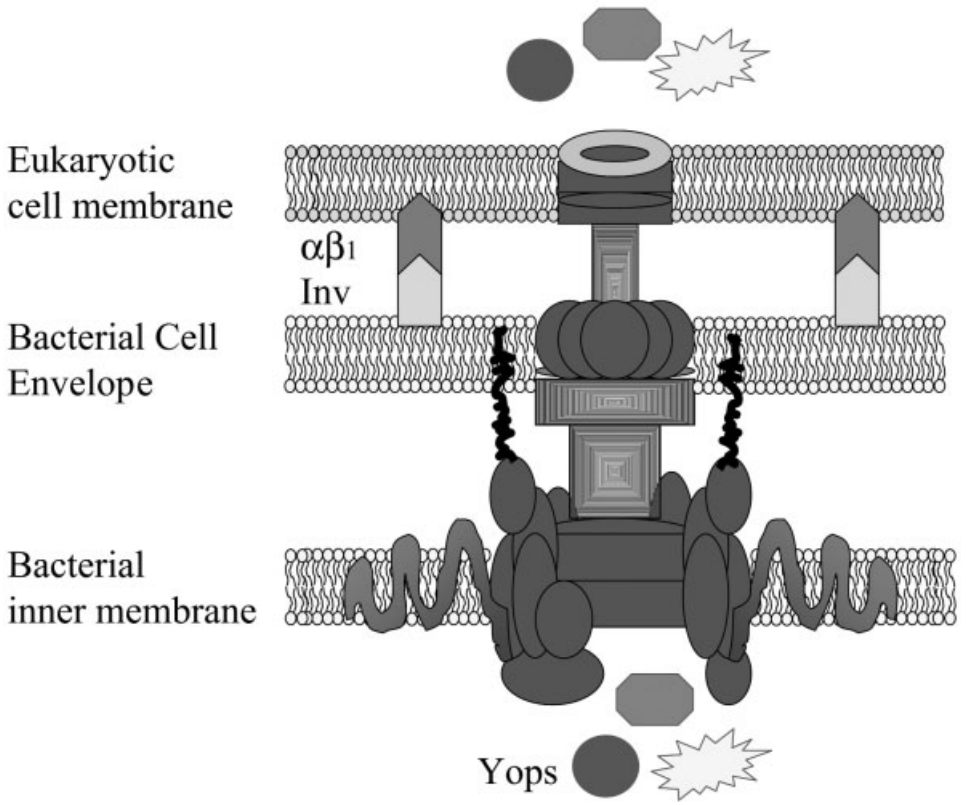


Figure 21.2. Type III secretion system and Yop translocation into mammalian cells. *Yersinia* binds via an adhesin: Invasin-$\alpha\beta$1 interaction is shown and Yops are injected into cells. The Type III secretion apparatus consists of about 25 proteins and spans the inner membrane, cell envelope, and mammalian membrane.

Yersinia spp., 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, synthesis of the genes encoding the secretion apparatus and Yops increases. VirF, a DNA-binding protein, positively regulates the induction of synthesis. VirF is encoded by *virF* on the pYV plasmid and is, itself, regulated by a chromosomally encoded factor, Ymo.

Yops are translocated into mammalian cells when a bacterium binds to mammalian cells, via one of the adhesins, Invasin or YadA, or when the bacterium is opsonized. Several proteins are necessary for translocation of Yops into host cells, but not for secretion into the extracellular milieu, which can occur when the bacteria are grown in media containing low concentrations of Ca2+ (see below). Two proteins, YopB and YopD, are thought to form a pore in the mammalian cell membrane through which the Yops travel. In addition, LcrV is required for Yop translocation into mammalian cells and may, itself, be translocated into mammalian cells. LcrV is one of the two components, along with F1, of an acellular vaccine under development in England. LcrV also has the property of preventing a pro-inflammatory response, which appears to be critical for allowing *Y. pestis* to replicate to high levels in lymph tissues and organs without inducing systemic failure.

Two proteins, YopN and TyeA, which are physically associated with the type III secretion structure, prevent secretion of Yops until the bacteria come into contact with mammalian membranes. Presumably YopN and TyeA sense components on mammalian membranes, and in the absence of such contact, they act as a plug on the apparatus, preventing secretion of the Yops through the structure. *Yersinia* grown in a medium with low concentrations of Ca2+, YopN, and TyeA, secrete Yops regardless of the presence of host cells. This observation was very useful for researchers in identifying and evaluating mutants in Yops and the type III secretion apparatus, although it remains unclear if the so-called low Ca2+ response has any relevance to disease progression in an infected animal.

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 systems found in *Y. enterocolitica* differ from those found in *Y. pestis* and *Y. pseudotuberculosis*, suggesting that the chromosomally encoded systems were acquired independently and after the enteric *Yersinia* spp. 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. However, those strains that do 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. 2003).

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*.

YOP FUNCTION

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). 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, neutrophils, and/or epithelial cells (Grosdent et al. 2002). In contrast to *Yersinia* Yops, effector molecules secreted by *Salmonella* induce host cells to take up the bacterium and allow *Salmonella* to grow intracellularly in host-cell vacuoles.

It is interesting that 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 that cluster at focal adhesion complexes upon $\alpha\beta1$ integrin binding to Invasin. The rapid dephosphorylation of these proteins results in a cessation of normal signal-transduction cascade between the outside of a cell and a response. In addition, 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.

Three Yops—YopE, YopT, and YopO—cause perturbations in the actin cytoskeleton after infection, and all three interact with Rho proteins, which are involved in controlling actin cytoskeleton dynamics. YopE has GTPase activity on Rho family proteins and thus catalyzes the conversion of the active, GTP-bound form of Rho into their inactive, GDP-bound form. Outcomes of YopE activities on cells include the destruction of actin stress fibers and an inability to phagocytose *Yersinia* or other particles. YopT targets RhoA and uncouples it from the membrane, leading to an increase in its inactive RhoGDI-bound form. YopO (YpkA) has serine-threonine kinase activity; it has been shown to bind to proteins in the Rho family, but its effect has not been further characterized.

YopJ (YopP in *Y. enterocolitica*) has the striking ability to cause murine macrophages to undergo apoptosis. 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. 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 mammalian cells and how it alters RSK function(s) has not yet 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. These genes include *rovA* and the genes that encode superantigens found in some enteric strains, dam methylases, ureases, and pili.

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* spp. 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 subcutaneous inoculation, causing the infected nodes to swell up. These large, swollen nodes are called "buboes," the word from which "bubonic" plague was derived. Although all three pathogenic *Yersinia* spp. contain a 70-kb plasmid, which is not found in *Y. ruckeri* or the seven nonpathogenic *Yersinia* spp., the presence of pYV does not completely account for the tropism for the lymph nodes, as strains lacking pYV have been reported to colonize the mesenteric lymph nodes in both people and animals.

Extracellular or Intracellular Pathogen?

The question of whether *Yersinia* spp. are primarily intracellular or extracellular pathogens when infecting a host has been discussed for several decades. 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 lungs. 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 cells with microvilli. In addition, there is evidence that *Y. pestis* replicates in macrophages and speculation that intracellular replication is important during the initial stages of infection after subcutaneous inoculation (Straley and Harmon 1984). Finally, there is indirect immunological evidence that enteric *Yersinia* spp. 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* spp. are extracellular or intracellular pathogens may be "both" and depends upon the stage of the infection process.

Y. 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 fourteenth century; and the Modern Orientalis pandemic, which started in 1894 and killed about ten million people by the early twentieth century. Recent outbreaks of bubonic plague continue to occur throughout the world including one in India in 1994 and one in Ecuador in 1998.

House rats and their fleas play a significant role as plague vectors during human epidemics. The recent outbreak in India followed a natural disaster,

which led to 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 often associated with the sporadic cases of human plague reported in the United States. In the United States, outbreaks in rodent populations occur in the states west of the Mississippi River. Rock squirrels and ground squirrels (*Spermophilus* spp.) and their fleas are the most common sources of human infections in these areas, whereas domestic cat and dog fleas (*Ctenocephalides* spp.) 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 areas of 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 also are 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. Cats may 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. Human to human transmission occurs rarely; however, this form of transmission is highly fatal. 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 treatment.

Three forms of the plague exist in humans and have been documented in cats as well. The most common and least fatal form is bubonic plague. After subcutaneous inoculation of the skin, *Y. pestis* disseminates to the regional lymph nodes, which become enlarged and painful. These nodes form abscesses (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 lungs in cats (Gage et al. 2000. Watson et al. 2001). In cats, 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 frequently fatal type of *Y. pestis* infection. 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 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 or underlying liver ailments can develop severe, potentially lethal systemic infections with *Y. pseudotuberculosis*. Infection of humans with *Y. enterocolitica* more often results in frank diarrhea and gastroenteritis, although mesenteric lymphadenitis

can occur (Metchock et al. 1991). Mesenteric lymphadenitis can be misdiagnosed as appendicitis. A common sequela from enteric *Yersinia* infections, particularly in people carrying the HLA B27 MHC allele, is reactive arthritis (Bottone 1997).

Y. 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, sheep, goats, and cattle. *Y. pseudotuberculosis* is prevalent in poultry flocks. Turkeys and young birds are most frequently affected. Clinical disease can range from sudden death to chronic ill thrift with persistent diarrhea. As with *Y. pestis*, carnivores, including cats and dogs, are considered 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.

Y. 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 often is 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* cause 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 to the muscularis mucosa. The abscessation and infiltration of inflammatory cells cause 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 nonhemorrhagic unless fulminant yersiniosis occurs. Both *Y. pseudotuberculosis* and *Y. enterocolitica* can disseminate via the portal circulation and mesenteric lymph nodes to major lymphatics to cause liver and splenic micro-abscesses and acute death. If mucosal damage already exists (e.g., due to parasites), animals may be predisposed to septic yersiniosis and reproductive disease. Mesenteric lymph nodes are usually edematous. In severe acute infections, 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 coccobacilli. *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 micro-abscesses in the liver.

Y. pseudotuberculosis, but not *Y. entercolitica*, has been documented to cause mastitis in does. Mastitis occurs as a result of direct invasion of the teat canal, rather than as a sequel to bacteremia. Udders are edematous and may have clots in the milk or blood in acute and chronic mastitis.

Y. ruckeri in Fish

Yersinia ruckeri is the causative agent of enteric redmouth disease in marine and freshwater fish. *Y. ruckeri* has also been isolated from other animals, including birds, otters, and humans. Enteric redmouth is a hemorrhagic inflammation of the perioral subcutis on rainbow trout and occasionally 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 both on the skin and internal organs. Acute death without clinical signs is common in young fish.

As with the other enteric *Yersinia* pathogens, 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 factors associated with stress are poor water quality, including an increased load of organic matter often from overcrowding and increased water temperatures, which results in lowered oxygen content. Commercial bacterin vaccines have been used successfully to control yersiniosis in aquaculture. Systemic infection and significant mortality can occur in fish hatcheries.

IMMUNITY

Acquired immunity to infection by *Yersinia* can be provided in a number of ways. For instance, there is evidence that prior infection by a different *Yersinia* sp. is often sufficient to provide immunity (Fukushima et al. 2001). While V antigen is often cited as a major protective antigen, there are reports that pYV-cured strains of *Y. pseudotuberculosis* induce protection (Simonet et al. 1985) as do *Y. pseudotuberculosis* mutants in the dam methylase gene (Julio et al. 2002) and *Y. pestis* strains that lack the HPI. In fact, EV7651f, a *Y. pestis* strain lacking the HPI and *hms* genes, has been the most widely used vaccine strain in humans and animals. A killed vaccine strain has also been developed, although it is not available for use in the United States at this time (Russell et al. 1995).

Recently, an acellular *Y. pestis* vaccine has been developed in the United Kingdom and is showing much promise in protecting against bubonic, systemic, and pneumonic forms of plague (Williamson et al. 2000). This vaccine is composed of two antigens, namely the FI capsule and V antigen. Nonetheless, there is still potential cause for concern with the potential of bioengineering of *Y. pestis*, the apparent rapid evolution of *Y. pestis,* and the finding that the FI antigen is not necessary for virulence. Nature or people could create 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 acellular vaccines and therapeutics to use against possible future outbreaks of the deadly *Y. pestis*.

FUTURE DIRECTIONS

In the past 20 years, much progress has been made in understanding the proteins involved in the pathogenesis of *Yersinia* spp., particularly the pYV-encoded type III secretion system apparatus, Yops, iron-acquisition mechanisms, and adherence factors in the enteric *Yersinia* spp. However, there remain areas that are still not well understood. For instance, the adhesins that function in *Y. pestis* have not been completely characterized, and the genes that are critical for lung colonization by *Y. pestis* are unknown. Regrettably, almost nothing is known 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, involving determination of the genes needed to markedly enhance virulence, touches upon some ethically and, currently, politically sensitive areas of research. While addressing this question may provide researchers with the knowledge or means to generate a more lethal form of enteric *Yersinia* spp., or even plague, not addressing these questions seems ultimately more foolish. An understanding of how pathogens establish footholds in the host 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 providing resistance to antibiotics. This acquisition of antibiotic resistant genes presumably occurred in the flea (Hinnebusch et al. 2002b). 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.

REFERENCES

Achtman, M., K. Zurth, G. Morelli, G. Torrea, A. Guiyoule, and E. Carniel. 1999. *Yersinia pestis*, the

cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. U S A 96: 14043–14048.

Alibek, K., and S. Handleman. 1999. Biohazard: The chilling true story of the largest covert biological weapons program in the world—told from inside by the man who ran it. New York: Random House, pp. 36–37, 164–173.

Autenrieth, I. B., U. Vogel, S. Preger, B. Heymer, and J. Heesemann. 1993. Experimental *Yersinia enterocolitica* infection in euthymic and T-cell-deficient athymic nude C57BL/6 mice: comparison of time course, histomorphology, and immune response. Infect. Immun. 61:2585–2595.

Bearden, S. W., and R. D. Perry. 1999. The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. Mol. Microbiol 32: 403–414.

Bliska, J. B., and S. Falkow. 1992. Bacterial resistance to complement killing mediated by the Ail protein of *Yersinia enterocolitica*. Proc. Natl. Acad. Sci. U S A 89:3561–3565.

Bliska, J. B., M. C. Copass, and S. Falkow. 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into HEp-2 cells. Infect. Immun. 61:3914–3921.

Bottone, E. J. 1997. *Yersinia enterocolitica*: the charisma continues. Clin. Microbiol. Rev. 10:257–276.

Carniel, E. 2001. The *Yersinia* high-pathogenicity island: an iron-uptake island. Microbes Infect. 3:561–569.

Cornelis, G. 2002. *Yersinia* type III secretion: send in the effectors. J. Cell. Biol. 158:401–408.

Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier. 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol. Mol. Biol. Rev. 62:1315–1352.

Drozdov, I. G., A. P. Anisimov, S. V. Samoilova, I. N. Yezhov, S. A. Yeremin, A. V. Karlyshev, V. M. Krasilnikova, and V. I. Kravchenko. 1995. Virulent non-capsulate *Yersinia pestis* variants constructed by insertion mutagenesis. J. Med. Microbiol. 42:264–268.

Foultier, B., P. Troisfontaines, D. Vertommen, M. N. Marenne, M. Rider, C. Parsot, and G. R. Cornelis. 2003. Identification of substrates and chaperone from the *Yersinia enterocolitica* 1B Ysa type III secretion system. Infect. Immun. 71:242–253.

Foultier, B., P. Troisfontaines, S. Muller, F. R. Opperdoes, and G. R. Cornelis. 2002. Characterization of the *ysa* pathogenicity locus in the chromosome of *Yersinia enterocolitica* and phylogeny analysis of type III secretion systems. J. Mol. Evol. 55:37–51.

Fukushima, H., Q. Hao, K. Wu, X. Hu, J. Chen, Z. Guo, H. Dai, C. Qin, S. Lu, and M. Gomyoda. 2001. *Yersinia enterocolitica* O9 as a possible barrier against *Y. pestis* in natural plague foci in Ningxia, China. Curr. Microbiol. 42:1–7.

Gage, K. L., D. T. Dennis, et al. 2000. Cases of cat-associated human plague in the Western US, 1977–1998. Clin. Infect. Dis. 30:893–900.

Gong, S., S. W. Bearden, V. A. Geoffroy, J. D. Fetherston, and R. D. Perry. 2001. Characterization of the *Yersinia pestis* Yfu ABC inorganic iron transport system. Infect. Immun. 69:2829–2837.

Grosdent, N., I. Maridonneau-Parini, M. P. Sory, and G. R. Cornelis. 2002. Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. Infect. Immun. 70:4165–4176.

Hinnebusch, B. J., A. E. Rudolph, P. Cherepanov, J. E. Dixon, T. G. Schwan, and A. Forsberg. 2002a. Role of *Yersinia murine* toxin in survival of *Yersinia pestis* in the midgut of the flea vector. Science 296:733–735.

Hinnebusch, B. J., M. L. Rosso, T. G. Schwan, and E. Carniel E. 2002b. High-frequency conjugative transfer of antibiotic resistance genes to *Yersinia pestis* in the flea midgut. Mol. Microbiol. 46:349–354.

Hinnebusch, B. J., R. D. Perry, and T. G. Schwann. 1996. Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. Science 273:367–370.

Hinnebusch, J., P. Cherepanov, Y. Du, A. Rudolph, J. D. Dixon, T. Schwan, and A. Forsberg. 2000. Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice. Int. J. Med. Microbiol. 290:483–487.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62:379–433.

Isberg, R. R., and S. Falkow 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature 317:262–264.

Julio, S. M., D. M. Heithoff, R. L. Sinsheimer, D. A. Low, and M. J. Mahan. 2002. DNA adenine methylase overproduction in *Yersinia pseudotuberculosis* alters YopE expression and secretion and host immune responses to infection. Infect. Immun. 70:1006–1009.

Lahteenmaki, K., M. Kukkonen, and T. K. Korhonen. 2001. The Pla surface protease/adhesin of *Yersinia pestis* mediates bacterial invasion into human endothelial cells. FEBS Lett 504:69–72.

Makoveichuk, E., P. Cherepanov, S. Lundberg, A. Forsberg, and G. Olivecrona. 2003. pH6 antigen of *Yersinia pestis* interacts with plasma lipoproteins and cell membranes. J. Lipid Res. 44:320–330.

Marra, A., and R. R. Isberg. 1997. Invasin-dependent and invasin-independent pathways for translocation of *Yersinia pseudotuberculosis* across the Peyer's patch intestinal epithelium. Infect. Immun. 65:3412–3421.

McDonald, C., P. O. Vacratsis, J. B. Bliska, and J. E. Dixon. 2003. The *Yersinia* virulence factor YopM forms a novel protein complex with two cellular kinases. J. Biol. Chem. 278:18514–18523.

Mecsas, J., I. Bilis, and S. Falkow. 2001. Identification of attenuated *Yersinia pseudotuberculosis* strains and characterization of an orogastric infection in BALB/c mice on day 5 postinfection by signature-tagged mutagenesis. Infect. Immun. 69:2779–2787.

Metchock, B., D. R. Lonsway, G. P. Carter, L. A. Lee, J. E. McGowan, Jr. 1991. *Yersinia enterocolitica*: a frequent seasonal stool isolate from children at an urban hospital in the southeast United States. J. Clin. Microbiol. 29:2868–2869.

Miller, V. L., and S. Falkow. 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. 56:1242–1248.

Pepe, J. C., M. R. Wachtel, E. Wagar, and V. L. Miller. 1995. Pathogenesis of defined invasion mutants of *Yersinia enterocolitica* in a BALB/c mouse model of infection. Infect. Immun. 63:4837–4848.

Perry, R. D., J. Abney, I. Mier, Jr., Y. Lee, S. W. Bearden, and J. D. Fetherston. 2003. Regulation of the *Yersinia pestis* Yfe and Ybt iron transport systems. Adv. Exp. Med. Biol. 529:275–283.

Russell, P., S. M. Eley, S. E. Hibbs, R. J. Manchee, A. J. Stagg, and R. W. Titball. 1995. A comparison of Plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model. Vaccine 13:1551–1556.

Simonet, M., P. Berche, D. Mazigh, and M. Veron. 1985. Protection against *Yersinia* infection induced by non-virulence-plasmid-encoded antigens. J. Med. Microbiol. 20:225–231.

Sodeinde, O. A., Y. V. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. D. Goguen. 1992. A surface protease and the invasive character of plague. Science 258: 1004–1007.

Straley, S. C. and P. A. Harmon. 1984. *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. Infect. Immun. 45:655–659.

Watson, R. P., T. W. Blanchard, M. G. Mense, and P. W. Gasper. 2001. Histopathology of experimental plague in cats. Vet. Pathol. 38:165–172.

Williamson, E. D., S. M. Eley, A. J. Stagg, M. Green, P. Russell, and R. W. Titball. 2000. A single dose sub-unit vaccine protects against pneumonic plague. Vaccine 19: 566–571.

Young, B. M., and G. M. Young. 2002. Evidence for targeting of Yop effectors by the chromosomally encoded Ysa type III secretion system of *Yersinia enterocolitica*. J. Bacteriol. 184:5563–5571.

22
Brucella

S. C. Olsen, C. O. Thoen, and N. F. Cheville

Brucella spp. cause zoonoses of worldwide importance. Brucellae that cause disease in goats, cattle, swine, sheep, dogs, dolphins, marine mammals, and humans are small, nonmotile, gram-negative, coccobacillary rods. The organisms are aerobes that require enriched media for primary isolation; in addition, *B. abortus* and *B. ovis* usually require an atmosphere of increased carbon dioxide (CO_2) tension. The six recognized species—*B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*—are named based on host preference, however, additional undesignated *Brucella* strains have been isolated from marine mammals. *Brucella* spp. can be differentiated on the basis of CO_2 requirement, production of hydrogen sulfide (H_2S), phage susceptibility, cell-surface antigens, and sensitivity to azo dyes. DNA polymorphism due to highly repeated DNA has been found in *Brucella* spp. Differences in restriction-fragment patterns were found when five *Brucella* species were analyzed by pulsed-field gel electrophoresis (Allardet-Servent et al. 1988).

Identification of certain isolates may require tests to determine their ability to utilize selected amino acid and carbohydrate substrates as sources of carbon and energy as measured by oxygen uptake. The differential sensitivity of *Brucella* spp. to dyes is due to slight but detectable pore-diameter differences due to alteration in group 2 outer-membrane proteins. Despite considerable phenotypic differences among the *Brucella* spp., all share greater than 90% DNA homology, and *B. melitensis* and *B. suis* share 90 to 100% identity at the nucleotide level (Paulsen et al. 2002). DNA hybridization studies suggest that *Brucellae* are part of a monospecific genus (DelVecchio et al. 2001). A small number of nucleotide polymorphisms (7,307 single nucleotide polymorphisms within a shared backbone of 3.24 Mb) do exist between *B. melitensis* and *B. suis*, and most likely other brucellae (Paulsen et al. 2002). Regions unique to each *Brucella* species have been identified and used in polymerase chain-reaction amplification techniques for detection and differentiation of brucellae (Bricker and Halling 1994).

Brucella are facultative intracellular pathogens that are able to survive and multiply within phagocytic cells and reticuloendothelial tissues. The persistence of brucellae within host cells is of prime importance in the evolution of the granulomatous reaction typical of brucellosis. The lipopolysaccharide (LPS) of *Brucella* may activate nuclear factor κB (NFκB) and induce cytokines, which recruit monocytes and contribute to granuloma formation (Lopez-Urrutia et al. 2001).

The *B. melitensis* and *B. suis* genomes have recently been published (DelVecchio et al. 2001; Paulsen et al. 2002) and sequencing of the *B. abortus* genome is under way.

VIRULENCE

Brucella do not display obvious virulence factors such as capsules, fimbriae, flagella, exotoxins, exoproteases, or other exoenzymes, cytolysins, resistance forms, antigenic variation, plasmids, or lysogenic phages (Moreno and Moriyon 2001). Analysis of the genomes of three *Brucella* species has confirmed the absence of functional sequences for most of the "classical" virulence factors, pathogenic islands, as well as the lack of a complete set of genes to mount types I, II, or III secretion systems (Moreno and Moriyon 2001). However, potential virulence genes

encoding for hemolysins, adhesins, invasins, ureases, iron regulators, and a type IV secretion system have been identified from *B. suis* or *B. melitensis* genomes.

The level of virulence of *Brucella* spp. and strains has been determined by experimental infection of various hosts. Within a given *Brucella* sp., it has repeatedly been demonstrated that strains with smooth colonial morphology (bacteria with surface expression of O-side chains [perosamine] on the LPS) are more virulent than those with rough colonial morphology (bacteria lacking expression of the LPS O-side chain) (Roop et al. 1991). The basis for this association of virulence with smooth LPS has been investigated in a variety of *in vitro* culture systems with both phagocytic and nonphagocytic cells from a number of hosts (Kreutzer et al. 1979; Riley and Robertson 1984; Canning et al. 1985; Bertram et al. 1986; Detilleux et al. 1990). These studies clearly demonstrate that both smooth (virulent) and rough (attenuated) brucellae are able to enter host cells. In studies utilizing phagocytic cells (both neutrophils and macrophages), the smooth brucellae survive and even multiply while the rough bacteria are eventually eliminated. Survival of smooth bacteria has been related to their ability to prevent or limit lysosome-phagosome fusion, and their ability to resist the destructive effects of lysosomal enzymes after fusion has occurred (see above references).

An important property of *Brucella* spp. associated with virulence is the ability to survive and multiply intracellularly in host phagocytes (Cheville 1994). *In vitro* studies show that the organisms are ingested by macrophages but are partially protected in the phagosome and can multiply. Electron microscopy has demonstrated the presence of an external layer (envelope) on *B. abortus*, *B. melitensis,* and *B. suis*. The biologic significance of an external envelope interfering with attachment of brucellae to mucous membranes of the host is unclear. In studies using murine macrophages, internalization and intracellular replication of *B. abortus* is influenced by plasma membrane cholesterol, and intracellular cholesterol trafficking is essential to establish *B. abortus* infection (Watari et al. 2002). Ketaconazole, which blocks the trafficking of cholesterol into lipid raft microdomains on the macrophage plasma membrane, blocks internalization of *B. abortus,* and bacteria that are internalized do not replicate.

The survivability of brucellae within phagocytic cells is associated with the ability to scavenge iron (Parent et al. 2002; Almiron et al. 2001), incorporation into phagosomes containing correct intracellular cholesterol trafficking (Watari et al. 2002), and the composition of the cell wall of the bacteria. Initially, it was thought that the inability of smooth brucellae to resist intracellular killing by neutrophils was due to the bacteria's inability to stimulate oxidant production by the phagocytic cell. The lack of respiratory burst activity observed when neutrophils were exposed to smooth *Brucella* spp. was thought to be due to the fact that the bacteria were not opsonized with fresh serum (Kreutzer et al. 1979). Canning et al. (1985) demonstrated that opsonized *B. abortus* stimulated oxidant production in both bovine neutrophils and mammary gland macrophages. Irradiated *B. abortus* strain 2308, opsonized with either pooled bovine reactor serum, normal bovine serum, or normal fetal calf serum, caused bovine neutrophils to produce moderate levels of hydrogen peroxide (H_2O_2); however, the opsonized bacteria resulted in very low levels of H_2O_2 being produced by bovine blood-derived macrophages (Bounous et al. 1992). The data on bovine macrophages are less clear and suggest that brucellae are not able to stimulate oxidant production necessary to initiate killing of the bacteria. This may be important; all evidence to date suggests that brucellae persist within macrophages and not within neutrophils.

Elimination of bacterial agents by phagocytes typically requires fusion of lysosomes with phagosomes. *Brucella* spp. appear to passively arrest phagosome maturation between the steps of acidification and phagosome-lysosome fusion, and brucellae survival is dependent on maintaining an acidic intraphagosomal pH and/or close contact with the phagosomal wall (Rittig et al. 2001). Studies have demonstrated that smooth brucellae inhibit phagosome-lysosome fusion; however, the bacterial factors responsible for the inhibition are not known. In one study, a water extract of smooth *B. abortus* inhibited phagosome-lysosome fusion, while water extracts from a nonvirulent *B. abortus* failed to inhibit fusion (Bertram et al. 1986). The only significant differences found within the aqueous extracts from both the virulent and nonvirulent strains were in the total amounts of sugars in each extract. Others have shown that crude supernatants from heat-killed *B. abortus*, but not from washed, heat-killed *B. abortus*, prevented lysosome-phagosome fusion in bovine neutrophils. Two low-molecular-weight components of the crude supernatants were determined to be 5'-guanosine monophosphate and adenine. These two compounds

inhibited the fusion of primary granules with phagosomes in bovine neutrophils (Canning et al. 1985; Bertram et al. 1986). Proteins produced by *B. abortus* in response to stress have been identified; however, their role in survival of bacteria in the host is unclear (Mayfield et al. 1988; Lin et al. 1992; Roop et al. 1992).

Virulent brucellae may also escape the killing mechanism of leukocytes by resisting the effects of both oxygen-dependent and -independent bactericidal systems of these cells. In a murine model, elimination of the nitrous oxide pathway had no effect on infection or persistence of pathogenic *Brucella abortus* (Sun et al. 2002). Smooth pathogenic strains of *B. abortus* have been shown to be more resistant to killing by the myeloperoxidase systems than were rough strains. The basis for this differential susceptibility is unknown, but it is thought to be related to LPS components on the surface of the smooth bacteria (Riley and Robertson 1984). Studies conducted on the interactions of brucellae with African green monkey kidney (Vero) cells have demonstrated that both smooth and rough brucellae are internalized. Rough brucellae, by virtue of the increased hydrophobicity of their cell wall, adhere more readily than smooth bacteria; however, the smooth bacteria demonstrate a greater rate of replication. Differences in the infectivity of smooth and rough brucellae for Vero cells are related to the ability of smooth cells to gain access to the rough endoplasmic reticulum (RER) of the host cells. Even though more rough bacteria than smooth bacteria entered these cells, the increased susceptibility of the rough bacteria to damage by lysosomal products may have prevented them from gaining access to the RER (Detilleux et al. 1990).

Gene-replacement and gene-deletion techniques have been widely used to construct vaccine strains that can be distinguished by marker deletions or additions. These vaccine strains have been used in work with *B. abortus*. Construction and testing of a Cu-Zn superoxide dismutase (SOD) deletion mutant of *B. abortus* strain 19 suggested that this enzyme plays a role in survival and pathogenicity (Tatum et al. 1992). However, injection of SOD-deficient mutants into mice failed to demonstrate important differences in survivability of the mutants (Sriranganathan et al. 1991). Overexpression of SOD in the *B. abortus* strain RB51 vaccine enhanced protection in a murine model (Vemulapalli et al. 2000). Others have suggested that genomic vaccines encoding the sequences of the *B. abortus* L7/L12 ribosomal protein or luminazine synthase genes can induce protection in a murine model of brucellosis (Kurar and Splitter 1997; Velikovsky et al. 2002).

DISEASE

Establishment of infection by *Brucella* spp. depends on the number and virulence of bacteria and the relative resistance of a host, as determined by innate and acquired specific immune mechanisms. Approximately 18% of cross-bred cattle have been estimated to be innately resistant to infections with *B. abortus* (Templeton and Adams 1990). As the resistance conferred in cattle appears to be a quantitative difference, rather than qualitative (Derr et al. 2002), other genes may influence resistance. Phenotypically, this resistance is manifested by the ability of macrophages from resistant cattle to limit the replication of *B. abortus* (Price et al. 1990). One gene identified as playing a role in the bovine macrophage is Slc11a1 (NRAMP1), the homologue of the murine Bcg gene (Barthel et al. 2001). A similar pattern of inheritance has been demonstrated in swine that are resistant to *B. suis* infections (Cameron et al. 1941). Innate resistance to other intracellular pathogens in mice is controlled by multiple genes and is very similar to the pattern of resistance to *Brucella* spp. observed in both cattle and swine (Ho and Cheers 1982; Skamene et al. 1982). In contrast, acquired resistance to brucellae infections in mice is influenced by a large complex of genes (Cheers 1984).

The host mechanisms responsible for increased susceptibility to infection as pregnancy advances are not known, but they may be related to the differential susceptibility of placental trophoblasts during the middle and late stages of pregnancy (Samartino and Enright 1992). The probability of isolation of *B. abortus* at parturition increased from 0.22 to 0.9 as fetal age at the time of challenge of nonvaccinated heifers increased from 60 to 150 days of gestation (Crawford et al. 1987). Organisms may penetrate the mucosa of nasal or oral cavities. Following penetration of mucosal epithelium, bacteria are transported, either free or within phagocytic cells, to regional lymph nodes, which become enlarged due to lymphatic and reticuloendothelial hyperplasia and inflammation. These changes may require several weeks to develop and may persist for months. If bacteria do not become localized and are not killed in regional lymph nodes, spread to other organs via lymph and blood may occur. Brucellae gain access to the uterus via a hematogenous route,

and the bacteria initially localize within erythrophagocytic trophoblasts of the placentome. Adjacent chorioallantoic trophoblasts become infected and support massive growth of the bacteria. These cells eventually rupture and ulcerate the chorioallantoic membrane. Bacteria and inflammatory cells both are present within the lumen of the uterus. Bacteria spread via a hematogenous route to the fetus and to the placentome. Fetuses may also ingest amniotic fluid containing brucellae. It is important to note that the endometrium is not infected with brucellae and, other than a diffuse submucosal inflammatory reaction, it remains largely intact.

The ability to utilize erythritol has been proposed as a virulence factor for brucellae, and genes for erythritol metabolism have been identified in a pathogenic *B. abortus* strain (Sangari et al. 2000). Localization of brucellae in the reproductive tract of both sexes may be due to a high concentration of erythritol but definitive information is not available (Keppie et al. 1965). The presence of elevated amounts of erythritol in uterine tissues of cattle and other species, including sheep, goats, and swine, suggests an important role for the ability to utilize erythritol in the tissue tropism of certain *Brucella* spp. Moreover, extracts of fetal fluids, placenta, and chorion have been shown to stimulate growth of *B. abortus*, *B. melitensis*, and *B. suis* (Keppie et al. 1965).

BRUCELLA ABORTUS

Brucella abortus infection in pregnant cattle involves the placenta, fetus, and mammary gland. One investigation indicated initial lesions occurred within the placenta as early as 4 weeks after challenge; at 5 weeks, a more extensive endometritis with erosion of endometrial epithelium was evident (Payne 1959; Anderson and Cheville 1986). Following experimental infection of pregnant cattle via the conjunctival route, bacteria were first detected in the uterus 4–5 weeks postinoculation.

Chorionic epithelium becomes infected and infection extends into the placenta. Considerable variation occurs in distribution and severity of placental lesions in natural and experimental *B. abortus* infections. Seldom are all placentomes involved, and frequently only portions of individual placentomes develop lesions. Moreover, fetuses that become infected during late gestation may be aborted, with an absence of grossly recognizable placental lesions. In such cases, microscopic examination of placental tissues often reveals mild focal inflammation (Enright et al. 1984). Other investigators report that extensive fetal inflammatory disease involving multiple organs leads to fetal stress, which results in premature deliveries. Bovine fetuses of a known gestation age were inoculated per os or intramuscularly with low doses of *B. abortus* strain 2308 and examined after abortion or delivery by cesarean section at intervals of 6–16 days postinoculation (Enright et al. 1984). Infected fetuses developed lymphoreticular hyperplasia of lymph nodes as early as 6 days postinoculation. Progression of lymph node changes to primary-follicle formation and germinal center formation was noted at 9 and 12 days postinoculation, respectively. Morphologic alterations of lymph nodes correlated with elevations of IgM and IgG immunoglobulin levels by day 9 postinoculation. Granulomatous responses, composed of macrophages, epithelioid cells, and giant cells, were present in lymphatic tissues, liver, and occasionally within the kidney interstitium as early as 6 and 9 days postinoculation. Inflammatory lesions were extensive in fetal lungs characterized by thickened interlobular septae and focal to diffuse, exudative interstitial pneumonia. Pulmonary lesions were composed primarily of macrophages and a few neutrophils.

Measurement of fetal cortisol levels may serve as an indicator of fetal stress. Also, as fetal corticosteroids can induce the physiological process of fetal expulsion/parturition (Gaverick and Smith 1993), they may also serve as an early indicator of a physiologic process that could lead to abortion. Fetal cortisol levels were elevated substantially above normal values and were present as early as 6 days postinoculation (Enright et al. 1984). Experimentally infected bovine fetuses were aborted between 7 and 19 days postinoculation. This relatively short and consistent interval between infection and abortion suggests that the bovine fetus is a sensitive indicator of *in utero* infection with *B. abortus*. The character and early development of granulomatous inflammatory reaction and a rapid development of the fetal immune response demonstrate that the fetus is capable of reaction to *B. abortus* infection similar to that of mature animals.

Mammary gland infection is often clinically inapparent, and gross lesions may not be present. Histologically, there is a lobular and periductal, lymphoplasmacytic, and histiocytic interstitial mastitis with leukocytes in alveoli and ducts. Brucellae localize and replicate primarily in macrophages in mammary secretions or in phagocytes in the inter-

stitium. Lesions are not seen in all infected mammals (Meador et al. 1989a). Supramammary lymph nodes are typically enlarged and are characterized by edema, lymphofollicular hyperplasia, medullary plasmacytosis, and sinus histiocytosis. Localization of *B. abortus* in the mammary gland is markedly influenced by nursing, that is, milk retention in the mammary gland correlates with the degree of infection. Studies in goats have shown that failure to nurse or release milk enhances localization and replication of bacteria in mammary glands after parturition (Meador et al. 1989b). In turn, mammary infection may result in increased systemic spread and persistence of brucellae in the host.

BRUCELLA MELITENSIS

Due to its high pathogenicity in humans, *B. melitensis* is one of the most serious zoonoses in the world with distribution in the Mediterranean, Arabian peninsula, and Asia, and parts of Africa, Latin America, and South America (Alton 1990). *Brucella melitensis* is the principal cause of brucellosis in sheep and goats, is endemic in camels in some areas (Abbas and Agab 2002), and is becoming problematic as a cause of cattle brucellosis in some countries (Corbel 1997). It is the least species specific of the brucellae, and transmits to many other species (Alton 1990). With the exception of small ruminants, camels, and cattle, most species are considered to be end hosts for *B. melitensis* (Alton 1990).

The pathogenesis of *B. melitensis* infection in goats and early localization within the mammary gland and pregnant uterus of sheep is similar to that of *B. abortus* in cattle. In sheep and goats, excretion in milk is a significant route of *B. melitensis* transmission to offspring (Grilló et al. 1997). Following intravenous injection in sheep, *B. melitensis* induced necrosis and edema of placentomes and glandular endometritis (Mollelo et al. 1963). In general, *B. melitensis* is considered to cause more necrosis in placental tissue and less exudation than *B. abortus*.

BRUCELLA OVIS

B. ovis is an important cause of epididymitis in rams, but appears to be the least pathogenic of the *Brucella* that affects animals. Venereal exposure is probably the most frequent route of transmission (Buddle 1955). Lesions in rams are most often located in the tail of the epididymis. Initial localization in the epididymis is accompanied by perivascular edema and infiltration of peritubular tissue by lymphocytes and monocytes; subsequently, neutrophils infiltrate the exudate. Previously inflamed tubular epithelium develops papillary hyperplasia and local hydropic degeneration, with subsequent formation of inflammatory reaction leading to an extravasation of spermatozoa. Host responses to extravasated spermatozoa lead to the formation of large spermatic granulomas, which may result in complete blockage of the epididymis; testicular degeneration and fibrosis are secondary to this blockage. *B. ovis* can be cultured from spleens of infected ewes in which no lesions are observed. Placental pathology experimentally induced by *B. ovis* tends to localize in the intercotyledonary placenta and is often less severe than the placentitis caused by *B. melitensis* or *B. abortus*.

Pregnant ewes inoculated with *B. ovis* in the conjunctiva on days 30 to 90 of gestation usually develop uterine infections and abort or deliver infected lambs. In contrast to the limited period of susceptibility of fetuses in the intraconjunctivally infected ewes, fetuses exposed to *B. ovis in utero* are susceptible to infection throughout pregnancy. Intervals between *in utero* infection with *B. ovis* and abortion range from 23 to 80 days postinoculation; by comparison, bovine fetuses infected *in utero* by *B. abortus* usually abort by 12 days postinoculation. This difference may be attributed to the low pathogenicity of *B. ovis*. The immunologic and granulomatous inflammatory responses of *B. ovis*-infected bovine fetuses are similar to those observed in *B. abortus*-infected bovine fetuses.

BRUCELLA SUIS

Brucellosis in swine is usually caused by *B. suis*, which may be transmitted by ingestion, although venereal transmission occurs (McMillan 1992). Boars are as susceptible to infection as sows, and many infected boars develop lesions in the testicles and accessory reproductive organs and shed *B. suis* organisms in semen for extended periods. There is a tendency for focal granulomatous inflammation to develop in the endometrium and extend to the entire nongravid uterus. *B. suis* also tends to secondarily localize in a greater variety of tissues than does *B. abortus*. The organism demonstrates a predilection for localization in bone and joints, spleen, liver, kidney, and brain. Prolonged bacteremia often occurs with *B. suis* infection and may contribute to localization in a wide variety of tissues.

The early stages in pathogenesis of *B. suis* infection in swine are comparable to the early stages of

B. abortus infection in cattle. However, the character of the response of swine to *B. suis* differs slightly from the response of cattle to *B. abortus*. *B. suis* multiplies in mononuclear phagocytes and produces granulomatous lesions composed of macrophages and epitheliod cells. The granulomas tend to undergo caseous necrosis and become encapsulated by fibrous connective tissue.

BRUCELLA CANIS

Brucellosis in dogs is characterized by abortions in females, and epididymitis, testicular atrophy, and infertility in males (Carmichael 1990). *Brucella canis* has a limited host range with canids most susceptible and with cats, laboratory animals, and humans being less susceptible to infection. The disease is rapidly transmitted via vaginal discharges, semen, urine, milk, and other body fluids. The principle clinical sign is abortion, however, many dogs do not demonstrate prominent clinical signs. Diagnosis is based on serologic tests. There is no acceptable vaccine for canine brucellosis.

HOST RESPONSE

The alimentary tract is the major route in the transmission of *B. abortus* in cattle. Licking aborted fetuses and placental membranes or ingesting contaminated milk by calves introduces brucellae to the oral mucosa, tonsils, and gastrointestinal mucosa. Passage of *B. abortus* through epithelial barriers results in acute regional lymphadenitis and bacteremia.

Epithelium covering domes of ileal Peyer's patches, an important site of entry for several bovine pathogens that traverse the intestinal mucosa, is important in uptake of brucellae. Studies of infected ligated ileal loops in calves have shown that transepithelial migration of *B. abortus* occurs chiefly by dome lymphoepithelial cell endocytosis and transport, and that bacteria are degraded by macrophages and neutrophils of the gut-associated lymphoid tissue (Ackermann et al. 1988).

The interaction of bacteria with serum components (i.e., antibody or complement), neutrophils, mononuclear macrophages, fibroblasts, and epithelial cells results in the production of a variety of biologically active substances that activate macrophages, expand clones of antigen-recognizing T lymphocytes, stimulate lymphocytes to secrete cytokines, stimulate hematopoiesis, and induce inflammation.

Several fractions have been isolated from *B. abortus* that generate chemotactic factors derived from serum (Bertram et al. 1986). A carbohydrate-rich, aqueous methanol fraction of *B. abortus* inhibited chemotactic activity at high concentration; however, a nondialyzable component of this fraction contained a potent stimulator of chemotaxis. Preheating the serum at 56°C for 30 minutes prevented generation of chemotactic activity by different fractions. Protein-rich fractions of *B. abortus* strain 2308 or *B. abortus* strain 19 failed to stimulate chemotaxis.

Neutrophils are considered an important line of defense against infection with the *Brucella* spp. (Riley and Robertson 1984). A component of *B. abortus* is capable of inhibiting release of myeloperoxidase by dose-dependent preferential inhibition of primary granule release from bovine neutrophilic leukocytes (Bertram et al. 1986). Failure of polymorphonuclear leukocytes (PMNs) to kill the organism at the primary site of infection may be responsible in part for the dissemination of *Brucella* spp. to other tissues of the body. Lysates from granules of guinea pig, human, or bovine PMNs were highly toxic to smooth *B. abortus* strain 45/0 or to rough *B. abortus* 45/20 (Riley and Robertson 1984). However, an oxygen-dependent killing system appeared to be lethal to both strains. Iodine was more active than chlorine in the presence of H_2O_2 and granule lysate in killing the organism. Ingestion of either strain by PMNs failed to stimulate the hexose monophosphate shut; therefore, *Brucella* spp. survive possibly because certain surface properties fail to generate a suitable stimulus to activate killing mechanisms during interaction with the plasma membrane.

Long-term protective immunity against intracellular pathogens such as *Brucella* is associated with development of strong cell-mediated responses with production of cytokines such as IL-2, IL-12, IL-18, and IFN-γ from CD4+ T cells that are associated with Th1-type responses (Manetti et al. 1993; Ismail et al. 2002). Production of IFN-γ and TNF-α from activated CD8+ cells contribute to macrophage activation and killing of brucellae (Ismail et al. 2002; Murphy et al. 2001). Although antibody responses may be beneficial, they do not appear to be associated with long-term protection. Cell-mediated responses have been evaluated in cattle infected with virulent *B. abortus* and in cattle vaccinated with *B. abortus* strain 19. Lymphocyte-stimulation responses were significantly greater in cattle infected with virulent *B. abortus* as compared to responses observed in cattle vaccinated with attenuated *B. abortus* strain 19. However, no correlation was

found between lymphocyte responses to specific antigen and humoral antibody responses in *B. abortus*-infected cattle.

Differences in subpopulations of lymphocytes and mononuclear macrophages may explain in part how an organism stimulates bactericidal activity of macrophages in one instance and suppresses this activity at other times. The chronic persistence of *B. abortus* infections may be due to intracellular localization of brucellae in macrophages whose bactericidal mechanisms are resistant or refractory to activation. Overcoming bactericidal incompetence in these cells may be necessary for elimination of brucellae. Studies on the nonspecific and specific immunity to brucellae in rodents have provided valuable information on host cell-cell interactions and on host cell-brucellae interactions, which cannot be adequately studied in domestic animals due to lack of genetically identical individuals (Cheers 1984).

The protective effects of submucosal immune responses and inflammatory reactions against invading organisms may substantially alter the ability of *Brucella* spp. to colonize the local lymph nodes. Bacterial and host factors that allow brucellae to penetrate intact mucosa should be examined. Submucosal inflammatory responses composed of macrophages, lymphocytes, plasma cells, and large numbers of eosinophils and neutrophils have been observed as early as 2 and 4 days after conjunctival inoculation of cattle with *B. abortus*. These reactions are present in the submucosa of the conjunctiva, lachrymal duct, and in tonsils. Alteration of submucosal inflammatory reactions by presensitization and their effectiveness in destroying brucellae are unknown. Some pregnancy-associated or pregnancy-specific factors that suppress immune responses may alter the effectiveness of vaccines. The responses of a pregnant host to *Brucella* spp. may represent a useful model to elucidate various mechanisms of immune suppression during pregnancy.

DISEASE CONTROL

Efforts to control and eliminate brucellosis in cattle in the United States rely on (1) the use of vaccine prepared from *B. abortus* strain RB51, (2) screening of milk of dairy herds for antibodies using the *Brucella* ring test, (3) detection of infected cattle herds by the serologic testing of slaughter cows and bulls and tracing reacting animals to the herd of origin, and (4) identification and removal of *B. abortus*-infected cattle from a herd by use of serologic tests. Due to the fact that brucellosis has nearly been eliminated from cattle in the United States, herd depopulation is also being conducted to facilitate eradication efforts. The occurrence of agglutinating substances in sera of cattle not known to have been exposed to *Brucella* spp. stimulated the development of tests for differentiating between reactions due to exposure to *Brucella* spp. and those originating from other sources. These tests are often subdivided into screening tests with high sensitivity and low specificity (buffered acid plate agglutination, card, or rose bengal plate tests), or confirmatory tests with high specificity (rivanol agglutination, standard tube agglutination, complement fixation, or fluorescence polarization assays [FPA]). Purified LPS extracted from *B. abortus* has also been used in enzyme-linked immunosorbent assays (ELISAs) for detecting cows infected with field strains of *B. abortus*.

The *Brucella* ring test (BRT), which detects brucellae antibodies in milk, is useful for monitoring dairy herds for possible *B. abortus* infection. ELISA and FPA tests have also been developed for detecting antibodies against brucellae in milk (Thoen et al. 1983).

A high percentage of *B. abortus*-infected cows shed large numbers of bacteria in vaginal secretions and fetal tissues following abortion or delivery of an infected calf; therefore, efforts have been made to detect *Brucella* spp. in these specimens using bacteriologic procedures. Although bacteriologic examinations are suitably sensitive and highly reliable, there was a need to develop more rapid and sensitive procedures. Recently, it has been shown that polymerase chain reaction (PCR) techniques can be used with high sensitivity in detecting brucellae in blood of infected individuals, and in milk, tissues, or other secretions (Gallien et al. 1998; Zerva et al. 2001).

Vaccination of calves 4–12 months of age with 10^{10} CFU of *B. abortus* strain RB51 protects cattle against abortion or infection after experimental challenge (Cheville et al. 1996; Olsen et al. 2000a). As strain RB51 has low expression of the O-side chains (perosamine) of the LPS (Schurig et al. 1991), it does not induce antibody responses that cause positive responses on conventional brucellosis surveillance tests. A reduced dosage of strain RB51 is safe when administered to pregnant cattle (Palmer et al. 1997) and provides protection against experimental challenge during the subsequent gestation (Olsen et al. 2000b).

Control of *B. melitensis* has predominantly been through vaccination in combination with serologic

or delayed-type hypersensitivity testing to facilitate removal of *B. melitensis*-infected individuals. The *B. melitensis* Rev1 strain has proven to be superior to other vaccines in small ruminants (Kolar 1987), although it has not been fully evaluated in cattle (Corbel 1997) or other large ruminants. When administered conjunctivally to sheep at the recommended dose, the Rev1 vaccine is capable of inducing protection and does not stimulate a prolonged humoral response (Garin-Bastuji et al. 1998). Subcutaneous vaccination of sheep with the Rev1 strain does induce prolonged serologic responses, which interfere with serologic tests and detection of infected animals (Garin-Bastuji et al. 1998). Both subcutaneous and conjunctival vaccination of sheep and goats with Rev1 can induce abortions (Blasco 1997). Therefore, Rev1 vaccination should not be used indiscriminately in pregnant animals.

In general, vaccines composed of killed *Brucella* or *Brucella* subunits, alone or combined with adjuvants, have not been effective in inducing protective immune responses in cattle. This may be related to intracellular pathways of antigen processing, surface expression with major histocompatibility antigens, or other causes. The specific proteins or genes of brucellae that induce protective cell-mediated responses are currently unknown, but work has suggested that protective antigens may be conserved across *Brucella* spp. (Olsen et al. 1997, 1998). Evaluation of recombinant vaccines in murine models has suggested protective roles for several genes (Kurar and Splitter 1997; Vemulapalli et al. 2000; Velikovsky et al. 2002). New approaches utilizing genetic engineering techniques to produce vaccines with increased immunogenicity are a subject of ongoing research.

Alum-precipitated bacteria prepared from *B. ovis* have been used to stimulate immune responses in sheep. Vaccines against *B. canis* or *B. suis* have not been shown to adequately stimulate host immunity to provide protection against challenge by natural exposure. Serologic tests and cultural examinations should be used for identifying infected dogs and swine so they can be removed from a population.

The major cycle of transmission of *B. abortus* is from an aborted fetus to mature females. *Brucella abortus* infection of farm dogs has been reported; clinical signs are uncommon, but abortion, epididymitis, and arthritis occur. Duration of infection varies but may be as long as 464 days. Lymph nodes associated with the alimentary canal have the highest incidence of infection (Forbes 1990). Infected dogs may shed bacteria via urine, vaginal exudates, feces, or aborted fetuses. Cattle-to-dog transmission occurs through ingestion of infected bovine placental tissue. Dog-to-cattle transmission has been reported, but only experimentally. Infected vaginal discharges have been reported up to 42 days after abortion in dogs.

Insects that suck blood or feed on body fluids are capable of transmitting pathogenic *B. abortus*. Life cycles of the face fly (*Musca autumnalis*) are closely tied to cattle and other ruminants. This fly requires semifluid bovine feces for ova deposition, and selectively feeds on bovine body fluids including blood, tears, and placental exudates. Experimentally, face flies will take up, hold, and excrete brucellae in their feces (Cheville et al. 1989). Although this mechanical transmission is probable in nature, it is unlikely to play a major role in transmission and maintenance of the disease in cattle.

REFERENCES

Abbas, B., and H. Agab. 2002. A review of camel brucellosis. Prev. Vet. Med. 55:47–56.

Ackermann, M. R., N. F. Cheville, and B. L. Deyoe. 1988. Bovine ileal dome lymphoepithelial cells: Endocytosis and transport of *Brucella abortus* strain 19. Vet. Pathol. 25:226–237.

Allardet-Servent, A., G. Bourg, M. Ramuz, M. Pages, M. Bellis, and G. Roizes. 1988. DNA polymorphism in strains of the genus *Brucella*. J. Bacteriol. 170:4603–4607.

Almiron, M., M. Martinez, N. Sanjuan, and R. A. Ugalde. 2001. Ferrochelatase is present in *Brucella abortus* and is critical for its intracellular survival and virulence. Infect. Immun. 69: 6225–6230.

Alton, G. G. 1990. *Brucella melitensis*. In Animal brucellosis. K. Nielsen and J. R. Duncan (eds.). Boston: CRC Press, pp. 383–410.

Anderson T. D., and N. F. Cheville. 1986. Ultrastructural morphometric analysis of *Brucella abortus* infected trophoblasts in experiental placentitis. Am. J. Pathol. 124:226–237.

Barthel, R., J. Feng, J. A. Piedrahita, D. N. Murray, J. W. Templeton, and L. G. Adams. 2001. Stable transfection of the bovine NRAMP1 gene into murine RAW264.7 cells: effect on *Brucella abortus* survival. Infect. Immun. 69:3110–3119.

Bertram, T. A., P. C. Canning, and J. A. Roth. 1986. Preferential inhibition of primary granule release from bovine neutrophils by a *Brucella abortus* extract. Infect. Immun. 52:285–292.

Blasco, J. M. 1997. A review of the use of *B. melitensis* Rev1 vaccine in adult sheep and goats. Prev. Vet. Med. 31:271–283.

Bounous, D. I., F. M. Enright, K. A. Gossett, C. M. Berry, and M. T. Kearney. 1992. Comparison of oxidant production by bovine neutrophils and monocyte-derived macrophages stimulated with *Brucella abortus* strain 2308. Inflam. 16:215–218.

Bricker, B. J., and S. M. Halling. 1994. Differentiation of *Brucella abortus* (biovars 1,2, and 4), *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* (biovar 1) by the polymerase chain reaction. J. Clin. Microbiol. 32:2660–2666.

Buddle, M. B. 1955. Observations on the transmission of *Brucella* infection in sheep. N. Z. Vet. J. 3:10–19.

Cameron, H. S., P. W. Gregory. and E. H. Hughes. 1941. Studies in genetic resistance in swine to *Brucella* Infection. II. A bacteriological examination of resistant stock. Cornell Vet. 31:21.

Canning, P. C., J. A. Roth, L. B. Tabatabai, and Deyoe, B. L. 1985. 1985. Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. J. Infect. Dis. 152:913–921.

Carmichael, L. E. 1990. *Brucella canis*. In Animal brucellosis. K. Neilsen and J. Robert Duncan (eds.). Ann Arbor: CRC Press, pp. 335–350.

Cheers, C. 1984. Pathogenesis and cellular immunity in experimental murine brucellosis. In Biological standardization, vol. 56. Third International Symposium on Brucellosis. L. Vallette and W. Heunessen (eds.). Basel: S. Karger, pp. 237–246.

Cheville, N. F. 1994. Ultrastructure pathology: An introduction to interpretation. Ames: Iowa State University Press.

Cheville, N. F., D. G. Rogers, B. L. Deyoe, E. S. Krafsur, and J. C. Cheville. 1989. Uptake and excretion of *Brucella abortus* in tissues of the face fly (*Musca autumnalis*). Am. J. Vet. Res. 50:1302–1306.

Cheville, N. F., S. C. Olsen, A. E. Jensen, M. G. Stevens, M. V. Palmer, and A. M. Florance. 1996. Effects of age at vaccination on efficacy of *Brucella abortus* strain RB51 to protect cattle against brucellosis. Am. J. Vet. Res. 57:1153–1156.

Corbel, M. J. 1997. Brucellosis: an overview. Emerg. Infect. Dis. 3:213–221.

Crawford, R. P., L. G. Adams, and J. D. Williams. 1987. Relationship of fetal age at conjunctival exposure of pregnant heifers and *Brucella abortus* isolation. Am. J. Vet. Res. 48:755–757.

DelVecchio, V. G., V. Kapatral, R. J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernal, M. Mazur, E. Goltsman, E. Selkov, P. H. Elzer, S. Hagius, D. O. Callaghan, J.-J. Letesson, R. Haselkorn, N. Kyrpides, and R. Overbeek. 2001. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc. Natl. Acad. Sci. U S A 99:443–448.

Derr, J., C. Seabury, C. Scutta, and J. W. Templeton. 2002. Genetic resistance to disease in bison. In Brucellosis in elk and bison in the greater Yellowstone area. T. J. Kreeger (ed.). Cheyenne: Wyoming Game and Fish Department, pp. 97–98.

Detilleux, P. G., B. L. Deyoe, and N. F. Cheville. 1990. Penetration and intracellular growth o *Brucella abortus* in nonphagocytic cells in vitro. Infect. Immun. 58:2320–2328.

Enright, F. M., J. V. Walker, G. W. Jeffers, and B. L. Deyoe. 1984. Cellular and humoral responses of *Brucella abortus* infected bovine fetuses. Am. J. Vet. Res. 45:424–430.

Forbes, L. B. 1990. *Brucella abortus* infection in 14 farm dogs. J. Am. Vet. Med. Assoc. 196:911–916.

Gallien, P., C. Dorn, G. Alban, C. Staak, and D. Protz. 1998. Detection of *Brucella* species in organs of naturally infected cattle by the polymerase chain reaction. Vet. Rec. 142:512–514.

Garin-Bastuji, B., J-M. Blasco, M. Grayon, and J-M. Verger. 1998. *Brucella melitensis* infection in sheep: present and future. Vet. Res. 29:255–274.

Gaverick, H. A., and M. F. Smith. 1993. Female reproductive physiology and endocrinology of cattle. Vet. Clinics of North America 9:223–247.

Grilló, M. J., M. Barberán, and J. M. Blasco. 1997. Transmission of Brucella melitensis from sheep to lambs. Vet. Rec. 140:602–605.

Ho, M., and C. Cheers. 1982. Resistance and susceptibility of mice to bacterial infection. IV. Genetic and cellular basis of resistance to chronic infection with *Brucella abortus*. J. Infect. Dis. 146:381–387.

Ismail, N., J. P. Olano, H.-M., Feng, and D. H. Walker. 2002. Current status of immune mechanisms of killing of intracellular microorganisms. FEMS Microbiol. Letts 207:111–120.

Keppie, J., A. E. Williams, K. Witt, and H. Smith. 1965. The role of erythritol in tissue localization of the *Brucellae*. Br. J. Exp. Pathol. 46:104–108.

Kolar, J. 1987. Control of Brucella melitensis brucellosis in developing countries. Ann. Inst. Pasteur. Microbiol. 138:122–126.

Kreutzer, D. L., L. A. Dreyfus, and D. C. Robertson. 1979. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infect. Immun. 23:737–742.

Kurar, E., and G. A. Splitter. 1997. Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. 15:1851–1857.

Lin, J., L. G. Adams, and T. A. Ficht. 1992. Characterization of the heatshock response in

Brucella abortus and isolation of the genes encoding the GroE heatshock proteins. Infect. Immun. 60:2425–2431.

Lopez-Urrutia, L., A. Alonso, Y. Bayon, M. L. Nieto, A. Orduna, and M. Sanchez Crespo. 2001. *Brucella* lipopolysaccharides induce cyclooxygenase-2 expression in monocytic cells. Biochem. Biophys. Res. Commun. 289:372–375.

Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinn, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J. Exp. Med. 177:1199–1204.

Mayfield, J. E., B. J. Bricker, H. Godfrey, R. M. Crosby, D. J. Knight, S. M. Halling, D. Balinsky, and L. B. Tabatabai. 1988. The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. Gene 63:1–9.

McMillan, A. P. 1992. Brucellosis. In Diseases of swine, 7th ed. A. D. Leman, B. E. Straw, W. L. Mengeling, S. D. Allaire, and D. J. Taylor (eds.). Ames: Iowa State University Press, pp. 446–453.

Meador, V. P., B. L. Deyoe, and N. F. Cheville. 1989a. Effect of nursing on *Brucella abortus* infection of mammary glands of goats. Vet. Pathol. 26:369–375.

———. 1989b. Pathogenesis of *Brucella abortus* infection of the mammary gland and supramammary lymph node of the goat. Vet. Pathol. 26:357–368.

Mollelo, J. A., J., C. Flint, J. R. Collier, and R. Jensen. 1963. Placental Pathology. II. Placental lesions of sheep experimentally infected with *Brucella melitensis*. Am. J. Vet. Res. 24:905–913.

Moreno, E., and I. Moriyon, I. 2001. *Brucella melitensis*: A nasty bug with hidden credentials for virulence. PROC. Natl. Acad. Sci. U S A 99:1–3.

Murphy, E. A., M. Parent, J. Sathiyaseelan, X. Jiang, and C. L. Baldwin. 2001. Immune control of *Brucella abortus* 2308 infections in BALB/c mice. FEMS Immunol. Med. Microbiol. 32:85–88.

Olsen, S. C. 2000a. Immune responses and efficacy after administration of a commercial *Brucella abortus* strain RB51 vaccine to cattle. Vet. Therapeutics 1:183–191.

———. 2000b. Responses of adult cattle to vaccination with a reduced dose of *Brucella abortus* strain RB51. Res. Vet. Sci. 69:135–140.

Olsen, S. C., A. E. Jensen, M. V. Palmer, and M. G. Stevens. 1998. Evaluation of serologic responses, lymphocyte proliferative responses, and clearance from lymphatic organs after vaccination of bison with *Brucella abortus* strain RB51. Am. J. Vet. Res. 59:410–415.

Olsen, S. C., N. F. Cheville, M. G. Stevens, H. H. Houng, E. S. Drazek, T. L. Hadfield, R. L., Warren, and D. L. Hoover. 1997. Lymphocyte proliferative responses of goats vaccinated with *Brucella melitensis* 16M or a δpurE201 strain. Infect. Immun. 67:2987–2991.

Palmer, M. V., S. C. Olsen, and N. F. Cheville. 1997. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. Am. J. Vet. Res. 58:472–477.

Parent, M. A., B. H. Bellaire, E. A. Murphy, R. M. Roop, P. H. Elzer, and C. L. Baldwin. 2002. *Brucella abortus* siderophore 2,3-dihydroxybenzoic acid (DHBA) facilitates intracellular survival of the bacteria. Microb. Pathog. 32:239–248.

Paulsen, I. T., R. Seshadri, K. E. Nelsen, J. A. Eisen, J. F. Heidelber, T. D. Read, R. J. Dodson, L. Umayam, L. M. Brinkac, M. J. Beanan, S. C. Daugherty, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, W. C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. E. Van Aken, S. Riedmuller, H. Tettelin, S. R. Gil, O. White, S. L. Salzberg, D. L. Hoover, L. E. Lindler, S. M. Halling, S.M. Boyle, and C. M. Fraser. 2002. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. U S A 99:13148–13153.

Payne, J. M. 1959. The pathogenesis of experimental brucellosis in the pregnant cow. J. Pathol. Bacteriol. 78:447–463.

Price, R. E., J. W. Templeton, R. Smith, and L. G. Adams. 1990. Ability of resistant phagocytes from cattle naturally resistant or susceptible to brucellosis to control in vitro intracellular survival of *Brucella abortus*. Infect. Immun. 58:879–886.

Riley, L. K., and D. C. Robertson. 1984. Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. Infect. Immun. 46:224–230.

Rittig, M. G., M. T. Alvarez-Martinez, F. Porte, J. P. Liautard, and B. Rouot. 2001. Intracellular survival of *Brucella* spp. in human monocytes involves conventional uptake but special phagosomes. Infect. Immun. 69:3995–4006.

Roop, R. M., G. Jeffers, T. Bagchi, J. Waler, F. M. Enright, and G. G. Schurig. 1991. Experimental infection of goat fetuses in utero with a stable rough mutant of *Brucella abortus*. Res. Vet. Sci. 51:123–127.

Roop, R. M., M. L. Price, B. E. Dunn, S. M. Boyle, N. Sriranganathan, and G. G. Schurig. 1992. Molecular cloning and nucleotide sequence analy-

sis of the gene encoding the immunoreactive *Brucella abortus* Hsp60 protein, BA60K. Microb. Pathog. 12:47–62.

Samartino, L. E., and F. M. Enright. 1992. Interaction of bovine chorioallantoic membrane explants with three strains of *Brucella abortus*. Am. J. Vet. Res. 53:359–363.

Sangari, F. J., J. Agueero, and J. M. Garcia-Lobo. 2000. The genes for erythritol catabolism are organized as an inducible operon in *Brucella abortus*. Microbiol. 146:487–495.

Schurig, G. G., R. M. Roop, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan. 1991. Biological properties of RB51: a stable rough strain of *Brucella abortus*. Vet. Microbiol. 28:171–188.

Skamene, E., P. Gros, A. Forget, P. A. L. Kongshavn, C. St. Charles. 1982. Genetic regulation of resistance to intracellular pathogens. Nature 297:506–509.

Sriranganathan, N., S. M. Boyle, G. Schurig, and H. Misra. 1991. Superoxide dismutases of virulent and avirulent strains of *Brucella abortus*. Vet. Microbiol. 26:359–366.

Sun, Y. H., A. B. den Hartigh, L. RdE Santos, L. G. Adams, and R. M. Tsolis. 2002. VirB-mediated survival of *Brucella abortus* in mice and macrophages is independent of a functional inducible nitric oxide synthase or NADPH oxidase in macrophages. Infect. Immun. 70:4826–4832.

Tatum, F. M., P. G. Detilleux, J. M. Sacks, and S. M. Halling 1992. Construction of CuZn superoxide dismutase deletion mutants of *Brucella abortus*: Analysis of survival in vitro in epithelial and phagocytic cells and in vivo in mice. Infect. Immun. 60:2863–3869.

Templeton, J. W., and L. G. Adams. 1990. Natural resistance to bovine brucellosis. In Advances in brucellosis research. L. G. Adams (ed.). College Station: Texas A&M University Press, pp. 144–150.

Thoen, C. O., J. A. Bruner, D. W. Luchsinger, and D. E. Pietz. 1983. Detection of *Brucella* antibodies of different immunoglobulin classes in cow milk by enzyme-linked immunosorbent assay. Am. J. Vet. Res.44: 306–308.

Velikovsky, C. A., J. Cassataro, G. H. Giambartolomei, F. A. Goldbaum, S. Estein, R. A. Bowden, L. Bruno, A. A. Fossati, and M. Spitz. 2002. A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. Infect. Immun. 70:2507–2511.

Vemulapalli, R., Y. He, S. M. Boyle, N. Sriranganathan, and G. G. Schurig. 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect. Immun. 68:3286–3289.

Watarai, M., S-I. Makino, M. Michikawa, Y. Yanagisawa, S. Murakami, and T. Shirahata. 2002. Macrophage plasma membrane cholesterol contributes to *Brucella abortus* infection of mice. Infect. Immun. 70:4818–4825.

Zerva, L., K. Bourantas, S. Mitka, A. Kansouzidou, and N. J. Legakis. 2001. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. J. Clin. Microbiol. 39:1661–1664.

23
Pseudomonas

M. Matewish and J. S. Lam

In the family Pseudomonadaceae, *Pseudomonas aeruginosa* is the predominant species that is associated with diseases in humans and animals. Four other species of pathogens, namely *P. pseudomallei*, *P. mallei*, *P. cepacia*, and *P. maltophilia*, have recently been reclassified as *Burkholderia pseudomallei*, *B. mallei*, *B. cepacia,* and *Stenotrophomonous maltophilia,* respectively. Therefore, this chapter will focus on the significance of *P. aeruginosa* as a pathogen of animals and humans.

P. aeruginosa is a gram-negative, nonsporulating, rod-shaped bacterium which is ~ 0.3–0.5 μm by 1–2 μm and motile by one or several polar flagella. The secretion of the fluorescent-green pigment pyoverdin and the blue-green pyocyanin is diagnostic. The optimal growth temperature is 37°C, but the bacterium can thrive at temperatures between 10°C 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 remarkably versatile in its nutrition and is capable of utilizing over 76 organic compounds as the sole source of carbon and energy.

P. aeruginosa is ubiquitous in the environment and can be isolated from most natural habitats including water, soil, plants, and sewage. Its physiological versatility allows it to survive in virtually any kind of moist environment, even in distilled water. In the hospital setting, *P. aeruginosa* is found wherever there is water including respiratory equipment, cleaning solutions, medicines, disinfectants, sinks, and floors, and it accounts for about 10% of human nosocomial infections. Differentiation of clinical isolates is important for epidemiological studies and is facilitated by numerous typing schemes, including biotyping, antibiogram analysis, O-antigen serotyping, pyocin typing, and bacteriophage typing (Rhame 1980). These phenotyping methods are being replaced with more accurate and sensitive genotyping/DNA fingerprinting methods that include pulsed-field gel electrophoresis (Grothues et al. 1988), ribotyping (Denamur et al. 1991), and PCR-based techniques such as amplified fragment length polymorphism analysis (AFLP) (De Vos et al. 1993) and random amplified polymorphic DNA analysis (RAPD) (Mahenthiralingam et al. 1996).

DISEASES

P. aeruginosa is an opportunistic pathogen and generally requires a defect or alteration in the normal host defenses to establish an infection. When these barriers are breached by injury, trauma, surgery, burns, or indwelling devices such as intravenous lines or urinary catheters, it makes the host susceptible to *P. aeruginosa* colonization. In humans, this pathogen can cause life-threatening infections in individuals who are immunocompromised, and those with burn wounds, cancer, cystic fibrosis, AIDS, or in intensive care. The range of diseases includes pneumonia, bacteremia, and infections of the urinary tract and surgical sites.

P. aeruginosa causes a variety of diseases in animals (table 23.1), which includes 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. *P. aeruginosa* infections in which there is no obvious breach in host defense include mastitis in cattle and genital tract infections in horses. The source of a severe outbreak of mastitis in dairy herds was

found to be teat wipes that were contaminated with *P. aeruginosa* (and stored in 70% alcohol) (Daly et al. 1999). The wipes were used to sterilize the teats before injection of antibiotics into the mammary glands and provided a direct route for transfer of *P. aeruginosa* into the tissues. Other predisposing factors to infection 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.

Sporadic outbreaks of septicemic disease occur in poultry, mink, and chinchillas. Mink in particular are highly susceptible to *P. aeruginosa* infection, as shown by an intratracheal LD_{50} of 10^3–10^4, as compared to 10^6–10^9 in other species (Long et al. 1980).

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 three general groups: (1) cell-surface components, (2) extracellular products, and (3) genetic regulatory systems. Our understanding of the pathogenic mechanisms contributed by virulence factors of *P. aeruginosa* is derived mostly from studies on human diseases.

VIRULENCE FACTORS OF *P. AERUGINOSA*

Cell-Associated Factors

Flagella

Flagella are important for initial colonization of altered epithelium because they provide motility and function as an adhesin. The flagellar filament is composed of flagellin proteins encoded by *fliC* and has a cap protein, FliD. Two distinct types of FliD proteins (types A and B) are involved in binding to secreted respiratory mucin glycoconjugates bearing Lewis X or sialyl-Lewis X determinants (Scharfman et al. 2001). In addition, the flagellin itself adheres to secreted mucin glycoconjugates bearing Lewis X determinants (Scharfman et al. 2001) and to a cell-associated membrane-tethered mucin, Muc1, which is expressed by respiratory epithelial cells (Lillehoj et al. 2002).

Flagellar synthesis, bacterial motility, and adhesion to mucin are regulated by a two-component regulatory system, FleS-R (Ritchings et al. 1995), and by the sigma factor RpoN (Ramphal et al. 1996). Expression of the cap protein FliD requires an additional transcriptional regulator FleQ (Jyot et al. 2002).

Studies in a neonatal mouse model of pneumonia demonstrated that *fliC* mutants of *P. aeruginosa* had decreased virulence (Feldman et al. 1998). Histopathological studies showed that the *fliC* mutant caused focal inflammation and, unlike the parent strain, did not spread throughout the lungs. Flagella mutants were less invasive than motile strains in a mouse burn infection model and the addition of antiflagellum antibody had a significant protective effect (Montie et al. 1987). Flagella are a target for nonopsonic bacterial clearance by macrophages and polymorphonuclear neutrophils (PMNs) (Mahenthiralingam and Speert 1995) and may contribute to host clearance mechanisms by facilitating phagocytosis. Flagella also bind to epithelial glycolipid receptors, which are important stimuli for the production of interleukin-8 (IL-8) (Dimango et al. 1995). IL-8 serves to target PMN migration and activate PMNs and macrophages.

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
Cattle	Respiratory infections, mastitis, enteritis
Horses	Genital tract infection, abortion, ulcerative keratitis
Swine	Respiratory infections
Poultry	Septicemia, keratitis
Sheep	Pneumonia, mastitis, fleecerot
Mink	Hemorrhagic pneumonia, septicemia
Chinchillas	Pneumonia, septicemia
Laboratory rodents	Septicemia, enteritis

Source: Reproduced with permission from Gyles and Thoen (1993).

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 protease	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, Pyoverdin)	Acquisition of iron, enhances survival in low-iron environments such as host tissues

Thus, flagella may play a role in modulating the immune response during infection.

Pili

P. aeruginosa pili are the dominant adhesins involved in the initial phase of attachment to host tissues and are the prototypes of the type 4 family of pili (Paranchych et al. 1990; Hahn 1997). These structures are composed of pilin monomers encoded by *pilA* and are regulated by the sensor-regulator system PilS-R (Hobbs et al. 1993) and the sigma factor RpoN (Ishimoto and Lory 1992). Although each pilin protein contains a functional receptor-binding site (Hazes et al. 2000), only the binding sites of monomers displayed at the pilus tip are functional (Lee et al. 1994). 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 (Doig et al. 1990; Farinha et al. 1994; Hazes et al. 2000). 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 mediate adherence to many epithelial cell types *in vitro* including human buccal cells (Woods et al. 1980), lung pneumocytes (Farinha et al. 1994), exfoliating tracheal cells (Zoutman et al. 1991), immortalized human airway cells (Feldman et al. 1998), and cystic fibrosis airway cells (Saiman and Prince 1993). 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). 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).

Lipopolysaccharide

Lipopolysaccharide (LPS) of *P. aeruginosa* consists of hydrophobic lipid A, which anchors LPS in the

outer membrane, a core oligosaccharide that contains highly conserved sugar residues, and the long chain polysaccharide O antigen or O polysaccharide. 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 (Tobias et al. 1986; Lamping et al. 1996) and transferred to the CD14 receptor (Wright et al. 1990) and Toll-like receptor 4 (Qureshi et al. 1999) on macrophages, inducing the production of inflammatory mediators including tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6, IL-8, and IL-10 (Lynn and Golenbock 1992). It has been suggested that the lipid A of *P. aeruginosa* is less biologically potent than that from *Escherichia coli* (and other gram-negative bacteria) (Kropinski et al. 1985), but the difference in *in vivo* biological activity may be relatively small.

P. aeruginosa produces an "uncapped" core oligosaccharide (lacking attached O antigen) referred to as "rough LPS" and a "capped" form with O polysaccharide that is referred to as "smooth LPS" (Sadovskaya et al. 1998, 2000). The structure of the core region is highly conserved among *P. aeruginosa* strains including clinical isolates (Knirel et al. 2001; Bystrova et al. 2002) and rough LPS is required for adherence to and invasion of corneal epithelial cells *in vitro* and whole mice eyes *in situ* (Zaidi et al. 1996), as well as airway respiratory epithelial cells (Pier et al. 1996). *P. aeruginosa* LPS-mediated internalization within epithelial cells is followed by replication, and a reservoir of intracellular bacteria is created that can cause further infection (Fleiszig et al. 1995). 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 protein (CFTR) (Pier et al. 1996; Zaidi et al. 1999), asialo-GM1 (Gupta et al. 1994), and galectin-3 (Gupta et al. 1997).

The O-antigen polysaccharide is also referred to as B-band LPS and is composed of di- to pentasaccharide repeating units of various monosaccharides and confers serospecificity. Biosynthesis and assembly of this heteropolymer are mediated by numerous genes within the *wbp* loci (reviewed by Rocchetta et al. 1999). The O-antigen is antiphagocytic and confers resistance to complement-mediated killing (Dasgupta et al. 1994). It is required for virulence as shown in a murine burn wound sepsis model study where the LD_{50} for the O-antigen deficient mutant was at least 1,000-fold higher than that of the wild-type strain (Cryz et al. 1984). In a separate study, Tang et al. (1996) 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. *In vitro* studies have revealed that different forms of LPS affect microcolony formation in the initial stages of biofilm generation (Rocchetta et al. 1999). Production of different forms of LPS that favor the biofilm mode of growth is controlled by the gene, *migA*, which encodes a glycosyltransferase and modulates the assembly of the core oligosaccharide region (Yang et al. 2000). The *migA* gene is highly expressed in respiratory tract infections (Wang et al. 1996a) and is regulated by the *rhlR-rhlI* quorum-sensing system (Yang et al. 2000). 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, and to the development of biofilms.

Alginate and Biofilm Mode of Growth

P. aeruginosa growing on tissues or abiotic surfaces such as catheters can be induced to produce a slimelike mucoid exopolysaccharide called alginate 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. The secreted exopolysaccharide forms a viscous gel around the bacteria and facilitates the formation of microcolonies or biofilms. These mucoid strains are most often isolated from the sputum of human patients with cystic fibrosis, and the *P. aeruginosa* biofilms in the lungs of these patients have been observed *in vivo* (Lam et al. 1980). Exopolysaccharide-enclosed microcolonies attached to bladder epithelial cells from catheterized patients have also been observed (Marrie et al. 1980). Thus, it is proposed that the biofilm mode of growth occurs in other *P. aeruginosa* infections (Donlan and Costerton 2002).

The production of alginate is controlled by a complex regulatory hierarchy (Wozniak and Ohman 1994) involving several proteins that control transcription of *algD*, the first gene in the alginate

biosynthetic operon. AlgD is a GDP-mannose dehydrogenase involved in synthesis of the nucleotide-activated precursor sugar, GDP-mannuronic acid. Other genes involved in the synthesis of the mannuronic acid include *algA* (Shinabarger et al. 1991) and *algC* (Ye et al. 1994). The alternative sigma factor σ^{22} (known as AlgT)(DeVries and Ohman 1994) is the product of the first gene of another cluster, the *algT* operon, which includes the *mucABCD* genes. The *muc* gene products appear to modulate the activity of AlgT (Mathee et al. 1997) and, together with another set of response regulators, AlgB and AlgR (Wozniak and Ohman 1994), control transcription of *algD.*

The production of alginate and, consequently, the biofilm mode of growth have numerous effects on pathogenesis of *P. aeruginosa* infections (Govan and Deretic 1996). The mechanisms by which alginate interferes with host immune defenses include: acting as a direct physical and chemical barrier against phagocytic cells, interfering with both nonopsonic and opsonic phagocytosis, quenching reactive oxygen intermediates and scavenging hypochlorite generated by phagocytic cells, and stimulating the production of pro-inflammatory cytokines including IL-1, TNFα, and IL-8. The biofilm mode of growth also provides a two to three order of magnitude decrease in susceptibility to antimicrobial agents in comparison to planktonic cells (Olson et al. 2002). It is interesting that bacterial cells within a biofilm show reduced metabolic activities, making them less susceptible to certain antibiotics that target primarily metabolically active cells (Anwar et al. 1992).

Extracellular Products

Exotoxin A

Exotoxin A, encoded by *toxA*, is an ADP-ribosyltransferase that exerts cytotoxic activity on a wide variety of mammalian cells and is similar to the diptheria toxin. Exotoxin A binds to and is internalized by the α-2-macroglobulin receptor (Kounnas et al. 1992). After internalization, the enzymatic subunit is translocated to the cytosol (Ogata et al. 1990) where it transfers adenosine diphosphate (ADP)-ribose from nicotinamide adenine dinucleotide (NAD) to elongation factor-2 (EF-2), and inhibits protein synthesis (Iglewski and Kabat 1975) resulting in cell death. Crystallographic structure analysis of ToxA indicates that the protein has an overall tertiary fold with three distinct domains (I, II, III) individually responsible for receptor binding, transmembrane targeting, and ADP-ribosyl transferase activity, respectively (Wedekind et al. 2001). A catalytic site loop in domain III has the critical diphthamide-specific ribosyltransferase structural motif (Yates and Merrill 2001). ToxA is secreted in response to iron starvation (Frank and Iglewski 1988) and is under the regulatory control of the *lasR-lasI* quorum-sensing system (Storey et al. 1998).

ToxA is apparently the most potent virulence factor of *P. aeruginosa* as determined by lethality in animals. For example, the LD_{50} of ToxA in mice is 0.2 μg/animal by the intraperitoneal route (Liu 1973) and 0.062 μg by the intravenous route (Callahan 1976). ToxA induces cell death in many mammalian cell lines that is characteristic of apoptosis (Morimoto and Bonavida 1992) and necrosis (Plotkowski et al. 2002). Local tissue damage, bacterial invasion, and possible immunosuppression in the infected host are largely attributable to ToxA. *In vivo* studies have shown exotoxin A to be critical for ocular damage, corneal ulceration, persistance of infection, and cell death in a mouse model of bacterial keratitis (Pillar and Hobden 2002).

The host defense system is affected by ToxA as it inhibits proliferation of human granulocyte and macrophage progenitor cells *in vitro* (Pollack and Anderson 1978; Stuart and Pollack 1982), alters the production of TNFα by human leukocytes (Staugas et al. 1992), and interferes with murine IL-1 production by peritoneal macrophages (Misfeldt et al. 1990). ToxA is toxic to murine and human PMNs and inhibits phagocytosis and killing of *P. aeruginosa* by PMNs *in vitro* (Miyazaki et al. 1995).

Rhamnolipid

The heat-stable hemolytic glycolipid (rhamnolipid) secreted by *P. aeruginosa* is composed of one (mono-rhamnolipid) or two (di-rhamnolipid) L-rhamnose sugars linked to one or two fatty acid branches of β-hydroxy-decanoic acid. The synthesis of mono-rhamnolipids is catalyzed by a rhamnosyltransferase system, RhlAB (Ochsner et al. 1994), while di-rhamnolipid production requires RhlC, a second rhamnosyltransferase (Rahim et al. 2001). 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 (Pearson et al. 1997) or phosphate limitation (Zhang and Miller 1992). Rhamnolipid has hemolytic activity and at high doses produces generalized cell membrane damage (Hingley et al. 1986). Macrophages are lysed within minutes when

incubated with 6–13 μg/ml of purified rhamnolipids, and a variety of different 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 to 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 allowing it to be more accessible to degradation by other enzymes such as phospholipase C (Liu 1974). The loss of lung surfactant is a possible contributing factor for the atelectasis associated with both acute and chronic *P. aeruginosa* lung infection (Liu 1979). Host respiratory clearance mechanisms are also impaired by rhamnolipids, which inhibit mucociliary transport of respiratory epithelium *in vitro* and *in vivo* (Read et al. 1992).

Phospholipase C

Hemolytic phospholipase C (PlcH) is encoded by *plcH*, which is part of the *plcHR* operon that includes the genes *plcR1* and *plcR2*, whose products are necessary for secretion and solubility of PlcH (Cota-Gomez et al. 1997). PlcH hydrolyzes phosphatidylcholine and sphingomyelin (Ostroff et al. 1990) and causes tissue necrosis and cell death *in vivo* (Coutinho et al. 1988). Lung surfactant found in the bronchial alveolar tissues consists of 70% phosphatidylcholine (Caminiti and Young 1991), and its hydrolysis by PlcH results in the degradation products diacylglycerol and phosphorylcholine. Diacylglycerol may have toxic effects on the animal 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 (Besterman et al. 1986; Needleham et al. 1986; Exton 1990). 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 may facilitate chronic persistent infections because it is capable of suppressing the neutrophil respiratory burst response to whole bacteria (Terada et al. 1999). In a rabbit model of chronic lung infection, a *plcH* mutant resulted in lower levels of the inflammatory mediators 6-keto-PGF1α and thromboxane B2 (Graham et al. 1990). The *plcH* mutants were less virulent in mouse burn models of sepsis (Ostroff et al. 1989) and produced less lung injury and dissemination than the wild-type strain in a rabbit model of pneumonia (Wiener-Kronish et al. 1993).

Proteases: LasB Elastase, LasA Elastase, and Alkaline Protease

Elastase (LasB or pseudolysin) is a metalloprotease that has both proteolytic and elastase activities (Galloway 1991). Although its proteolytic activity by far exceeds its elastase activity, elastolysis is more important for pathogenesis because many tissues contain elastin and require it for their physiological function. Lung tissues contain 28% elastin, which allows expansion and contraction of the organ. In addition, vascular tissues require elastin for their resilience. 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, TNFα, IL-2, components of the complement cascade, immunoglobulin (Ig) G, IgA, secretory IgA, and interferon-γ (IFN-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 (Kawaharajo et al. 1975; Wretlind and Wadstrom 1977). 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 (staphylolysin) is a serine-type protease that nicks elastin, making it more susceptible to the elastase activity of LasB (Peters et al. 1992). 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 (Baumann et al. 1993) 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 pro-inflammatory cytokines IFN-γ and TNFα (Parmely et al. 1990). *In situ* and *in vivo* experiments have shown AprA to increase vascular permeability (Molla et al. 1989) and to disrupt

the function of normal respiratory cilia (Hingley et al. 1986; Molla et al. 1989). AprA contributed to virulence in a burned mouse model of infection (Holder and Haidaris 1979). Early studies demonstrated that AprA was an important virulence factor associated with tissue damage in *P. aeruginosa* corneal infections (Howe and Iglewski 1984). However, results from a more-recent study contradict these findings. Pillar et al. (2000) reported that a defined *aprA* mutant was equally as effective as the parent strain in virulence and in adherence to corneal tissues.

The Type III Secretion System

The type III secretion system is a specialized protein complex that acts as a molecular syringe to deliver cytotoxins directly from the bacterium into the cytoplasm of eukaryotic cells. There are over 20 genes required for secretion, translocation, and regulation of this system (Frank 1997). The secretion of four cytotoxic proteins, 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 characterized of the effector proteins is exoenzyme S (ExoS), a 49-kDa bifunctional cytotoxin that has two independent catalytic activities. The carboxy-terminus domain has a cytotoxic FAS (factor activating exoenzyme S)-dependent ADP-ribosyltransferase activity that preferentially ribosylates several 21- to 25-kDa GTP-binding proteins of the H- and K-Ras family and the cytoskeletal protein vimentin (Coburn et al. 1989; Coburn and Gill 1991). The amino terminus of ExoS contains a GTPase-activating domain for small-molecular-weight GTPases of the Rho subfamily and stimulates actin reorganization that results in disruption of actin microfilaments (Goehring et al. 1999). The Rho and Ras proteins of eukaryotic cells control wound healing and tissue regeneration and interference with these proteins by ExoS may contribute to the establishment of infection and chronic disease by maintaining sites of colonization. ExoS causes cell injury and reduces proliferation and viability of host cells *in vitro* (McGuffie et al. 1998; Pederson and Barbieri 1998) and is important for tissue damage and bacterial dissemination in animal studies (Nicas and Iglewski 1985; Kudoh et al. 1994). Inhibition of Ras and Rho proteins may also interfere with both innate and aquired immunity, as both types of proteins are required for phagocytosis (Caron and Hall 1998) and Ras is required in T-cell activation (Cantrell et al. 1994).

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). ExoT 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. The specific mechanism of action of the third effector protein exoenzyme U (ExoU) is unknown; however, it is cytotoxic to a variety of mammalian cell types *in vitro* (Hauser et al. 1998). 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). The fourth effector protein exoenzyme Y (ExoY) is an adenylate cyclase, which induces elevation of intracellular cyclic AMP *in vitro* and *in vivo* (Yahr et al. 1998). Its role in pathogenesis is also not known.

Regulatory Systems

Cell-to-cell Signaling/Quorum Sensing

Quorum sensing is a bacterial cell-to-cell signaling system whereby bacteria communicate with each other in a cell-density-dependent manner 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,* which are 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-cell signaling autoinducer molecules 3-oxo-C12-HSL (N-[3-oxododecanoyl]-L-homoserine lactone) and C4-HSL (N-butyrylhomoserine lactone), respectively. As the bacterial cell density increases, the concentration of autoinducer increases proportionally and is released in the immediate area and 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 up-regulating their expression.

There is a highly complex cell-to-cell signaling regulatory network in *P. aeruginosa* (fig. 23.1), and it has been reviewed by Van Delden and Iglewski (1998). 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* system (Reimmann et al. 1997; Albus et al. 1997). In addition, the *rsaL* gene, which is located directly downstream of *lasR*, negatively regulates *lasR* (de Kievit et al. 1999). The *las* system controls expression of the virulence factors *lasB* (elastase), *lasA* (LasA protease), *toxA* (exotoxin A), 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, pyocyanin, hydrogen cyanide, and alkaline protease. A more detailed review of these interrelated regulatory systems has been published by Miller and Bassler (2001).

Quorum sensing is essential for pathogenesis of *P. aeruginosa* infections. This is substantiated by evidence that *lasR*, *lasI,* and *rhlI* mutants have reduced virulence compared to the wild-type strain in animal models of infection (Smith and Igleweski 2003). In addition, the *las* cell-cell signaling system

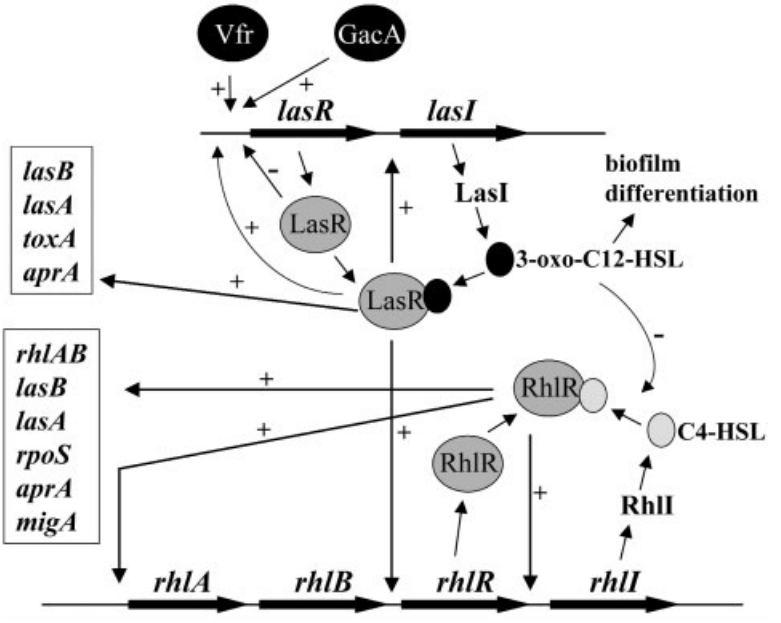


Figure 23.1. Model of the cell-to-cell signaling system in *P. aeruginosa*. The cascade of events begins with the transcription of *lasR* and ends with the activation of RhlR/C4-HSL controlled genes. LasR binds to the *P. aeruginosa* autoinducer 3-oxo-C12-HSL, which is synthesized by LasI. The LasR/3-oxo-C12-HSL complex induces expression of *lasI*, *lasR*, *lasB, lasA, toxA, aprA, rhlR,* and *rhlI*. RhlR forms a complex with C4-HSL, which is synthesized by RhlI. The RhlR/C4-HSL complex activates transcription of *rhlAB, rpoS, migA, lasA*, *lasB*, *aprA*, and *rhlI*. The 3-oxo-C12-HSL is required for biofilm differentiation, and at the translational level it blocks the interaction of RhlR and C4-HSL, which inhibits the ability of RhlR to activate transcription of other genes. The *las* system itself is positively controlled by GacA and Vfr. The plus (+) and minus (-) symbols represent positive or negative regulation, respectively. (Modified from Van Delden and Iglewski [1998]. Abbreviations used: *lasI/rhlI*: autoinducer synthases; *lasR/rhlR*: transcriptional activators; *lasB*: LasB elastase; *lasA*: LasA elastase; *toxA*: exotoxin A; *aprA*: alkaline protease; *rhlAB*: rhamnosyltransferase system for synthesis of rhamnolipid; *rpoS*: stationary sigma factor; *migA*: rhamnosyltransferase for LPS biosynthesis)

is required for normal biofilm formation (Davies et al. 1998) and *lasI* and *rhlI* mutants produce abnormal biofilms that are susceptible to biocides.

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 up-regulating production of *N*-butyryl-L-homoserine lactone, RhlR and LasR (Reimmann et al. 1997). Therefore, in a defined hierarchial manner, it indirectly regulates the expression of the virulence factors controlled by the *las* and *rhl* system. GacA is essential for biofilm formation as a *gacA* mutant showed a tenfold reduction in biofilm formation relative to that of the wild-type strain. Furthermore, *gacA* mutant biofilms showd a moderate decrease in resistance to a range of antibiotics (Parkins et al. 2001). In a burn mouse model, 77% of the animals inoculated with the wild-type strain succumbed to the infection in 36–48 hours but no mortality was seen in the animals infected with the *gacA* mutant (Rahme et al. 2000).

RpoN

rpoN encodes an alternate sigma factor, which enables RNA polymerase to recognize a unique set of promoter sequences in response to various environmental factors. It regulates the expression of diverse genes, including those required for biosynthesis of pili, flagella, and alginate (Woods et al. 1980; Totten et al. 1990; Goldberg and Dahnke 1992). An *rpoN* mutant showed reduced pathogenicity and was less virulent in a burn mouse model (Hendrickson et al. 2001), a murine corneal scratch model (Preston et al. 1995), and a model of acute pneumonia (Comolli et al. 1999a). In addition, *rpoN* mutants showed reduced ability to colonize tissues, as demonstrated in a chronic murine intestinal mucosal model (Pier et al. 1992).

ANTIBIOTIC RESISTANCE

P. aeruginosa is highly resistant to most antibiotics and disinfectants because of its intrinsic multidrug resistance (MDR) attributes including low permeability of the outer membrane and secondary resistance mechanisms, which include multidrug efflux pumps and the production of chromosome-encoded β-lactamases. This organism is usually resistant to penicillin, ampicillin, tetracycline, first- and second-generation cephalosporins, sulfonamides, neomycin, streptomycin, kanamycin, chloramphenicol, nitrofurans, and trimethoprim-sulfonamide. Only a few antibiotics are effective and include fluoroquinolones, amikacin, gentamicin, certain broad-spectrum β-lactam antibiotics, such as imipenem and carbapenem, and a fourth-generation cephalosporin, cefepime.

Outer-Membrane Permeability

Antibiotics first come into contact with the outer membrane, and the overall outer-membrane permeability of *P. aeruginosa* is 12–100-fold lower than that of *E. coli* (Nikaido and Hancock 1986). The permeability properties of the outer membrane are determined mainly by the properties of porins, which are trans-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 minor outer-membrane proteins that have been reported to act as porins include OprB, OprC, OprD, and OprE. The large exclusion limit of the outer membrane is thought to be mostly due to OprF because it is an inefficient route for taking up antibiotics (Hancock and Worobec 1998).

Porin OprD mediates the passage of carbapenem β-lactams such as imipenem and meropenem (Huang and Hancock 1993). The major resistance mechanism to these antibiotics is the loss of the OprD porin, which occurs in as many as 50% of *P. aeruginosa* infections treated for longer than 1 week with imipenem (Quinn et al. 1988).

Penetration of polycationic antibiotics such as gentamicin, tobramycin, and colistin into the cell is mediated by a self-promoted uptake mechanism, which involves the interaction of the polycationic antibiotics with divalent cation binding sites in the LPS molecules (Hancock and Bell 1988). The cation binding sites in the LPS are normally occupied by divalent cations that stabilize the outer membrane by cross-linking the LPS molecules. Polycationic antibiotics are much larger and by binding to the LPS, these drugs disrupt the normal permeability barrier of the outer membrane to promote "self entry" into the bacteria.

Multidrug Efflux Pumps

Four multidrug "Mex" efflux pumps have been characterized in *P. aeruginosa* and are named MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM. The three proteins of each system form a channel that allows substrates to be effluxed directly from the bacterial cytoplasm to the extracellular environment. These pumps contribute to the

intrinsic drug resistance because their activity can easily outpace the slow 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, and mutations in the repressor gene causes overproduction of their respective pumps, which results in increased drug resistance.

MexAB-OprM is the best-characterized pump system. 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 (Poole et al. 1996). A mutation in *mexR* leads to overexpression of the MexAB-OprM system resulting in clinically relevant levels of resistance. The other three efflux systems are only expressed in *P. aeruginosa* regulatory mutants, which are obtained after exposure to antibiotics. Expression of MexCD-OprJ occurs in strains that have a mutation in the *nfxB* repressor gene, and *nfxB* mutant strains are resistant to chloramphenicol, macrolides, novobiocin, quinolones, tetracycline, and cephems, but show hypersusceptibility to many β-lactams (Masuda et al. 1995). The *mexEF-oprN* operon is expressed in *nfxC* repressor mutants, and these strains show increased resistance to chloramphenicol, quinolones, trimethoprim, and carbapenems (Fukuda et al. 1990; Kohler et al. 1997). 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 (Masuda et al. 2000).

It is not known what proportion of resistant clinical isolates corresponds to efflux mutants. There is increasing evidence in human patient populations that efflux systems play a role in posttherapeutic bacterial antibiotic resistance (Ziha-Zarifi et al. 1999; Pumbwe and Piddock 2000). In a study by Beinlich et al. (2001), 12 *P. aeruginosa* isolates from various animal infection sites were identified to have a multidrug resistant phenotype. It is interesting that all isolates 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. Efflux systems also play a role in virulence, as it was shown by Hirakata et al. (2002) that a *mexAB-oprM* deletion mutant failed to kill mice, whereas the wild-type strain induced lethal septicemia in a murine model of endogenous *P. aeruginosa* bacteremia.

"GENOME-MINING" FOR ESSENTIAL GENES

To identify novel and unbiased genes essential for virulence, exquisite selection systems have been developed. One of these is the *in vivo* expression technology (IVET) system that positively selects for bacterial genes that are specifically induced during host infection (Mahan et al. 1993). Using the IVET system to examine virulence genes of *P. aeruginosa* in a neutropenic mouse infection model, Wang et al. (1996b) 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 new virulence factors. Another system is signature-tagged mutagenesis in which predetermined "tagged" sequences are designed as components of transposons, which cause random mutations by insertion into genes. Mutants with reduced virulence are identified in infection studies (Handfield and Levesque 1999).

The most advanced technique for analysis of global gene expression profiles of pathogens under different conditions is DNA microarrays and GeneChip technology (Debouck and Goodfellow 1999). These systems have been used successfully to study host-pathogen interactions, identify target genes of various regulators, and discover novel genes required for pathogenesis of *P. aeruginosa* (Ochsner et al. 2002).

PATHOGENESIS

The pathogenesis of *P. aeruginosa* infections can be divided into three distinct stages: (1) bacterial colonization, (2) local invasion, and (3) dissemination and systemic disease (fig. 23.2). The first step to establishment of an infection is the initial colonization of host tissues. The first line of host defense against bacterial invasion is the skin and mucous membranes. However, if these tissues are damaged by injury or compromised by the insertion of a urinary catheter or intravenous line, bacteria will attach to the epithelial cells predominantly by the cell-associated pili, flagella, and LPS. It is interesting that damaged and regenerating respiratory epithelium express greater amounts of receptors on

their cell surface for *P. aeruginosa* when compared to normal intact epithelial cells. For example, asialo-GM1 is a receptor for pili-mediated adhesion of *P. aeruginosa,* and asialo-GM1 is highly expressed on damaged epithelium (de Bentzmann et al. 1996). Following adherence, bacteria begin to proliferate. Survival 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 inside the cell. Alginate has also been shown to mediate attachment of mucoid *P. aeruginosa* cells to cilia of respiratory epithelial cells and to the glycoprotein-rich mucociliary blanket (Ramphal and Pier 1985; Baker and Svanborg-Eden 1989); however, adhesion by alginate occurs after initial colonization.

Once the tissue becomes colonized, a biofilm forms (Costerton et al. 1999). The infection process evolves into either an acute infection characterized by the high production of extracellular virulence factors or a chronic infection characterized by persistent infection accompanied by the production of low amounts of extracellular products. It is proposed that in an acute infection, the bacterial population uses the quorum cell-to-cell signaling systems (*las* and *rhl*) to coordinate the mass production of high levels of extracellular virulence factors that overwhelm host defenses. The extracellular products include exotoxin A, LasA and LasB elastases, alkaline protease, rhamnolipids, and exoenzymes secreted by the type III secretion system. These factors are primarily responsible for tissue-damaging effects and further impairment of host defenses, thereby facilitating local tissue invasion. Systemic infection and dissemination of *P. aeruginosa* from the local site of infection are presumably mediated by the same extracellular products that are responsible for local tissue damage.

Optimal bacterial clearance requires specific IgG antibodies, intact classical and alternative complement pathways, and adequate numbers of functioning PMNs. The proteases of *P. aeruginosa* circumvent

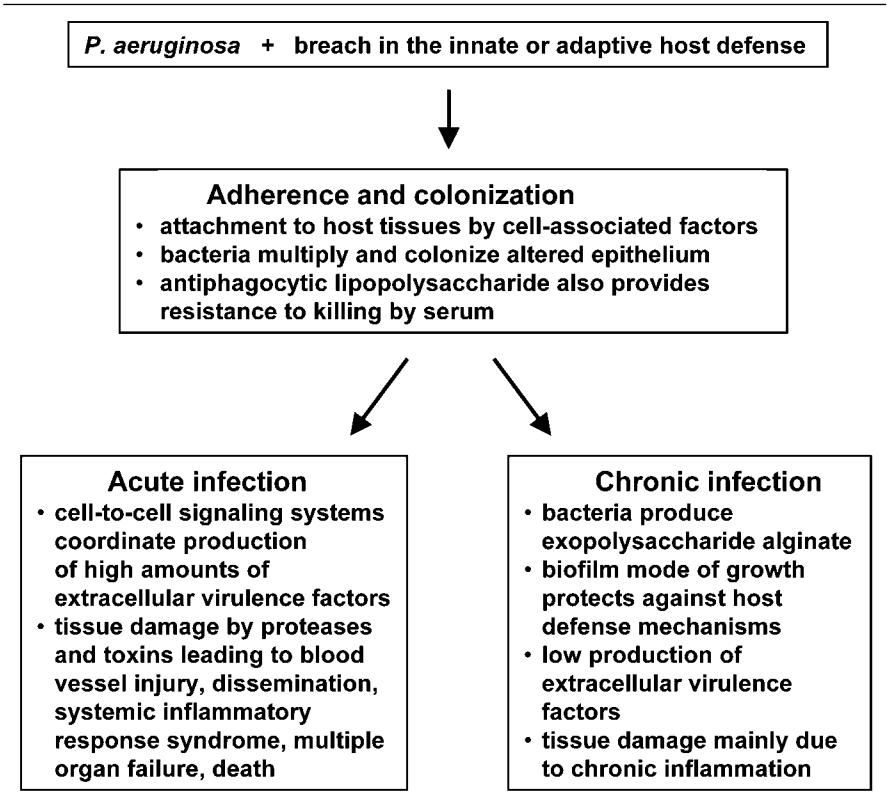


Figure 23.2. Overview of pathogenesis of infection due to *P. aeruginosa*. (Modified from Van Delden and Iglewski 1998)

host defense mechanisms and mediate Hageman factor activation (Holder and Neely 1989), immunoglobulin and complement degradation (Heck et al. 1990; Hong and Ghebrehiwet 1992), cytokine inactivation (Parmely et al. 1990), and host protease activation (Twining et al. 1993).

Systemic illness caused by *P. aeruginosa* is largely mediated by the lipid-A component of the LPS, which is also referred to as endotoxin. Once the bacteria have invaded the bloodstream, host immune defense mechanisms exert bactericidal effects. The release of 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 ARDS. Lipid A also activates the clotting, fibrinolytic, kinin, and complement systems and stimulates the production of arachidonic acid metabolites including prostaglandins and leukotrienes, and induces the production of cytokines including TNFα. All of these factors implicate endotoxin in clinical syndromes associated with septicemia and have been reviewed by Alexander and Rietschel (2001). Although *P. aeruginosa* produced less endotoxemia when compared with *E. coli* in a canine model of septic shock, more cardiovascular dysfunction and mortality were found in the animals infected with *P. aeruginosa* (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.

Exotoxin A has been proposed to play a significant systemic role because purified exotoxin A is highly lethal to animals and produces shock in dogs and rhesus monkeys (Pavlovskis et al. 1975). It has been shown that patients with high levels of serum antibodies to exotoxin A at the onset of septicemia have a better survival rate than those with low antibody titers (Pollack and Young 1979).

As a general 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 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 including high activity of elastase, alkaline protease, and possessing the gene for exoenzyme U. Interestingy, *P. aeruginosa* isolates from canine otitis externa were found to possess an elastase-deficient phenotype (Petermann et al. 2001).

VACCINES

Immunologic approaches for the preparation of *P. aeruginosa* vaccines have been reviewed by Holder (2001). Early immunotherapeutic approaches used whole cells or cell-wall-associated materials as antigens to generate opsonizing antibodies against infecting strains. Many multivalent LPS vaccines have been developed and commercially produced. Pseudogen and PEV-01 provided significant protection against acute pneumonia (Pennington and Pier 1983). Although LPS-based vaccines for *P. aeruginosa* have never gained clinical acceptance due to their endotoxic nature, a liposomal complete-core LPS vaccine has recently been determined to be nontoxic, nonpyrogenic, and immunogenic against a wide variety of pathogens (Bennett-Guerrero et al. 2000; Erridge et al. 2002).

The discovery of various virulence factors has led to the development of alternative immunological approaches. High levels of specific antibody have resulted from immunization with single-component vaccines prepared from exotoxin A, ribosomes and ribosomal RNA, flagella, pili, alginate, and outer membrane protein F (Price et al. 2002). However, the concern is that neutralization of any one specific virulence factor by immunologic means may not be successful against all the different types of disease that *P. aeruginosa* causes. Multicomponent vaccines that contain combinations of toxoids including elastase, alkaline protease, exotoxin A, and outer-membrane protein F have been effective in animal models (Gilleland et al. 1993; Matsumoto et al. 1998). Active and passive immunization using the *P. aeruginosa* type III translocation protein PcrV

enhanced survival in an acute lung infection model (Sawa et al. 1999), and antibodies against PcrV have been protective in animal models of *P. aeruginosa* sepsis (Shime et al. 2001).

The only animal vaccine of practical relevance against *P. aeruginosa* infections is for use in mink and chinchillas. This multicomponent vaccine consists of 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* (Homma et al. 1983). There are no other *Pseudomonas* vaccines that have made their way into common clinical use for humans or animals.

CONCLUSIONS AND FUTURE PROSPECTS

Due to its remarkable physiological versatility, *P. aeruginosa* is ubiquitous. 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. The innate antibiotic resistance of *P. aeruginosa* has posed serious problems in the treatment of pseudomonal infections. Therefore, the impetus of current research is directed toward identifying and characterizing novel genes/proteins that are required by the organism to establish an infection. The fact that many of the newly discovered genes/proteins have no known function is exciting, since their characterization will lead to a better understanding of pathogenic mechanisms and identification of new targets for therapeutic or prophylactic interventions.

In conclusion, this chapter has described the state-of-the-art knowledge of virulence mechanisms of *P. aeruginosa* infections and highlighted some new and exciting discoveries—including the multiple multidrug efflux pumps that mediate drug resistance, quorum-sensing systems that facilitate cell-to-cell signaling and coordination of virulence factor expression, the type III secretion system—and a better understanding of the biosynthesis of LPS. *P. aeruginosa* possesses the largest bacterial genome that has been completely sequenced (www.pseudomonas.com; Stover et al. 2000). The genome database in combination with new innovative technologies such as DNA microarrays, proteomics, high-resolution mass spectrometry, and sophisticated data analysis tools will undoubtedly lead to discovery of novel genes and proteins that are required for *P. aeruginosa* to establish an infection and to the development of new therapeutics.

REFERENCES

Albus, A. M., E. C. Pesci, L. J. Runyen-Janecky, S. E. West, and B. H. Iglewski. 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. 179:3928–3935.

Alexander, C., and E. T. Rietschel. 2001. Bacterial lipopolysaccharides and innate immunity. J. Endotoxin Res. 7:167–202.

Allewelt, M., F. T. Coleman, M. Grout, G. P. Priebe, and G. B. Pier. 2000. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect. Immun. 68:3998–4004.

Anwar, H., J. L. Strap, K. Chen, and J. W. Costerton. 1992. Dynamic interactions of biofilms of mucoid *Pseudomonas aeruginosa* with tobramycin and piperacillin. Antimicrob. Agents. Chemother. 36:1208–1214.

Azghani, A. O., E. J. Miller, and B. T. Peterson. 2000. Virulence factors from *Pseudomonas aeruginosa* increase lung epithelial permeability. Lung 178:261–269.

Baker, N. R., and C. Svanborg-Eden. 1989. Role of alginate in the adherence of *Pseudomonas aeruginosa*. Antibiot. Chemother. 42:72–79.

Baumann, U., S. Wu, K. M. Flaherty, and D. B. McKay. 1993. Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel b roll motif. EMBO J. 12:3357–3364.

Beinlich, K. L., R. Chuanchuen, and H. P. Schweizer. 2001. Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary clinical isolates of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 198:129–134.

Bennett-Guerrero, E., T. J. McIntosh, G. R. Barclay, D. S. Snyder, R. J. Gibbs, M. G. Mythen, and I. R. Poxton. 2000. Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine. Infect. Immun. 68:6202–6208.

Berk, R. S., D. Brown, I. Coutinho, and D. Meyers. 1987. *In vivo* studies with two phospholipase C fractions from *Pseudomonas aeruginosa*. Infect. Immun. 55:1728–1730.

Besterman, J. M., V. Duronio, and P. Cuatrecasas. 1986. Rapid formation of diacylglycerol from phosphatidylcholine: a pathway for generation of a

second messenger. Proc. Natl. Acad. Sci. USA 83:6785–6789.

Bystrova, O. V., A. S. Shashkov, N. A. Kocharova, Y. A. Knirel, B. Lindner, U. Zähringer, and G. B. Pier. 2002. Structural studies on the core and the O-polysaccharide repeating unit of *Pseudomonas aeruginosa* immunotype 1 lipopolysaccharide. Eur. J. Biochem. 269:2194–2203.

Callahan, L. T., 3rd. 1976. *Pseudomonas aeruginosa* exotoxin: purification by preparative polyacrylamide gel electrophoresis and the development of a highly specific antitoxin serum. Infect. Immun. 14:55–61.

Caminiti, S. P., and S. L. Young. 1991. The pulmonary surfactant system. Hosp. Pract. (Off. Ed). 26:87–90, 94–100.

Cantrell, D., M. Izquierdo-Pastor, K. Reif, and M. Woodrow. 1994. Signal transduction by the T-cell antigen receptor: regulation and function of p21ras and PtdIns-3 kinase. Chem. Immunol. 59:115–127.

Caron, E., and A. Hall. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science 282:1717–1721.

Chin, J. C., and J. E. Watts. 1988. Biological properties of phospholipase C purified from a fleecerot isolate of *Pseudomonas aeruginosa*. J. Gen. Microbiol. 134:2567–2575.

Coburn, J., and D. M. Gill. 1991. ADP-ribosylation of p21ras and related proteins by *Pseudomonas aeruginosa* exoenzyme S. Infect. Immun. 59:4259–4262.

Coburn, J., S. T. Dillon, B. H. Iglewski, and D. M. Gill. 1989. Exoenzyme S of *Pseudomonas aeruginosa* ADP-ribosylates the intermediate filament protein vimentin. Infect. Immun. 57:996–998.

Comolli, J. C., A. R. Hauser, L. Waite, C. B. Whitchurch, J. S. Mattick, and J. N. Engel. 1999a. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity *in vitro* and virulence in a mouse model of acute pneumonia. Infect. Immun. 67:3625–3630.

Comolli, J. C., L. L. Waite, K. E. Mostov, and J. N. Engel. 1999b. Pili binding to asialo-GM1 on epithelial cells can mediate cytotoxicity or bacterial internalization by *Pseudomonas aeruginosa*. Infect. Immun. 67:3207–3214.

Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322.

Cota-Gomez, A., A. I. Vasil, J. Kadurugamuwa, T. J. Beveridge, H. P. Schweizer, and M. L. Vasil. 1997. PlcR1 and PlcR2 are putative calcium-binding proteins required for secretion of the hemolytic phospholipase C of *Pseudomonas aeruginosa*. Infect. Immun. 65:2904–2913.

Coutinho, I. R., R. S. Berk, and E. Mammen. 1988. Platelet aggregation by a phospholipase C from *Pseudomonas aeruginosa*. Thromb. Res. 51:495–505.

Cryz, S. J., Jr., E. Furer, and R. Germanier. 1984. Protection against fatal *Pseudomonas aeruginosa* burn wound sepsis by immunization with lipopolysaccharide and high-molecular-weight polysaccharide. Infect. Immun. 43:795–799.

Daly, M., E. Power, J. Bjorkroth, P. Sheehan, A. O'Connell, M. Colgan, H. Korkeala, and S. Fanning. 1999. Molecular analysis of *Pseudomonas aeruginosa*: epidemiological investigation of mastitis outbreaks in Irish dairy herds. Appl. Environ. Microbiol. 65:2723–2729.

Danner, R. L., C. Natanson, R. J. Elin, J. M. Hosseini, S. Banks, T. J. MacVittie, and J. E. Parrillo. 1990. *Pseudomonas aeruginosa* compared with *Escherichia coli* produces less endotoxemia but more cardiovascular dysfunction and mortality in a canine model of septic shock. Chest 98:1480–1487.

Dasgupta, T., T. R. de Kievit, H. Masoud, E. Altman, J. C. Richards, I. Sadovskaya, D. P. Speert, and J. S. Lam. 1994. Characterization of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* derived from serotypes O3, O5, and O6. Infect. Immun. 62:809–817.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

de Bentzmann, S., C. Plotkowski, and E. Puchelle. 1996. Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. Am. J. Respir. Crit. Care Med. 154:S155–162.

Debouck, C., and P. N. Goodfellow. 1999. DNA microarrays in drug discovery and development. Nat. Genet. 21:48–50.

de Kievit, T., P. C. Seed, J. Nezezon, L. Passador, and B. H. Iglewski. 1999. RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. J. Bacteriol. 181:2175–2184.

Denamur, E., B. Picard, P. Goullet, E. Bingen, N. Lambert, and J. Elion. 1991. Complexity of *Pseudomonas aeruginosa* infection in cystic fibrosis: combined results from esterase electrophoresis and rDNA restriction fragment length polymorphism analysis. Epidemiol. Infect. 106:531–539.

De Vos, D., A. Lim, Jr., P. De Vos, A. Sarniguet, K. Kersters, and P. Cornelis. 1993. Detection of the outer membrane lipoprotein I and its gene in fluorescent and non-fluorescent pseudomonads: implications for taxonomy and diagnosis. J. Gen. Microbiol. 139:2215–2223.

DeVries, C. A., and D. E. Ohman. 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. J. Bacteriol. 176:6677–6687.

DiMango, E., H. J. Zar, R. Bryan, and A. Prince. 1995. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. J. Clin. Invest. 96:2204–2210.

Donlan, R. M., and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15:167–193.

Erridge, C., J. Stewart, E. Bennett-Guerrero, T. J. McIntosh, and I. R. Poxton. 2002. The biological activity of a liposomal complete core lipopolysaccharide vaccine. J. Endotoxin Res. 8:39–46.

Exton, J. H. 1990. Signaling through phosphatidylcholine breakdown. J. Biol. Chem. 265:1–4.

Farinha, M. A., B. D. Conway, L. M. Glasier, N. W. Ellert, R. T. Irvin, R. Sherburne, and W. Paranchych. 1994. Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte cells and decreased virulence in mice. Infect. Immun. 62:4118–4123.

Feldman, M., R. Bryan, S. Rajan, L. Scheffler, S. Brunnert, H. Tang, and A. Prince. 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infect. Immun. 66:43–51.

Fleiszig, S. M., T. S. Zaidi, and G. B. Pier. 1995. *Pseudomonas aeruginosa* invasion of and multiplication within corneal epithelial cells *in vitro*. Infect. Immun. 63:4072–4077.

Frank, D. W. 1997. The exoenzyme S regulon of *Pseudomonas aeruginosa*. Mol. Microbiol. 26:621–629.

Frank, D. W., and B. H. Iglewski. 1988. Kinetics of *toxA* and *regA* mRNA accumulation in *Pseudomonas aeruginosa*. J.Bacteriol. 170:4477–4483.

Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe. 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents. Chemother. 34:1757–1761.

Galloway, D. R. 1991. *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. Mol. Microbiol. 5:2315–2321.

Garrity-Ryan, L., B. Kazmierczak, R. Kowal, J. Comolli, A. Hauser, and J. N. Engel. 2000. The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. Infect. Immun. 68:7100–7113.

Gilleland, H. E., L. B. Gilleland, and M. R. Fowler. 1993. Vaccine efficacies of elastase, exotoxin A, and outer-membrane protein F in preventing chronic pulmonary infection by *Pseudomonas aeruginosa* in a rat model. J. Med. Microbiol. 38:79–86.

Goehring, U. M., G. Schmidt, K. J. Pederson, K. Aktories, and J. T. Barbieri. 1999. The N-terminal domain of *Pseudomonas aeruginosa* exoenzyme S is a GTPase- activating protein for Rho GTPases. J. Biol. Chem. 274:36369–36372.

Goldberg, J. B., and T. Dahnke. 1992. *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. Mol. Microbiol. 6:59–66.

Govan, J. R., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60:539–574.

Graham, L. M., A. Vasil, M. L. Vasil, N. F. Voelkel, and K. R. Stenmark. 1990. Decreased pulmonary vasoreactivity in an animal model of chronic *Pseudomonas pneumonia*. Am. Rev. Respir. Dis. 142:221–229.

Gray, L., and A. Kreger. 1979. Microscopic characterization of rabbit lung damage produced by *Pseudomonas aeruginosa* proteases. Infect. Immun. 23:150–159.

Gray, L. D., and A. S. Kreger. 1975. Rabbit corneal damage produced by *Pseudomonas aeruginosa* infection. Infect. Immun. 12:419–432.

Grothues, D., U. Koopmann, H. von der Hardt, and B. Tummler. 1988. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonization of cystic fibrosis siblings with closely related strains. J. Clin. Microbiol. 26:1973–1977.

Gupta, S. K., R. S. Berk, S. Masinick, and L. D. Hazlett. 1994. Pili and lipopolysaccharide of *Pseudomonas aeruginosa* bind to the glycolipid asialo GM1. Infect. Immun. 62:4572–4579.

Gupta, S. K., S. Masinick, M. Garrett, and L. D. Hazlett. 1997. *Pseudomonas aeruginosa* lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. Infect. Immun. 65:2747–2753.

Gyles, C. L. 1993. *Pseudomonas* and *Moraxella*. In Pathogenesis of bacterial infections of animals, 2nd ed. C. L. Gyles and C. O. Thoen (eds.). Ames: Iowa State University Press, pp. 248–261.

Hahn, H. P. 1997. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*-a review. Gene 192:99–108.

Hamood, A. N., J. A. Griswold, and C. M. Duhan. 1996. Production of extracellular virulence factors by *Pseudomonas aeruginosa* isolates obtained from tracheal, urinary tract, and wound infections. J. Surg. Res. 61:425–432.

Hancock, R. E., and A. Bell. 1988. Antibiotic uptake into gram-negative bacteria. Eur. J. Clin. Microbiol. Infect. Dis. 7:713–720.

Hancock, R. E., and F. S. Brinkman. 2002. Function of *Pseudomonas* porins in uptake and efflux. Annu. Rev. Microbiol. 56:17–38.

Hancock, R. E. W., and E. A. Worobec. 1998. Outer membrane proteins. In Pseudomonas. Biotechnology handbooks, vol. 10. T. Montie (ed.). London: Plenum, pp. 139–167.

Handfield, M., and R. C. Levesque. 1999. Strategies for isolation of *in vivo* expressed genes from bacteria. FEMS Microbiol. Rev. 23:69–91.

Hauser, A. R., and J. N. Engel. 1999. *Pseudomonas aeruginosa* induces type-III-secretion-mediated apoptosis of macrophages and epithelial cells. Infect. Immun. 67:5530–5537.

Hauser, A. R., P. J. Kang, and J. N. Engel. 1998. PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol. Microbiol. 27:807–818.

Hazes, B., P. A. Sastry, K. Hayakawa, R. J. Read, and R. T. Irvin. 2000. Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. J. Mol. Biol. 299:1005–1017.

Heck, L. W., P. G. Alarcon, R. M. Kulhavy, K. Morihara, M. W. Russell, and J. F. Mestecky. 1990. Degradation of IgA proteins by *Pseudomonas aeruginosa* elastase. J. Immunol. 144:2253–2257.

Hendrickson, E. L., J. Plotnikova, S. Mahajan-Miklos, L. G. Rahme, and F. M. Ausubel. 2001. Differential roles of the *Pseudomonas aeruginosa* PA14 *rpoN* gene in pathogenicity in plants, nematodes, insects, and mice. J. Bacteriol. 183:7126–7134.

Hingley, S. T., A. T. Hastie, F. Kueppers, M. L. Higgins, G. Weinbaum, and T. Shryock. 1986. Effect of ciliostatic factors from *Pseudomonas aeruginosa* on rabbit respiratory cilia. Infect. Immun. 51:254–262.

Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. J. Exp. Med. 196:109–118.

Hobbs, M., E. S. Collie, P. D. Free, S. P. Livingston, and J. S. Mattick. 1993. PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. 7:669–682.

Holder, I. A. 2001. *Pseudomonas* vaccination and immunotherapy: an overview. J. Burn Care Rehabil. 22:311–320.

Holder, I. A., and A. N. Neely. 1989. *Pseudomonas* elastase acts as a virulence factor in burned hosts by Hageman factor-dependent activation of the host kinin cascade. Infect. Immun. 57:3345–3348.

Holder, I. A., and C. G. Haidaris. 1979. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: extracellular protease and elastase as *in vivo* virulence factors. Can. J. Microbiol. 25:593–599.

Homma, J. Y., C. Abe, R. Yanagawa, and H. Noda. 1983. Effectiveness of immunization with multicomponent vaccines in protection against hemorrhagic pneumonia due to *Pseudomonas aeruginosa* infection in mink. Rev. Infect. Dis. 5 Suppl 5:S858–866.

Hong, Y. Q., and B. Ghebrehiwet. 1992. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin. Immunol. Immunopathol. 62:133–138.

Howe, T. R., and B. H. Iglewski. 1984. Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa in vitro* and in a mouse eye model. Infect. Immun. 43:1058–1063.

Huang, H., and R. E. Hancock. 1993. Genetic definition of the substrate selectivity of outer membrane porin protein OprD of *Pseudomonas aeruginosa*. J. Bacteriol. 175:7793–7800.

Iglewski, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U S A 72:2284–2288.

Ishimoto, K. S., and S. Lory. 1992. Identification of *pilR*, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. J. Bacteriol. 174:3514–3521.

Jyot, J., N. Dasgupta, and R. Ramphal. 2002. FleQ, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. J. Bacteriol. 184:5251–5260.

Kawaharajo, K., J. Y. Homma, Y. Aoyama, and K. Morihara. 1975. *In vivo* studies on protease and elastase from *Pseudomonas aeruginosa*. Jpn. J. Exp. Med. 45:89–100.

Knirel, Y. A., O. V. Bystrova, A. S. Shashkov, B. Lindner, N. A. Kocharova, S. N. Senchenkova, H. Moll, U. Zahringer, K. Hatano, and G. B. Pier. 2001. Structural analysis of the lipopolysaccharide core of a rough, cystic fibrosis isolate of *Pseudomonas aeruginosa*. Eur. J. Biochem. 268:4708–4719.

Kohler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L. K. Curty, and J. C. Pechere. 1997.

Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. 23:345-354.

Komori, Y., T. Nonogaki, and T. Nikai. 2001. Hemorrhagic activity and muscle damaging effect of *Pseudomonas aeruginosa* metalloproteinase (elastase). Toxicon 39:1327–1332.

Kounnas, M. Z., R. E. Morris, M. R. Thompson, D. J. FitzGerald, D. K. Strickland, and C. B. Saelinger. 1992. The a 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A. J. Biol. Chem. 267:12420–12423.

Krall, R., G. Schmidt, K. Aktories, and J. T. Barbieri. 2000. *Pseudomonas aeruginosa* ExoT is a Rho GTPase-activating protein. Infect. Immun. 68:6066–6068.

Kropinski, A. M. B., B. Jewel, J. Kuzio, F. Milazzo, and D. Berry. 1985. Structure and function of *Pseudomonas aeruginosa* lipopolyaccharide. Antibiot. Chemother. 36:58–73.

Kudoh, I., J. P. Wiener-Kronish, S. Hashimoto, J. F. Pittet, and D. Frank. 1994. Exoproduct secretions of *Pseudomonas aeruginosa* strains influence severity of alveolar epithelial injury. Am. J. Physiol. 267:L551–556.

Lam, J., R. Chan, K. Lam, and J. W. Costerton. 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. 28:546–556.

Lamping, N., A. Hoess, B. Yu, T. C. Park, C. J. Kirschning, D. Pfeil, D. Reuter, S. D. Wright, F. Herrmann, and R. R. Schumann. 1996. Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. J. Immunol. 157:4648–4656.

Lee, K. K., H. B. Sheth, W. Y. Wong, R. Sherburne, W. Paranchych, R. S. Hodges, C. A. Lingwood, H. Krivan, and R. T. Irvin. 1994. The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. Mol. Microbiol. 11:705–713.

Lillehoj, E. P., B. T. Kim, and K. C. Kim. 2002. Identification of *Pseudomonas aeruginosa* flagellin as an adhesin for Muc1 mucin. Am. J. Physiol. Lung Cell. Mol. Physiol. 282:L751–756.

Liu, P. V. 1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506–513.

———. 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. 130 Suppl:S94–99.

———. 1979. Toxins of *Pseudomonas aeruginosa*. In *Pseudomonas aeruginosa*: clinical manisfestations of infection and current therapy. R. G. Dogget (ed.). New York: Academic Press, pp. 63–88.

Lomholt, J. A., K. Poulsen, and M. Kilian. 2001. Epidemic population structure of *Pseudomonas aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. Infect. Immun. 69:6284–6295.

Long, G. G., A. M. Gallina, and J. R. Gorham. 1980. *Pseudomonas aeruginosa* of mink: pathogenesis, vaccination, and serologic studies. A. J. Vet. Res. 41:1720–1725.

Lynn, W. A., and D. T. Golenbock. 1992. Lipopolysaccharide antagonists. Immunol. Today 13:271–276.

Mahan, M. J., J. M. Slauch, and J. J. Mekalanos. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science 259:686–688.

Mahenthiralingam, E., and D. P. Speert. 1995. Nonopsonic phagocytosis of *Pseudomonas aeruginosa* by macrophages and polymorphonuclear leukocytes requires the presence of the bacterial flagellum. Infect. Immun. 63:4519–4523.

Mahenthiralingam, E., M. E. Campbell, J. Foster, J. S. Lam, and D. P. Speert. 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J. Clin. Microbiol. 34:1129–1135.

Marrie, T. J., J. Lam, and J. W. Costerton. 1980. Bacterial adhesion to uroepithelial cells: a morphologic study. J. Infect. Dis. 142:239–246.

Masuda, N., E. Sakagawa, and S. Ohya. 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 39:645–649.

Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino. 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44:2242–2246.

Mathee, K., C. J. McPherson, and D. E. Ohman. 1997. Posttranslational control of the algT (algU)-encoded sigma22 for expression of the alginate regulon in Pseudomonas aeruginosa and localization of its antagonist proteins MucA and MucB (AlgN). J Bacteriol 179:3711–3720.

Matsumoto, T., K. Tateda, N. Furuya, S. Miyazaki, A. Ohno, Y. Ishii, Y. Hirakata, and K. Yamaguchi. 1998. Efficacies of alkaline protease, elastase and exotoxin A toxoid vaccines against gut-derived *Pseudomonas aeruginosa* sepsis in mice. J. Med. Microbiol. 47:303–308.

McClure, C. D., and N. L. Schiller. 1992. Effects of *Pseudomonas aeruginosa* rhamnolipids on human monocyte-derived macrophages. J. Leukoc. Biol. 51:97–102.

McGuffie, E. M., D. W. Frank, T. S. Vincent, and J. C. Olson. 1998. Modification of Ras in eukaryotic cells by *Pseudomonas aeruginosa* exoenzyme S. Infect. Immun. 66:2607–2613.

Meyers, D. J., K. C. Palmer, L. A. Bale, K. Kernacki, M. Preston, T. Brown, and R. S. Berk. 1992. *In vivo* and *in vitro* toxicity of phospholipase C from *Pseudomonas aeruginosa*. Toxicon 30:161–169.

Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55:165–199.

Misfeldt, M. L., P. K. Legaard, S. E. Howell, M. H. Fornella, and R. D. LeGrand. 1990. Induction of interleukin-1 from murine peritoneal macrophages by *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. 58:978–982.

Miyazaki, S., T. Matsumoto, K. Tateda, A. Ohno, and K. Yamaguchi. 1995. Role of exotoxin A in inducing severe *Pseudomonas aeruginosa* infections in mice. J. Med. Microbiol. 43:169–175.

Molla, A., T. Yamamoto, T. Akaike, S. Miyoshi, and H. Maeda. 1989. Activation of hageman factor and prekallikrein and generation of kinin by various microbial proteinases. J. Biol. Chem. 264:10589–10594.

Montie, T. C., D. Drake, H. Sellin, O. Slater, and S. Edmonds. 1987. Motility, virulence, and protection with a flagella vaccine against *Pseudomonas aeruginosa* infection. Antibiot. Chemother. 39:233–248.

Morimoto, H., and B. Bonavida. 1992. *Diphtheria* toxin- and *Pseudomonas* A toxin-mediated apoptosis. ADP ribosylation of elongation factor-2 is required for DNA fragmentation and cell lysis and synergy with tumor necrosis factor-a. J. Immunol. 149:2089–2094.

Needleman, P., J. Turk, B. A. Jakschik, A. R. Morrison, and J. B. Lefkowith. 1986. Arachidonic acid metabolism. Annu. Rev. Biochem. 55:69–102.

Nicas, T. I., and B. H. Iglewski. 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. Can. J. Microbiol. 31:387–392.

Nikaido, H., and R. E. W. Hancock. 1986. Outer membrane permeability in *Pseudomonas aeruginosa*. In The Bacteria, vol. 10. J. Sokatch (ed.). Orlando, FL: Academic Press, p. 145–193.

Ochsner, U. A., A. Fiechter, and J. Reiser. 1994. Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J. Biol. Chem. 269:19787–19795.

Ochsner, U. A., P. J. Wilderman, A. I. Vasil, and M. L. Vasil. 2002. GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol. Microbiol. 45:1277–1287.

Ogata, M., V. K. Chaudhary, I. Pastan, and D. J. FitzGerald. 1990. Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. J. Biol. Chem. 265:20678–20685.

Olson, M. E., H. Ceri, D. W. Morck, A. G. Buret, and R. R. Read. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can. J. Vet. Res. 66:86–92.

Ostroff, R. M., A. I. Vasil, and M. L. Vasil. 1990. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. J. Bacteriol. 172:5915–5923.

Ostroff, R. M., B. Wretlind, and M. L. Vasil. 1989. Mutations in the hemolytic-phospholipase C operon result in decreased virulence of *Pseudomonas aeruginosa* PAO1 grown under phosphate-limiting conditions. Infect. Immun. 57:1369–1373.

Paranchych, W., B. L. Pasloske, and P. A. Sastry. 1990. Expression, processing, and assembly of *Pseudomonas aeruginosa N*-methylphenylalanine pilin. In Pseudomonas: biotransformations, pathogenesis, and evolving biotechnology. S. Silver, A. M. Chakrabarty, B. H. Iglewski, and S. Kaplan (eds.). Washington, DC: American Society for Microbiology, pp. 343–351.

Parkins, M. D., H. Ceri, and D. G. Storey. 2001. *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. Mol. Microbiol. 40:1215–1226.

Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W. W. Zhou. 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. Infect. Immun. 58:3009–3014.

Parsek, M. R., and E. P. Greenberg. 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. U S A 97:8789–8793.

Pavlovskis, O. R., L. T. Callahan, and M. Pollack. 1975. *Pseudomonas aeruginosa* exotoxin. In Microbiology. D. Schlessinger (ed.). Washington, DC: American Society for Microbiology, pp. 252–256.

Pearson, J. P., E. C. Pesci, and B. H. Iglewski. 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. 179:5756–5767.

Pederson, K. J., and J. T. Barbieri. 1998. Intracellular expression of the ADP-ribosyltransferase domain of *Pseudomonas exoenzyme* S is cytotoxic to eukaryotic cells. Mol. Microbiol. 30:751–759.

Pennington, J. E., and G. B. Pier. 1983. Efficacy of cell wall *Pseudomonas aeruginosa* vaccines for protection against experimental pneumonia. Rev. Infect. Dis. 5 Suppl 5:S852–857.

Petermann, S. R., C. Doetkott, and L. Rust. 2001. Elastase deficiency phenotype of *Pseudomonas aeruginosa* canine otitis externa isolates. Clin. Diagn. Lab Immunol. 8:632–636.

Peters, J. E., S. J. Park, A. Darzins, L. C. Freck, J. M. Saulnier, J. M. Wallach, and D. R. Galloway. 1992. Further studies on *Pseudomonas aeruginosa* LasA: analysis of specificity. Mol. Microbiol. 6:1155–1162.

Pier, G. B., G. Meluleni, and E. Neuger. 1992. A murine model of chronic mucosal colonization by *Pseudomonas aeruginosa*. Infect. Immun. 60:4768–4776.

Pier, G. B., M. Grout, T. S. Zaidi, J. C. Olsen, L. G. Johnson, J. R. Yankaskas, and J. B. Goldberg. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science 271:64–67.

Pillar, C. M., and J. A. Hobden. 2002. *Pseudomonas aeruginosa* exotoxin A and keratitis in mice. Invest. Ophthalmol. Vis. Sci. 43:1437–1444.

Pillar, C. M., L. D. Hazlett, and J. A. Hobden. 2000. Alkaline protease-deficient mutants of *Pseudomonas aeruginosa* are virulent in the eye. Curr. Eye Res. 21:730–739.

Plotkowski, M. C., H. C. Povoa, J. M. Zahm, G. Lizard, G. M. Pereira, J. M. Tournier, and E. Puchelle. 2002. Early mitochondrial dysfunction, superoxide anion production, and DNA degradation are associated with non-apoptotic death of human airway epithelial cells induced by *Pseudomonas aeruginosa* exotoxin A. Am. J. Respir. Cell. Mol. Biol. 26:617–626.

Pollack, M., and L. S. Young. 1979. Protective activity of antibodies to exotoxin A and lipopolysaccharide at the onset of *Pseudomonas aeruginosa* septicemia in man. J. Clin. Invest. 63:276–286.

Pollack, M., and S. E. Anderson, Jr. 1978. Toxicity of *Pseudomonas aeruginosa* exotoxin A for human macrophages. Infect. Immun. 19:1092–1096.

Poole, K., and R. Srikumar. 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr. Top. Med. Chem. 1:59–71.

Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco. 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. 40:2021–2028.

Preston, M. J., S. M. Fleiszig, T. S. Zaidi, J. B. Goldberg, V. D. Shortridge, M. L. Vasil, and G. B. Pier. 1995. Rapid and sensitive method for evaluating *Pseudomonas aeruginosa* virulence factors during corneal infections in mice. Infect. Immun. 63:3497–3501.

Price, B. M., J. Barten Legutki, D. R. Galloway, B. U. von Specht, L. B. Gilleland, H. E. Gilleland, Jr., and J. Staczek. 2002. Enhancement of the protective efficacy of an *oprF* DNA vaccine against *Pseudomonas aeruginosa*. FEMS Immunol. Med. Microbiol. 33:89–99.

Pumbwe, L., and L. J. Piddock. 2000. Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44:2861–2864.

Quinn, J. P., A. E. Studemeister, C. A. DiVincenzo, and S. A. Lerner. 1988. Resistance to imipenem in *Pseudomonas aeruginosa*: clinical experience and biochemical mechanisms. Rev. Infect. Dis. 10:892–898.

Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J. Exp. Med. 189:615–625.

Rahim, R., U. A. Ochsner, C. Olvera, M. Graninger, P. Messner, J. S. Lam, and G. Soberon-Chavez. 2001. Cloning and functional characterization of the *Pseudomonas aeruginosa rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol. Microbiol. 40:708–718.

Rahme, L. G., F. M. Ausubel, H. Cao, E. Drenkard, B. C. Goumnerov, G. W. Lau, S. Mahajan-Miklos, J. Plotnikova, M. W. Tan, J. Tsongalis, C. L. Walendziewicz, and R. G. Tompkins. 2000. Plants and animals share functionally common bacterial virulence factors. Proc. Natl. Acad. Sci. USA 97:8815–8821.

Ramphal, R., and G. B. Pier. 1985. Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. Infect. Immun. 47:1–4.

Ramphal, R., S. K. Arora, and B. W. Ritchings. 1996. Recognition of mucin by the adhesin-flagellar system of *Pseudomonas aeruginosa*. Am. J. Respir. Crit. Care Med. 154:S170–174.

Read, R. C., P. Roberts, N. Munro, A. Rutman, A. Hastie, T. Shryock, R. Hall, W. McDonald-Gibson, V. Lund, G. Taylor, and et al. 1992. Effect of *Pseudomonas aeruginosa* rhamnolipids on

mucociliary transport and ciliary beating. J. Appl. Physiol. 72:2271–2277.

Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski, and D. Haas. 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol. Microbiol. 24:309–319.

Rhame, F. S. 1980. The ecology and epidemiology of *Pseudomonas aeruginosa*. In *Pseudomonas aeruginosa*: the organism, disease it causes, and their treatment. L. D. Sabath (ed.). Bern, Switzerland: Hans Huber, pp. 38–51.

Ritchings, B. W., E. C. Almira, S. Lory, and R. Ramphal. 1995. Cloning and phenotypic characterization of *fleS* and *fleR*, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. Infect. Immun. 63:4868–4876.

Rocchetta, H. L., L. L. Burrows, and J. S. Lam. 1999. Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. Microbiol. Mol. Biol. Rev. 63:523–553.

Sadovskaya, I., J. R. Brisson, J. S. Lam, J. C. Richards, and E. Altman. 1998. Structural elucidation of the lipopolysaccharide core regions of the wild-type strain PAO1 and O-chain-deficient mutant strains AK1401 and AK1012 from *Pseudomonas aeruginosa* serotype O5. Eur. J. Biochem. 255:673–684.

Sadovskaya, I., J. R. Brisson, P. Thibault, J. C. Richards, J. S. Lam, and E. Altman. 2000. Structural characterization of the outer core and the O-chain linkage region of lipopolysaccharide from *Pseudomonas aeruginosa* serotype O5. Eur. J. Biochem. 267:1640–1650.

Saiman, L., and A. Prince. 1993. *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J. Clin. Invest. 92:1875–1880.

Sato, H., K. Okinaga, and H. Saito. 1988. Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection. Microbiol. Immunol. 32:131–139.

Sawa, T., T. L. Yahr, M. Ohara, K. Kurahashi, M. A. Gropper, J. P. Wiener-Kronish, and D. W. Frank. 1999. Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. Nat. Med. 5:392–398.

Scharfman, A., S. K. Arora, P. Delmotte, E. Van Brussel, J. Mazurier, R. Ramphal, and P. Roussel. 2001. Recognition of Lewis x derivatives present on mucins by flagellar components of *Pseudomonas aeruginosa*. Infect. Immun. 69:5243–5248.

Shime, N., T. Sawa, J. Fujimoto, K. Faure, L. R. Allmond, T. Karaca, B. L. Swanson, E. G. Spack, and J. P. Wiener-Kronish. 2001. Therapeutic administration of anti-PcrV F(ab')$_2$ in sepsis associated with *Pseudomonas aeruginosa*. J. Immunol. 167:5880–5886.

Shinabarger, D., A. Berry, T. B. May, R. Rothmel, A. Fialho, and A. M. Chakrabarty. 1991. Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J. Biol. Chem. 266:2080–2088.

Smith, R. S., and B. H. Iglewski. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Curr. Opin. Microbiol. 6:56–60.

Staugas, R. E., D. P. Harvey, A. Ferrante, M. Nandoskar, and A. C. Allison. 1992. Induction of tumor necrosis factor (TNF) and interleukin-1 (IL-1) by *Pseudomonas aeruginosa* and exotoxin A-induced suppression of lymphoproliferation and TNF, lymphotoxin, gamma interferon, and IL-1 production in human leukocytes. Infect. Immun. 60:3162–3168.

Storey, D. G., E. E. Ujack, H. R. Rabin, and I. Mitchell. 1998. *Pseudomonas aeruginosa lasR* transcription correlates with the transcription of *lasA*, *lasB*, and *toxA* in chronic lung infections associated with cystic fibrosis. Infect. Immun. 66:2521–2528.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406:959–964.

Stuart, R. K., and M. Pollack. 1982. *Pseudomonas aeruginosa* exotoxin A inhibits proliferation of human bone marrow progenitor cells *in vitro*. Infect. Immun. 38:206–211.

Sundin, C., M. L. Henriksson, B. Hallberg, A. Forsberg, and E. Frithz-Lindsten. 2001. Exoenzyme T of *Pseudomonas aeruginosa* elicits cytotoxicity without interfering with Ras signal transduction. Cell. Microbiol. 3:237–246.

Tang, H., M. Kays, and A. Prince. 1995. Role of *Pseudomonas aeruginosa* pili in acute pulmonary infection. Infect. Immun. 63:1278–1285.

Tang, H. B., E. DiMango, R. Bryan, M. Gambello, B. H. Iglewski, J. B. Goldberg, and A. Prince. 1996.

Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect. Immun. 64:37–43.

Terada, L. S., K. A. Johansen, S. Nowbar, A. I. Vasil, and M. L. Vasil. 1999. *Pseudomonas aeruginosa* hemolytic phospholipase C suppresses neutrophil respiratory burst activity. Infect. Immun. 67:2371–2376.

Tobias, P. S., K. Soldau, and R. J. Ulevitch. 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. 164:777–793.

Toder, D. S., M. J. Gambello, and B. H. Iglewski. 1991. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. Mol. Microbiol. 5:2003–2010.

Totten, P. A., J. C. Lara, and S. Lory. 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. 172:389–396.

Twining, S. S., S. E. Kirschner, L. A. Mahnke, and D. W. Frank. 1993. Effect of *Pseudomonas aeruginosa* elastase, alkaline protease, and exotoxin A on corneal proteinases and proteins. Invest. Ophthalmol. Vis. Sci. 34:2699–2712.

Van Delden, C., and B. H. Iglewski. 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg. Infect. Dis. 4:551–560.

Wang, J., A. Mushegian, S. Lory, and S. Jin. 1996b. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by in vivo selection. Proc. Natl. Acad. Sci. USA 93:10434–10439.

Wang, J., S. Lory, R. Ramphal, and S. Jin. 1996a. Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. Mol. Microbiol. 22:1005–1012.

Wedekind, J. E., C. B. Trame, M. Dorywalska, P. Koehl, T. M. Raschke, M. McKee, D. FitzGerald, R. J. Collier, and D. B. McKay. 2001. Refined crystallographic structure of *Pseudomonas aeruginosa* exotoxin A and its implications for the molecular mechanism of toxicity. J. Mol. Biol. 314:823–837.

Wiener-Kronish, J. P., T. Sakuma, I. Kudoh, J. F. Pittet, D. Frank, L. Dobbs, M. L. Vasil, and M. A. Matthay. 1993. Alveolar epithelial injury and pleural empyema in acute *P. aeruginosa* pneumonia in anesthetized rabbits. J. Appl. Physiol. 75:1661–1669.

Woods, D. E., D. C. Straus, W. G. Johanson, Jr., V. K. Berry, and J. A. Bass. 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. 29:1146–1151.

Wozniak, D. J., and D. E. Ohman. 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. 176:6007–6014.

Wretlind, B., and T. Wadstrom. 1977. Purification and properties of a protease with elastase activity from *Pseudomonas aeruginosa*. J. Gen. Microbiol. 103:319–327.

Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249:1431–1433.

Yahr, T. L., A. J. Vallis, M. K. Hancock, J. T. Barbieri, and D. W. Frank. 1998. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. Proc. Natl. Acad. Sci. U S A 95:13899–13904.

Yang, H., M. Matewish, I. Loubens, D. G. Storey, J. S. Lam, and S. Jin. 2000. *migA*, a quorum-responsive gene of *Pseudomonas aeruginosa*, is highly expressed in the cystic fibrosis lung environment and modifies low- molecular-mass lipopolysaccharide. Microbiology 146:2509–2519.

Yates, S. P., and A. R. Merrill. 2001. A catalytic loop within *Pseudomonas aeruginosa* exotoxin A modulates its transferase activity. J. Biol. Chem. 276:35029–35036.

Ye, R. W., N. A. Zielinski, and A. M. Chakrabarty. 1994. Purification and characterization of phosphomannomutase/phosphoglucomutase from *Pseudomonas aeruginosa* involved in biosynthesis of both alginate and lipopolysaccharide. J. Bacteriol. 176:4851–4857.

Zaidi, T. S., J. Lyczak, M. Preston, and G. B. Pier. 1999. Cystic fibrosis transmembrane conductance regulator-mediated corneal epithelial cell ingestion of *Pseudomonas aeruginosa* is a key component in the pathogenesis of experimental murine keratitis. Infect. Immun. 67:1481–1492.

Zaidi, T. S., S. M. Fleiszig, M. J. Preston, J. B. Goldberg, and G. B. Pier. 1996. Lipopolysaccharide outer core is a ligand for corneal cell binding and ingestion of *Pseudomonas aeruginosa*. Invest. Ophthalmol. Vis. Sci. 37:976–986.

Zhang, Y., and R. M. Miller. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. 58:3276–3282.

Ziha-Zarifi, I., C. Llanes, T. Kohler, J. C. Pechere, and P. Plesiat. 1999. *In vivo* emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system

MexA-MexB-OprM. Antimicrob. Agents Chemother. 43:287–291.
Zoutman, D. E., W. C. Hulbert, B. L. Pasloske, A. M. Joffe, K. Volpel, M. K. Trebilcock, and W. Paranchych. 1991. The role of polar pili in the adherence of *Pseudomonas aeruginosa* to injured canine tracheal cells: a semiquantitative morphologic study. Scanning Microsc. 5:109–124; discussion 124–126.

24
Moraxella

W. P. Michalski and J. L. Farn

There are ten species in the genus *Moraxella* but only *Moraxella bovis*, the agent of infectious bovine keratoconjunctivitis (IBK or pinkeye), is of veterinary importance. While it is accepted that both pili and a hemolytic exotoxin are required to produce infection, a number of additional factors produced by *M. bovis* may have a significant role in pathogenesis. Pinkeye is a highly contagious and painful eye disease that is common in cattle throughout the world. Its economic impact in the cattle industry is significant and results from losses due to treatment costs, reduced weight gain, decreased milk production, and devaluation of sale animals.

Much remains to be learned about pinkeye. Fortunately, most cattle recover from infectious pinkeye and acquire immunity for a considerable period of time after infection. This suggests vaccination should be a reasonable and cost-effective means of control.

CHARACTERISTICS OF THE ORGANISM

Classification

The exact taxonomic delineation of the genus *Moraxella* is the subject of debate. In the 1984 edition of *Bergey's Manual of Systematic Bacteriology,* the genus *Moraxella* consisted of two subgenera, *Moraxella* and *Branhamella,* in the family Neisseriaceae. More recent work based on DNA-rRNA hybridization studies and 16S rRNA analysis indicated that *Moraxella* does not belong in the family Neisseriaceae, and there is agreement that *Moraxella*, *Branhamella,* and other "false *Neisseria*" belong in the same family. Rossau et al. (1991) proposed Moraxellaceae as the family name. The nomenclature accepted in the latest edition of *Bergey's Manual of Systematic Bacteriology* recognizes the family Moraxellaceae with three genera, *Moraxella, Acinetobacter,* and *Psychrobacter,* with the genus *Moraxella* consisting of two subgenera, namely, *Moraxella (Moraxella)* and *Moraxella (Branhamella)*.

Moraxella organisms are short, plump, gram-negative rods (1.0–1.5 x 1.5–2.5 μm), which appear predominantly in pairs but may also be in short chains. They are generally nonflagellate, but under specialized conditions may show sluggish motility. They are strictly aerobic and are peroxidase and catalase-positive. Moraxellae are asaccharolytic, are negative for DNase, indole and H_2S production, and have variable reactions for nitrate reduction and gelatin liquefaction. *Moraxella lacunata* is the type species for the genus. Other species of the *Moraxella* subgenus are *M. nonliquefaciens, M. osloensis, M. atlantae, M. lincolnii,* and *M. bovis.*

CHARACTERISTICS OF *M. BOVIS*

The first isolations of *Moraxella* spp. from animals were reported by Mitter in 1915 and Allen in 1919 (Pugh 1981), who both observed a gram-negative diplobacillus in the ocular discharges of cattle affected with ophthalmia. *M. bovis* was first isolated by Jones and Little (1923).

M. bovis is most often observed microscopically as short, plump rods that are almost coccoid and usually occur in pairs and short chains. Biochemical characteristics of this species include a positive reaction in an oxidase test, an inability to reduce nitrate or to grow on MacConkey's agar, and the production of a characteristic reaction in litmus milk (Pugh 1981; Le Minor 1984). Additionally, *M.*

bovis strains may be piliated (Pedersen et al. 1972) and/or hemolytic on blood agar (Pugh and Hughes 1968; Gil-Turnes 1983). Cells of *M. bovis* are naturally competent for DNA uptake and the %G + C content has been estimated at between 41 and 44.5% (Le Minor 1984).

Phenotypic Characteristics of *M. bovis*

M. bovis is a nonmotile microorganism that does not express flagella, however "twitching motility" is observed. Twitching motility is seen as a peripheral zone surrounding an *M. bovis* colony that has been cultured for several days in a humid atmosphere (Henriksen 1976). This form of motility is only displayed when the *M. bovis* strain is expressing pili. The expression of pili has also been associated with alterations in colony morphology. A study by Bøvre and Frøholm (1972) demonstrated that two distinct colony types resulted after incubation of *M. bovis* on blood agar plates for 1 to 4 days in a humid atmosphere at the optimum growth temperature of 33°C. One colony type was small (1–2 mm), flat, and smooth with entire edges, and was capable of twitching motility and agar corrosion. These colonies were formed by cells that were piliated and capable of autoagglutination when cultured in liquid medium. The second colony type was larger (4–5 mm), convex, noncorroding, and formed by nonpiliated cells that did not display twitching motility or autoagglutination. Several studies have shown that only piliated strains are able to colonize the bovine conjunctival mucosa (Pedersen et al. 1972; Sandhu et al. 1974; Lepper and Power 1988).

The other common phenotypic characteristic of *M. bovis* is β-hemolysis, which is observed when the bacterium is cultured on blood agar media. Significantly, it has been illustrated that nonhemolytic strains are incapable of causing IBK in cattle (Pugh and Hughes 1968).

Sources of the Bacterium

M. bovis is found wherever cattle are raised (i.e., the organism is distributed worldwide). The reservoir of *M. bovis* is thought to be the conjunctiva, nasopharynx, or possibly vagina of asymptomatic cattle over 2 years of age. The bacterium is susceptible to desiccation, does not survive well away from the animal host, and is most frequently isolated from bovine eyes in cases of IBK (Le Minor 1984). Isolates of *M. bovis* have been classified into seven distinct pilus antigen serotypes (A to G)(Moore and Lepper 1991).

Virulence Factors of *M. bovis*

Type IV Pili

The pili of *M. bovis* are virulence determinants since only piliated bacteria are experimentally able to cause IBK (Pedersen et al. 1972; Jackman and Rosenbusch 1984; Gil-Turnes 1983; Jayappa and Lehr 1986; Lepper and Power 1988; fig. 24.1). Pili play an important role in the establishment of infection by enabling the bacteria to colonize the corneal/conjunctival surface (Jackman and

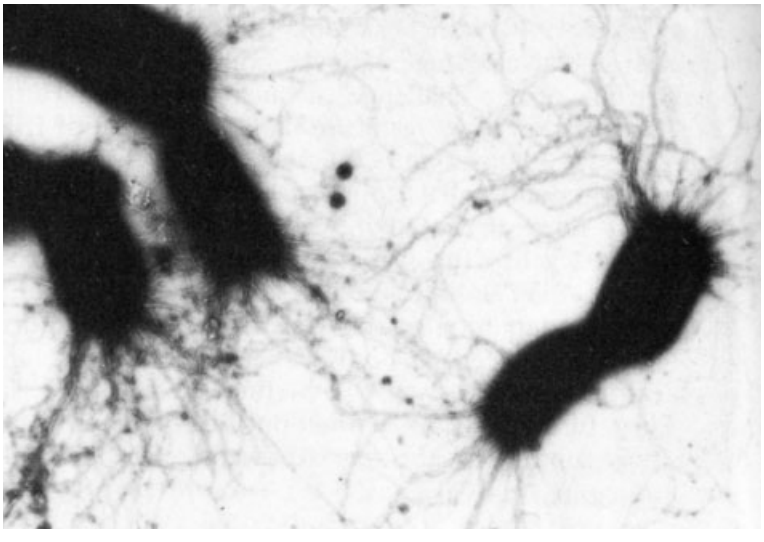


Figure 24.1. Negatively stained, piliated cells of *M. bovis* (electron micrograph, 14,000X magnification; Lepper and Hermans 1986, with permission).

Rosenbusch 1984). *M. bovis* pili belong to the type IV pili family, which includes pili from several veterinary pathogens, including *Dichelobacter nodosus* (Elleman 1988), *Pseudomonas aeruginosa* (Pasloske et al. 1985), and *Pasteurella multocida* (Ruffolo et al. 1997).

Type IV pili can range up to several micrometers in length and generally have a diameter of approximately 6 nm (reviewed in Tennent and Mattick 1994; fig. 24.1). Type IV pili are generally composed of a single repeating pilin subunit in the size range of 145 to 160 amino acids. The immature type IV pilins contain a short (six or seven residues), positively charged amino acid leader sequence, which is cleaved from the preprotein by a signal peptidase to form the mature protein. The mature proteins are characterized by an N-methylphenylalanine amino acid at the amino terminus. The N-terminal region of type IV pilins is highly homologous; variation between pilins of different strains and species occurs in the carboxy-terminal two-thirds of the pilin protein (Tennent and Mattick 1994). Type IV pili of *N. gonorrhoea* and some serotypes of *M. bovis* are O-glycosylated (Parge et al. 1995).

Although the receptor for the *M. bovis* pili on the surface of the host cell is unknown, it would appear that the binding is somewhat host specific (Ruehl et al. 1993; Tennent and Mattick 1994). The presence of type IV pili is also associated with the ability of bacteria to display twitching motility (Henriksen 1976), to corrode and create pits in agar (Bøvre and Frøholm 1972), and to become competent for the uptake of DNA.

The highly conserved nature of the type IV pilin subunit, and presumably the assembly and extrusion apparatus, has been exploited to allow the recombinant expression of type IV pili. Recombinant *P. aeruginosa* were engineered to produce pili from the Australian *M. bovis* isolate Dalton 2d (Elleman et al. 1990). Large amounts of recombinant pili were prepared in this way and used to successfully vaccinate and protect cattle from homologous challenge with *M. bovis* Dalton 2d (Lepper et al. 1993). However, for any piliated vaccine to be successful against IBK, it must contain pili from each of the seven known serotypes. Commercially useful production of recombinant pili of all seven serotypes for vaccine formulation has yet to be achieved.

Hemolysin

It has been unequivocally determined in studies comparing piliated, hemolytic and piliated, and nonhemolytic strains of *M. bovis* that β–hemolysin activity is also a requirement for the establishment of IBK (Hughes et al. 1968; Rogers et al. 1987a, b). Early analysis of the *M. bovis* hemolysin found that the maximum hemolytic activity was seen in the logarithmic phase of growth. Other characteristics noted were that the activity was filterable, heat labile, sensitive to formalin or trypsin treatments, and highly active against a range of animal red blood cells such as rabbit, sheep, cow, and horse, but of only low activity against human cells. The putative protein carrying the hemolytic activity was also found to have a requirement for a sulfhydryl group and divalent cations, supporting the theory that the hemolysin was an enzyme. The same study concluded that there was probably only one type of hemolysin expressed by *M. bovis* since identical hemolytic activities were detected in both whole organism preparations and in cell-free culture filtrates (Sandhu and White 1977).

To date, a pure preparation of β–hemolysin from *M. bovis* has not been obtained. The most highly purified preparations have been contaminated with other proteins and a considerable amount of carbohydrate (Beard and Moore 1994). Therefore, the majority of studies investigating the action and role of the hemolysin in IBK have used either a partially purified preparation or whole cells. Hoien-Dalen et al. (1990) made a comparison of the leukocidic and hemolytic activities of a hemolytic and nonhemolytic strain of *M. bovis*. This investigation suggested that the two activities, leukocidic and hemolytic, may have been due to the same enzyme. By inoculating calves intracorneally with a partially purified hemolytic preparation, it was shown that the resultant ocular damage, healing process, and microscopic pathology resembled published descriptions of naturally occurring IBK. Injection with an equivalent fraction from a nonhemolytic strain induced no change in the eye (Beard and Moore 1994).

In IBK, *M. bovis* can be observed in pits on the surface of the corneal and conjunctival cells (Chandler et al. 1983; Rogers et al. 1987a, b). The cause of these pits is unknown, but toxins such as the hemolysin and/or a cytotoxin may play a role in their development. Partially purified preparations from hemolytic and nonhemolytic *M. bovis* strains were used to demonstrate that a cytotoxic effect on bovine and ovine corneal epithelial cells was associated with the preparation that contained the hemolysin/cytolysin (Gray et al. 1995).

Using a monoclonal antibody raised against the *E. coli* α–hemolysin (RTX toxin), a single protein was recognized in a Western blot procedure carried

out on a partially purified *M. bovis* hemolytic preparation. This cross-reactivity indicated that the suspected *M. bovis* hemolysin/cytotoxin might be a member of the RTX toxin family (Gray et al. 1995).

Studies comparing the activity of the putative *M. bovis* hemolysin with representatives of the RTX toxin family suggest that there may be a common process whereby pores are formed in target cells. Studies of the *M. bovis* hemolytic fraction suggest that the following occurs: (1) the toxin disrupts the target cell plasma membrane by formation of defined-sized transmembrane pores; (2) the pores allow for the dissipation of target cell transmembrane ion gradients, which results in increased intracellular colloid-osmotic pressure; (3) water moves into the cells in response and the cells swell and lyse (Clinkenbeard and Thiessen 1991). This sequence and kinetics of events of the *M. bovis* hemolysin are similar to those of members of the RTX toxin family (Angelos et al. 2001) and are common to all serotypes (Farn et al. 1999; Farn 2000).

Potential Virulence Determinants of *M. bovis*

M. bovis produces a wide range of additional enzymes that has the potential to play a role in *M. bovis* infection. An extensive investigation of 13 reference strains of *M. bovis* found that all were capable of expressing the following enzyme activities: hemolysin, lipase, esterase, phosphoamidase, phosphatase, a protease capable of hydrolyzing casein and gelatin, a leucine and valine aminopeptidase, and a fibrinolysin. A number of other enzymes such as hyaluronidase, catalase, and cysteine aminopeptidase were also expressed by some of the *M. bovis* strains tested (Frank and Gerber 1981; Farn et al. 1999, 2001). It is not known whether the variation in enzyme activities expressed by these *M. bovis* strains correlated with differences in virulence. The isolation and a more thorough characterization of these potential virulence factors may ascertain their role, if any, in the onset and progression of IBK.

INFECTIOUS BOVINE KERATOCONJUNCTIVITIS (IBK) OR PINKEYE

The first report of infectious bovine keratoconjunctivitis (IBK), commonly known as pinkeye, was made in 1889 when it was described as a contagious inflammation of the cornea in cattle. It was observed that IBK did not spread from cattle to other species of animals housed in the same environment, such as horses, nor to the humans who were milking the IBK-infected herd. In this initial description of the disease, the animals became anorexic and emaciated, and their milk yield decreased (Billings 1889). More recent studies have shown that IBK is relatively widespread in cattle herds and of significant economic importance due to losses at market and costs associated with treatment (Thrift and Overfield 1974). Rapid dissemination among the herd results in up to 80% of at-risk animals having succumbed to the disease within 1 to 3 weeks (Bedford 1976).

An investigation into the impact of IBK on weaning and postweaning Hereford calves in Kentucky (U.S.) found that animals lost, on average, 17 kg from their 205-day weight if they contracted pinkeye (Thrift and Overfield 1974). A report by the National Animal Health Monitoring System of the United States Department of Agriculture found that the rate of IBK infection (1.1%) in the United States was second only to scours and diarrhea as the most prevalent condition affecting unweaned calves over 3 weeks of age. A 1993 study by the same body found that the cost attributable to decreases in weight gain, milk production, and treatment was $US150 million per annum (Kirkpatrick and Lalman 1993).

Pinkeye is also prevalent among Australian cattle herds. In a 1982 survey of Australian cattle producers, 81.3% reported pinkeye in their herds in the 3 years prior to the survey (Slatter et al. 1982). The total national production loss due to IBK infection is estimated at $23.6 million per year.

Epidemiology and Pathogenesis of IBK

IBK is a highly contagious disease of cattle. It has been clearly determined that *Moraxella bovis* is the species most commonly isolated from cattle suffering from IBK (Handl and Kubes 1977; Chandler et al. 1979). Transmission experiments demonstrated that *M. bovis* could most reliably produce typical IBK in the undamaged bovine eye (Lepper and Barton 1987). However, it appeared that *M. bovis* alone was not entirely responsible for the onset and progression of disease. Other factors such as environmental stress, season of the year, *M. bovis* strain, host immunity, and the presence of other pathogens may play integral roles in the onset and severity of IBK (Brown et al. 1998).

Initial signs of infection generally occur when the herd is exposed to conditions that irritate the corneal surface of the eye. For instance, if cattle are exposed to high levels of weed or grass seed during grazing, the surface of the eye may be damaged and facilitate

establishment of disease. Dust and factors such as a lack of mineral supplementation are also thought to play a role in the infection, but these observations have not yet been proven (Brown et al. 1998). Exposure to ultraviolet (UV) light is the major environmental factor that appears to play a role in the establishment of IBK. Conjunctival and corneal damage resulting from exposure to high levels of direct or indirect UV light has been shown to predispose cattle to *M. bovis*-associated IBK (Thrift and Overfield 1974). This correlation may explain the seasonal variation that is observed with the infection. The rate of isolation of *M. bovis* in one study increased from spring (21.4%) to summer (29.3%) with a maximum in autumn (Brown et al. 1998). A winter outbreak of IBK suggested that the combination of *M. bovis* and sunlight, albeit even if reflected from fresh snow, were contributing factors to the development of IBK (Hubbert and Hermann 1970). Several studies have reported that the highest prevalence of IBK infection is preceded by peak values of UV radiation (Hughes and Pugh 1970).

M. bovis is spread by direct contact, nasal and ocular secretions, and mechanical vectors (Kopecky et al. 1986). One such vector is the face fly, which has been implicated in a large increase in IBK cases in North America since its introduction to the country in 1952 (Coleman and Gerhardt 1987). *M. bovis* was found to remain viable on the external surfaces of the face fly for up to 3 days and was recovered from laboratory-reared flies that were exposed to lachrymal exudates on infected cattle in the field. It was also demonstrated that, following feeding on *M. bovis* infected broth, the fly is capable of transmitting IBK to calves (Glass and Gerhardt 1984).

A number of other infectious organisms have been implicated in IBK. The two most commonly associated pathogens are infectious bovine rhinotracheitis (IBR) virus and *Mycoplasma bovoculi*. Both of these agents have been isolated from cattle infected with *M. bovis* (Sykes et al. 1962; Kelly et al. 1983). A study involving the concomitant infection of calves with IBR virus and *M. bovis* found that there was an increased severity of IBK and a greater frequency of corneal perforation than when either agent was present alone (George et al. 1988).

Clinical Signs of IBK

In experimental trials, the initial clinical signs of *M. bovis* infection generally appear between 1 and 2 days postinoculation (Smith and George 1985). Early signs of infection include irritation of the anterior segment, causing rubbing of the eyes, pain, blepharospasm, photophobia, and a copious ocular discharge that can last up to 12 days. This discharge appears watery initially but progressively becomes purulent and begins to mat the eyelids. Within 24 to 48 hours of the onset of lachrymation/photophobia, the condition deteriorates and the conjunctiva becomes hyperemic and edematous, and blepharitis may be present. At this stage, the central area of the cornea (approx. 3 mm) exhibits a slight cloudiness. As the lesion enlarges, the opacity becomes more severe due to a buildup of edema within the corneal stroma. The central area begins to take on a yellowish color, and the opaque peripheral area becomes heavily vascularized. By approximately 6 days following infection, the peripheral areas of the cornea are covered by a mass of capillaries while the central area remains yellow. At this point the condition is very painful, and the animal may be either blind or suffer from impaired vision (Bedford 1976; Smith and George 1985). Corneal ulceration often follows at this stage, particularly in young cattle. Only the corneal epithelium and the basement membrane are involved in less-severe infections, however, in more serious cases, the area of ulceration enlarges to involve deeper stromal tissue.

Rupture of the corneal ulcer may be a spontaneous process or can result from blunt trauma. If perforation occurs, purulent inflammation of all parts of the eye may result; more commonly, uveal prolapse is observed with fibrin sealing of the wound and the retention of the shape of the globe with varying degrees of purulent inflammation. The healing time of an affected eye depends on the severity of the disease. In less severely infected eyes, the cornea vascularizes rapidly, and the eye may be healed in 2 weeks, with the majority being healed in 4 to 6 weeks (Punch and Slatter 1984). Depending on the degree of involvement of the corneal stroma, granulation tissue may form at the ulcer site. Once these severe stromal defects have healed, a dense corneal scar results and may last months to years (Aikman et al. 1985; Brown et al. 1998).

Immunological Responses in Cattle

The cornea and conjunctiva of the eye are mucosal surfaces and possess specific immunological and nonspecific mechanical defenses against invading bacteria. Current understanding of mucosal immunity of the eye is predominantly derived from human studies. In humans, conjunctival immunological defenses comprise intraepithelial lymphocytes and antigen-presenting cells, especially dendritic cells

and aggregations of lymphoid follicles in the mucosal layer. In addition, the conjunctiva may form part of the common mucosal immune system. The aggregations of lymphoid follicles, producing predominantly IgA, may be the conjunctival equivalent of mucosa-associated lymphoid tissue (Forrester et al. 1996). In contrast to the conjunctiva, the center of the cornea has fewer intraepithelial lymphocytes. Consequently, corneal tissue does not respond readily to antigenic insults, although immunoglobulins in the cornea do reflect past antigen exposure (Preston et al. 1992; Smolin 1993; Forrester et al. 1996).

Approximately 80% of total immunoglobulins in tears are of the IgA class. There may also be an important contribution of IgG to the secretory immune system either through selective transfer or local production (Butler et al. 1972; Duncan et al. 1972). However, the immunological response to *M. bovis* infection is not well understood. Efforts to measure specific anti-*M. bovis* antibodies have often yielded conflicting results, although protection induced by exposure of cattle to the bacterium suggest immunological defenses are important (Hughes et al. 1968; Bryan et al. 1973). Measurements of antibody responses in cattle following *M. bovis* infection indicate that anti-*M. bovis* antibodies are present in both tears and serum, although the role and contribution of different immunoglobulin classes in resistance is not well defined (Arora et al. 1976b; Killinger et al. 1978; Kopecky et al. 1983; Makinde et al. 1985). In serum, hemagglutinating antibody titers induced following vaccination with a *M. bovis* whole-cell bacterin demonstrate no correlation with protection from disease (Arora et al. 1976a). This may indicate a contribution by locally produced antibodies in the protective immune response.

Anti-*M. bovis* antibody titers in tears are predominantly of the IgA class, and locally produced IgA has been implicated in protecting cattle from IBK (Bishop et al. 1982; Smith et al. 1989). Furthermore, the observation of protection conferred to eyes following challenge in the contralateral eye (Kopecky et al. 1983) indicates a common mucosal immune system exists in bovine conjunctiva. However, selective transport of IgG antibodies from blood to lachrymal secretions may also contribute to the immune response against *M. bovis* (Killinger et al. 1978).

Treatment of IBK

IBK is a self-limiting disease, but early treatment will reduce scarring. Antimicrobial therapy, though widely applied, may not be effective in eradicating the carrier state or in causing clinical improvement in advanced disease (Brown et al. 1998). Most *M. bovis* isolates are susceptible to a range of antibiotics including, penicillin, streptomycin, gentamicin, trimethoprim-sulfonamide, cephalosporin, tetracycline, and nitrofurans (George et al. 1984). There appears to be increasing resistance among *M. bovis* isolates to antibiotics such as tylosin, lincomycin, streptomycin, erythromycin, and cloxacillin (Brown et al. 1998) suggesting that the variation in antimicrobial sensitivity among *M. bovis* isolates may necessitate culture and sensitivity testing of field isolates prior to the commencement of treatment (Fulks et al. 1990).

To reduce the spread of IBK, affected animals should be isolated. Irritating factors such as sunlight and flies should be limited by providing shaded shelters or eye patches. Third eyelid flaps or temporary tarsorrhaphy may facilitate healing in cases with severe ulceration. Little has been reported regarding the efficacy of periocular artificial pigmentation with sprays, dyes, and tattooing (Brown et al. 1998).

Vaccine Development

The fact that animals are immune to pinkeye for up to 12 months after infection suggests vaccination should be a reasonable means of control. Until now it has been impossible to make an effective preparation that guarantees immunity against any of the many strains of pinkeye prevalent in the cattle population.

Initial attempts to formulate a vaccine against IBK focussed on the type IV pili. Recombinant *M. bovis* pili were produced in *P. aeruginosa*, which is more amenable to fermentation than the parent bacterium (Beard et al. 1990; Elleman et al. 1990). However, lack of cross-protection between pili serotypes of *M. bovis* requires that a successful vaccine must contain pili from each serotype (Lepper et al. 1992). To date, recombinant pili from all serotypes have not been produced in quantities that would permit the commercial development of a multivalent pili-based vaccine.

An *M. bovis* hemolysin vaccine has long been considered an alternative vaccine candidate to pili (Ostle and Rosenbusch 1984; Billson et al. 1993, 1994), however the lack of genetic information about the toxin has hindered attempts to develop a vaccine based on this protein. Studies in cattle showed that a partially purified cell-free supernatant from a hemolytic isolate of *M. bovis* protected ani-

mals against a wild-type challenge, whereas a similar preparation from a nonhemolytic isolate did not (Billson et al. 1994).

Genes encoding *M. bovis* RTX toxin (Farn et al. 1999, 2001; Angelos et al. 2001) and two additional novel phospholipase b and protease antigens (Farn et al. 1999, 2001) have recently been identified in all serotypes. Extensive studies aiming at production of recombinant antigens are under way, and animal trials are planned for 2004 (W. P. Michalski, personal communication). The roles of the enzymes secreted from *M. bovis* in the pathogenesis of IBK have not been resolved, and it may be that a polyvalent vaccine consisting of multiple antigens such as inactivated hemolysin in the presence of the pili, lipase, and/or protease may be more effective than a vaccine containing only one determinant (Farn 2000; Farn et al. 2001).

M. bovis isolates specifically bind bovine lactoferrin and bovine transferrin and use these proteins as a source of iron to support the growth of iron-limited cells. Specific bacterial receptor proteins that are involved in iron acquisition from these proteins have been identified and their genes have been cloned from *M. bovis*. Lactoferrin receptor proteins are attractive vaccine targets since they are found in virtually all isolates of *M. bovis* and some antireceptor antibodies appear bactericidal. At present, however, data are scarce regarding the vaccine efficacy of these receptor proteins (Yu and Schryvers 2002).

REFERENCES

Aikman, J. G., E. M. Allan, and I. E. Selman. 1985. Experimental production of infectious bovine keratoconjunctivitis. Vet. Record. 117:234–239.

Angelos, J. A., J. F. Hess, and L. W. George. 2001. Cloning and characterisation of a *Moraxella bovis* cytotoxin gene. Am. J. Vet. Res. 62:1222–1228

Arora, A. K., A. H. Killinger, and M. E. Mansfield. 1976a. Detection of *Moraxella bovis* antibodies in infectious bovine keratoconjunctivitis. Am. J. Vet. Res. 37:803–805.

Arora, A. K., A. H. Killinger, and W. L. Meyers. 1976b. Detection of *Moraxella bovis* antibodies in infectious bovine keratoconjunctivitis. Am. J. Vet. Res. 37:1489–1492.

Beard, M. K., and L. J. Moore. 1994. Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of *Moraxella bovis*. Vet Microbiol. 42:15–33.

Beard, M. K., J. S. Mattick, L. J. Moore, M. R. Mott, C. F. Marrs, and J. R. Egerton. 1990. Morphogenetic expression of *Moraxella bovis* fimbriae (pili) in *Pseudomonas aeruginosa.* J. Bacteriol. 172:2601–2607.

Bedford, P. G. 1976. Infectious bovine keratoconjunctivitis. Vet. Rec. 98:134–135.

Billings, F. S. 1889. Contagious inflammation of the cornea in cattle. Bull. Agri. Expt. 3:247–252.

Billson, F. M., J. L. Hodgson, J. R. Egerton, A. W. Lepper, W. P. Michalski, C. L. Schwartzkoff, P. R. Lehrbach, and J. M. Tennent. 1994. A haemolytic cell-free preparation of *Moraxella bovis* confers protection against infectious bovine keratoconjunctivitis. FEMS Microbiol. Lett. 124:69–73.

Billson, F. M., J. M. Tennent, A. W. Lepper, J. R. Egerton, J. L. Hodgson, C. L. Schwartzkoff, and P. R. Lehrbach. 1993. Protective efficacy of a *Moraxella bovis* haemolytic fraction in a challenge trial. Australian Microbiologist 14:A–34.

Bishop, B., G. G. Schurig, and H. F. Trautt. 1982. Enzyme-linked immunosorbent assay for measurement of anti-*Moraxella bovis* antibodies. Am. J. Vet. Res. 43:1443–1445.

Bøvre, K., and L. O. Frøholm. 1972. Variation of colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M. nonliquefaciens*. Acta Pathol. Microbiol. Scand. [B] Microbiol. Immunol. 80:629–640.

Brown, M. H., A. H. Brightman, B. W. Fenwick, and M. A. Rider. 1998. Infectious bovine keratoconjunctivitis: a review. J. Vet. Intern. Med. 12:259–266.

Bryan, H. S., L. C. Helper, A. H. Killinger, H. E. Rhoades, and M. E. Mansfield. 1973. Some bacteriologic and ophthalmic observations on bovine infectious keratoconjunctivitis in an Illinois beef herd. J. Am. Vet. Assoc. 163:739–741.

Butler, J. E., C. F. Maxwell, C. S. Pierce, M. B. Hylton, R. Asofsky, and C. A. Kiddy. 1972. Studies of relative synthesis and distribution of IgA and IgG_1 in various tissues and body fluids of the cow. J. Immun. 109:38–46.

Chandler, R. L., P. J. Baptista, and B. Turfrey. 1979. Studies on the pathogenicity of *Moraxella bovis* in relation to infectious bovine keratoconjunctivitis. J. Comp. Pathol. 89:441–448.

Chandler, R. L., R. G. Bird, M. D. Smith, H. S. Anger, and B. A. Turfrey. 1983. Scanning electron microscope studies on preparations of bovine cornea exposed to *Moraxella bovis*. J. Comp. Pathol. 93:1–8.

Clinkenbeard, K. D., and A. E. Thiessen. 1991. Mechanism of action of *Moraxella bovis* hemolysin. Infect. Immun. 59:1148–1152.

Coleman, R. E., and R. R. Gerhardt. 1987. Isolation of *Moraxella bovis* from the crops of field-collected face flies. J. Agric. Entomol. 4:92–94.

Duncan, J. R., B. N. Wilkie, F. Hiestand, and A. J. Winter. 1972. The serum and secretory immunoglobulins of cattle: characterisation and quantitation. Infect. Immun. 57:983–988.

Elleman, T. C. 1988. Pilins of *Bacteroides nodosus*: molecular basis of serotypic variation and relationships to other bacterial pilins. Microbiol. Rev. 52:233–247.

Elleman, T. C., P. A. Hoyne, and A. W. Lepper. 1990. Characterization of the pilin gene of *Moraxella bovis* Dalton 2d and expression of pili from *M. bovis* in *Pseudomonas aeruginosa*. Infect. Immun. 58:1678–1684.

Farn, J. L. 2000. A genetic analysis of the virulence determinants of *Moraxella bovis*. Ph.D. thesis. The University of Melbourne, Department of Microbiology and Immunology.

Farn, J. L., J. M. Tennent, and R. A. Strugnell. 1999. Vaccine antigens of Moraxella. Patent PCT/AU00/01048.

Farn, J. L., R. A. Strugnell, P. A. Hoyne, W. P. Michalski, and J. M. Tennent. 2001. Molecular characterisation of a secreted enzyme with phospholipase activity from *Moraxella bovis*. J. Bacteriol. 183:6717–6720.

Forrester, J., A. Dick, P. McMenamin, and W. Lee. 1996. The eye: basic sciences in practice. London: W. B. Saunders Company Ltd., pp. 265–318.

Frank, S. K., and J. D. Gerber. 1981. Hydrolytic enzymes of *Moraxella bovis*. J. Clin. Microbiol. 13:269–271.

Fulks, K. A., C. F. Marrs, S. P. Stevens, and M. R. Green. 1990. Sequence analysis of the inversion region containing the pilin genes of *Moraxella bovis*. J. Bacteriol. 172:310–316.

George, L. W., A. Ardans, J. Mihalyi, and M. R. Guerra. 1988. Enhancement of infectious bovine keratoconjunctivitis by modified-live infectious bovine rhinotracheitis virus vaccine. Am. J. Vet. Res. 49:1800–1806.

George, L. W., W. D. Wilson, J. D. Baggot, and J. E. Mihalyi. 1984. Antibiotic treatment of *Moraxella bovis* infection in cattle. J. Am. Vet. Med. Assoc. 185:206–209.

Gil-Turnes, C. 1983. Hemagglutination, autoagglutination and pathogenicity of *Moraxella bovis* strains. Can. J. Comp. Med. 47:503–504.

Glass, H. W., Jr., and R. R. Gerhardt. 1984. Transmission of *Moraxella bovis* by regurgitation from the crop of the face fly (Diptera: Muscidae). J. Econ. Entomol. 77:399–401.

Gray, J. T., P. J. Fedorka-Cray, and D. G. Rogers. 1995. Partial characterization of a *Moraxella bovis* cytolysin. Vet. Microbiol. 43:183–196.

Handl, R., and O. Kubes. 1977. Significance of *Moraxella bovis* in the etiology of infectious bovine keratoconjunctivitis of cattle and its diagnosis. Folia Microbiol. 22:487.

Henriksen, S. D. 1976. Moraxella, Neisseria, Branhamella, and Acinetobacter. Annu. Rev. Microbiol. 30:63–83.

Hoien-Dalen, P. S., R. F. Rosenbusch, and J. A. Roth. 1990. Comparative characterization of the leukocidic and hemolytic activity of *Moraxella bovis*. Am. J. Vet. Res. 51:191–196.

Hubbert, W. T., and G. J. Hermann. 1970. A winter epizootic of infectious bovine keratoconjunctivitis. J. Am. Vet. Med. Assoc. 157:452–454.

Hughes, D. E., and G. W. Pugh. 1970. A five-year study of infectious bovine keratoconjunctivitis in a beef herd. J. Am. Vet. Med. Assoc. 157:443–457.

Hughes, D. E., G. W. Pugh, Jr., and T. J. McDonald. 1968. Experimental bovine infectious keratoconjunctivitis caused by sunlamp irradiation and *Moraxella bovis* infection: resistance to re-exposure with homologous and heterologous *Moraxella bovis*. Am. J. Vet. Res. 29:829–833.

Jackman, S. H., and R. F. Rosenbusch. 1984. In vitro adherence of *Moraxella bovis* to intact corneal epithelium. Curr. Eye Res. 3:1107–1112.

Jayappa, H. G., and C. Lehr. 1986. Pathogenicity and immunogenicity of piliated and nonpiliated phases of *Moraxella bovis* in calves. Am. J. Vet. Res. 47:2217–2221.

Jones, F. S., and R. B. Little. 1923. Infectious ophthalmia in cattle. J. Exp. Med. 38:139–48.

Kelly, J. I., G. E. Jones, and A. G. Hunter. 1983. Isolation of *Mycoplasma bovoculi* and *Acholeplasma oculi* from outbreaks of infectious bovine keratoconjunctivitis. Vet. Rec. 112:482.

Killinger, A. H., R. M. Weisiger, L. C. Helper, and M. E. Mansfield. 1978. Detection of *Moraxella bovis* antibodies in the IgA, IgG and IgM classes of immunoglobulin in bovine lachrymal secretions by an indirect fluorescent antibody test. Am. J. Vet. Res. 39:931–934.

Kirkpatrick, J. G., and D. Lalman. 1993. Pinkeye in cattle; infectious bovine keratoconjunctivitis. http://www.ansi.okstate.edu/EXTEN/cc-corner/pinkeyefs.html.

Kopecky, K. E., G. W. J. Pugh, and T. J. McDonald. 1983. Infectious bovine keratoconjunctivitis: evidence for general immunity. Am. J. Vet. Res. 44:260–262.

———. 1986. Infectious bovine keratoconjunctivitis: contact transmission. Am. J. Vet. Res. 47:622–624.

Le Minor, L. 1984. Genus Moraxella. In Bergey's manual of systematic bacteriology, vol.1. N. R. Krieg and J. G. Holt (eds.). Baltimore: Williams and Wilkins, pp. 427–428.

Lepper, A. W., and B. E. Power. 1988. Infectivity and virulence of Australian strains of *Moraxella bovis* for the murine and bovine eye in relation to pilus serogroup sub-unit size and degree of piliation. Aust. Vet. J. 65:305–309.

Lepper, A. W., and I. J. Barton. 1987. Infectious bovine keratoconjunctivitis: seasonal variation in cultural, biochemical and immunoreactive properties of *Moraxella bovis* isolated from the eyes of cattle. Aust. Vet. J. 64:33–39.

Lepper, A. W., and L. R. Hermans. 1986. Characterisation and quantitation of pilus antigens of *Moraxella bovis* by ELISA. Aust. Vet. J. 63:402–405.

Lepper, A. W., L. J. Moore, J. L. Atwell, and J. M. Tennent. 1992. The protective efficacy of pili from different strains of *Moraxella bovis* within the same serogroup against infectious bovine keratoconjunctivitis. Vet. Microbiol. 32:177–187.

Lepper, A. W., T. C. Elleman, P. A. Hoyne, P. R. Lehrbach, J. L. Atwell, C. L. Schwartzkoff, J. R. Egerton, and J. M. Tennent. 1993. A *Moraxella bovis* pili vaccine produced by recombinant DNA technology for the prevention of infectious bovine keratoconjunctivitis. Vet. Microbiol. 36:175–183.

Makinde, A. A., A. O. Ezeh, O. Onoviran, and I. Umo. 1985. Prevalence of serum antibodies to *Moraxella bovis* in cattle in Nigeria. Brit. Vet. J. 141:643–646.

Moore, L. J., and A. W. Lepper. 1991. A unified serotyping scheme for *Moraxella bovis*. Vet. Microbiol. 29:75–83.

Ostle, A. G., and R. F. Rosenbusch. 1984. *Moraxella bovis* hemolysin. Am. J. Vet. Res. 45:848–851.

Parge, H. E., K. T. Forest, M. J. Hickey, D. A. Christensen, E. D. Getzoff, and J. A. Tainer. 1995. Structure of the fibre-forming protein pili at 2.6Å resolution. Nature 378:32–38.

Pasloske, B. L., B.B. Finlay, and W. Paranchych. 1985. Cloning and sequencing of the *Pseudomonas aeruginosa* PAK pilin gene. FEBS Lett. 183:408–412.

Pedersen, K. B., L. O. Froholm, and K. Bovre. 1972. Fimbriation and colony type of *Moraxella bovis* in relation to conjunctival colonization and development of keratoconjunctivitis in cattle. Acta Pathol. Microbiol. Scand. [B] Microbiol Immunol. 80:911–918.

Preston, M. J., K. A. Kernacki, J. M. Berk, L. D. Hazlett, and R. S. Berk. 1992. Kinetics of serum, tear and corneal antibody response in resistant and susceptible mice intraocularly infected with *Pseudomonas aeruginosa*. Infect. Immun. 60:885–891.

Pugh, G. W. 1981. Moraxella. In Handbuch der bakteriellen infektionen bie tieren. Stuttgart: Gustav Fischer Verlag Jena, pp. 677–707.

Pugh, G. W., Jr., and D. E. Hughes. 1968. Experimental bovine infectious keratoconjunctivitis caused by sunlamp irradiation and *Moraxella bovis* infection: correlation of hemolytic ability and pathogenicity. Am. J. Vet. Res. 29:835–839.

Punch, P. I., and D. H. Slatter. 1984. A review of infectious bovine keratoconjunctivitis. Vet. Bull. 54:193–207.

Rogers, D. G., N. F. Cheville, and G. W. J. Pugh. 1987a. Conjunctival lesions caused by *Moraxella bovis* in gnotobiotic calves. Vet. Pathol. 24:554–559.

———. 1987b. Pathogenesis of corneal lesions caused by *Moraxella bovis* in gnotobiotic calves. Vet. Pathol. 24:287–295.

Rossau, R., A. Van Lanschoole et al. 1991. Taxonomy of Moreaxellaceae fam. nov., a new bacterial family to accommodate the genera Moraxella, Acinetobacter, and Psychrobacter and related organisms. Int. J. Sys. Bacteriol. 41:310–319.

Ruehl, W. W., C. Marrs, M. K. Beard, V. Shokooki, J. R. Hinojoza, S. Banks, D. Bieber, and J. S. Mattick. 1993. Q pili enhance the attachment of *Moraxella bovis* to bovine corneas *in vitro*. Mol. Microbiol. 7:285–288.

Ruffolo, C. G., J. M. Tennent, W. P. Michalski, and B. Adler. 1997. Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. Infect. Immun. 65:339–343.

Sandhu, T. S., and F. H. White. 1977. Production and characterization of *Moraxella bovis* hemolysin. Am. J. Vet. Res. 38:883–885.

Sandhu, T. S., F. H. White, and C. F. Simpson. 1974. Association of pili with rough colony type of *Moraxella bovis*. Am. J. Vet. Res. 35:437–439.

Slatter, D. H., M. E. Edwards, C. D. Hawkins, and G. E. Wilcox. 1982a. A national survey of the clinical features, treatment and importance of infectious bovine keratoconjunctivitis. Aust. Vet. J. 59:69–72.

———. 1982b. A national survey of the occurrence of infectious bovine keratoconjunctivitis. Aust. Vet. J. 59:65–68.

Smith, J. A., and L. W. George. 1985. Treatment of acute ocular *Moraxella bovis* infections in calves with a parenterally administered long-acting oxytetracycline formulation. Am. J. Vet. Res. 46:804–807.

Smith, J. A., W. H. Greene, and J. W. Allen. 1989. Antibodies related to resistance in bovine pinkeye. California Veterinarian. 43:7, 9–10, 18.

Smolin, G. 1993. Immunology of ocular tissues. In Biomedical foundation of ophthalmology. T. D. Duane and E. A. Jaeger (eds.). Philadelphia: J. B. Lippincott Company.

Sykes, J. A., L. Dmochowski, C. E. Grey, and W. O. Russell. 1962. Isolation of a virus from infectious

bovine kerato-conjunctivitis. Proc. Soc. Exp. Biol. 111:51–57.

Tennent, J. M., and J. S. Mattick. 1994. Type 4 fimbriae. In Fimbriae: adhesion, genetics, biogenesis and vaccines. P. Klemm (ed.). Boca Raton: CRC Press, Inc., pp. 127–146.

Thrift, F. A., and J. R. Overfield. 1974. Impact of pinkeye (infectious bovine kerato-conjunctivitis) on weaning and postweaning performance of Hereford calves. J. Anim. Sci. 38:1179–1184.

Yu, R. H., and A. B. Schryvers. 2002. Bacterial lactoferrin receptors: insights from characterizing the *Moraxella bovis* receptors. Biochem. Cell Biol. 80:81–90.

25
Campylobacter and *Helicobacter*

L. A. Joens

CAMPYLOBACTER

Campylobacter spp. are widely distributed among domestic animals, but in most cases live as commensals in the intestinal tract of mammals and birds. Food products from farm animals, especially poultry, have been implicated as the major source of *Campylobacter* spp. for infection of humans. *Campylobacter jejuni* is currently the leading cause of bacterial foodborne illness in the world, with *C. coli* involved in 5% of the cases. While the mechanisms of pathogenesis of *C. jejuni* and *C. coli* have received substantial investigation due to their roles in human disease, many food animals are apparently colonized without development of concomitant illness. The identification of non-*jejuni*, non-*coli* species, especially as culture techniques advance, has demonstrated a role for some *Campylobacter* spp. in animal disease.

CAMPYLOBACTER FETUS

Campylobacter spp. were recognized as etiological agents of abortion in sheep nearly one hundred years ago (McFaydean and Stockman 1913). Since then, two distinct subspecies have been described, each of which causes different pathology. *Campylobacter fetus* ss *fetus* causes sporadic abortion in sheep, often late in gestation, while ss *venerealis* is a cause of sexually transmitted bovine infertility and sporadic abortion in cattle.

Campylobacter fetus grows under microaerophilic conditions, similar to other *Campylobacter* spp., but is distinguished from *C. jejuni* by optimal growth at 37°C (but not 42°C) and susceptibility to cephalothin. *Campylobacter fetus* can also be identified by means of a PCR assay using primers to the 16S rDNA (Oyarzabal et al. 1997).

Campylobacter fetus ss *fetus* is transmitted by way of contaminated food or water. Bacteremia ensues, and the organism spreads to distal sites including the placenta. Abortion in the third trimester of ovine gestation results from placentitis (Thompson and Blaser 2000). Sporadic abortion due to *C. fetus* is found in cattle, goats, pigs, and horses (Skirrow 1981).

Campylobacter fetus ss *venerealis*, the cause of bovine venereal campylobacteriosis (BVC), is transmitted by infected bulls through normal breeding or artificial insemination with contaminated semen. Abortion may also result from infection with *C. fetus* ss *venerealis*, but occurs in less than 10% of infected cows (Garcia and Brooks 1993). The organism can be recovered from the glans penis and distal urethra of infected bulls (Smibert 1978). In cows, the organism colonizes the reproductive tract in an ascending fashion, moving from vagina to cervix, uterus, and oviducts (Smibert 1978; Corbeil 1999).

Studies by Blaser et al. (1988) demonstrated that encapsulated *C. fetus* is serum resistant. Mutants lacking a high-molecular-weight proteinaceous surface layer (S-layer) were serum sensitive. The *C. fetus* S-layer fails to bind complement component C3b, and prevents phagocytosis by neutrophils due to defective opsonization in serum. An acid-glycine extractable 135-kDa protein is responsible for resistance to phagocytosis (McCoy et al. 1975). Binding of O-specific antibody is prevented by the presence of this antigen and occurs after removal of the antigen by glycine extraction or by spontaneous mutation.

Bacteria lacking the S-layer have reduced virulence in a murine model of the disease (Blaser and Pei 1993). These data indicate that the S-layer shields the LPS, perhaps as a means to decrease the immunogenicity of the organism.

The product of *sapA* (surface array protein type A) is a predicted 97-kDa protein (Blaser and Gotschlich 1990) with an N-terminal amino acid sequence identical to that of the purified S-layer protein. Comparison of *sapA* alleles (*sapA*, *sapA1*, and *sapA2*) revealed 5' sequence conservation and 3' divergence (Dworkin and Blaser 1997a). The *sapB* and *sapB2* alleles encoding *C. fetus* type B S-layers also contain conserved 5' regions, which are different from those in the *sapA* alleles.

Immunoblots of the S-layer protein of *C. fetus* ss *venerealis* taken at weekly intervals from an experimentally infected heifer revealed putative antigenic shifts based upon the size of reactive proteins (Garcia et al. 1995). Furthermore, genomic digests probed with the 5' conserved region of the *sapA* gene indicated genomic rearrangement among the weekly isolates. Subsequent analysis of the molecular mechanisms of antigenic variation indicated that the *sapA* promoter is contained on an invertible segment of DNA that can allow expression of two oppositely oriented S-layer protein gene cassettes (Dworkin and Blaser 1997a). Alternative expression can also occur by recombination of the 5' conserved regions of the multiple *sapA* homologs, which are clustered on the chromosome (Dworkin and Blaser 1997b).

While the S-layer inhibits phagocytosis by blocking C3b binding and hinders immune recognition by antigenic variation, it is not required for adherence to epithelial cells *in vitro*, as an S-layer-deficient mutant and its parent strain adhered equally well to HEp-2 cells in similar numbers (Graham and MacDonald 1998).

CAMPYLOBACTER JEJUNI AND *CAMPYLOBACTER COLI*

Campylobacter jejuni and *C. coli* can be found in large numbers (10^7 CFU per g) in the intestinal tract of companion and food-producing animals. This relationship is usually commensal without apparent gross or pathologic consequences for the host. Humans become infected with *Campylobacter* by consuming undercooked meat products, especially poultry products, contaminated by either *C. jejuni* or *C. coli*. Ninety-five percent of the human infections are attributable to *C. jejuni*. The syndrome associated with campylobacteriosis is a severe diarrheal disease with feces containing blood and leukocytes. The host may have fever, vomiting, and abdominal pain. The incubation period of the disease is 24–72 h, and even untreated illness is usually self-limiting. Dogs and cats have been implicated in transmission of campylobacteriosis to humans but the prevalence of campylobacters in these companion animals is variable, with an inverse relationship existing between increasing age and colonization (Skirrow 1981). Although healthy dogs harbor *C. jejuni* (Bruce et al. 1980), a strong correlation exists between the presence of diarrhea in dogs and the recovery of *C. jejuni (*Fleming 1983; Nair et al. 1985). In cats, the prevalence of *C. jejuni* seems to be limited to clinically normal animals (Bruce et al. 1980). In food animals, symptoms of campylobacteriosis are mild, and consist of soft-to-watery feces with mucus and flecks of blood.

Extraintestinal infections with *C. jejuni* have been reported in sheep, goats, cattle, and pigs. Infection can result in abortion in sheep and goats, with symptoms similar to those seen in *C. fetus* abortions, and mastitis in cattle (Doyle and Roman 1982; Morgan et al. 1985). Mastitis was produced experimentally (Lander and Gill 1980). *Campylobacter jejuni* is frequently isolated from raw milk contaminated by either bovine fecal material or through mastitic infections, and is a major cause of campylobacteriosis in countries where raw milk is consumed (Doyle and Roman 1982; Humphrey and Beckett 1987; Humphrey and Hart 1988). *C. jejuni* and *C. coli* infections have occasionally been associated with abortion in sheep and pigs (Diker et al. 1988), but in these species, *Campylobacter* infections are usually more benign.

Pathogenesis of *C. jejuni* infections has received substantial investigation. For a disease state to exist, *C. jejuni* must penetrate the intestinal mucous lining (Szymanski et al. 1995) and bind to and invade the cells lining the small and large intestines. Epithelial cell binding allows the bacterium to occupy a niche that resists cleaning effects of the intestine, such as fluid flow and peristalsis. Attachment to epithelial cells is mediated by proteins that are constitutively expressed (Konkel and Cieplak 1992). These attachment factors include a fibronectin-binding protein (CadF) (Konkel et al. 1997), a lipoprotein (JlpA) (Jin et al. 2001), and Peb1A (Pei and Blaser 1993). Other adhesins that have been suggested for the binding of *C. jejuni* to epithelial cells are flagellin, pili, and LPS (Kervella et al. 1993). Binding of *C. jejuni* to host epithelial cells initiates events leading to invasion and subsequent disease. Invasion of host

cells has been established both *in vivo* in a neonatal piglet model (Babakhani et al. 1993) and in cultured epithelial cells (Konkel and Joens 1989). The ability of *C. jejuni* to invade epithelial cells is strain-dependent (Konkel and Joens 1989), with environmental isolates being far less invasive in HeLa cells than clinical isolates (Newell et al. 1985) and isolates from noninflammatory diarrhea being less invasive than those from colitis patients (Everest et al. 1992). Internalization of *C. jejuni* is brought about by directed endocytosis, as a result of bacterial metabolic processes. Cia proteins are synthesized and secreted upon contact via a type III secretion system (Konkel et al. 1999). The proteins are translocated into the cytoplasm of host cells, where they probably alter signaling pathways. Once internalized, *C. jejuni* can be found in membrane-bound vacuoles; they undergo an initial decline in numbers, followed by exponential growth over a 72 h period (Konkel and Cieplak 1992). The bacterium translocates through the intestinal epithelium of the host and thereby gains access to deeper tissue. It is in the deeper tissue of the lamina propria and submucosa that *Campylobacter* comes into contact with inflammatory cells. Light and electron microscopy have demonstrated the organism in granulocytes, parenchymal cells, and mononuclear cells in the lamina propria and submucosa (Ruiz-Palacios et al. 1981; Newell and Pearson 1984; Kiehlbauch et al. 1985; van Spreeuwel et al. 1985; Russell et al. 1989**;** Babakhani et al. 1993) of the infected host. Disease in the newborn piglet, infected orally with clinical isolates of *C. jejuni*, mimics human campylobacteriosis. Electron microscopy studies of infected porcine intestinal tissue have identified macrophages and neutrophils in the lamina propria and submucosa within 3 days of infection, with numerous cells containing internalized *C. jejuni* (Babakhani et al. 1993). These results correlate with those in the primate host and demonstrate an interaction of *C. jejuni* with the host macrophage; they point to a possible role for the macrophage in the disease process of campylobacteriosis.

Uptake of *C. jejuni* by macrophages appears to be independent of complement opsonization but is strain and host dependent. Avirulent strains of *C. jejuni* (those unable to invade the chorioallantoic membrane of chicken embryos) inoculated intravenously into Balb/c mice are cleared at a significantly higher rate than virulent strains; they are engulfed by peritoneal macrophages at a similarly higher rate *in vitro. Campylobacter jejuni* strains exposed to guinea pig resident peritoneal macrophages were also resistant to phagocytosis, while avirulent *C. coli* strains were engulfed in significantly higher numbers. Differences in uptake of the two strains may have been due to the presence of an antiphagocytic capsulelike material in *C. jejuni* that was absent in *C. coli* (Banfi et al. 1986). In contrast, Wassenaar et al. (1997) reported a linear correlation between infective dose and internalized *C. jejuni* in human peripheral blood monocytes. Supporting results were obtained by Myszewski and Stern (1991) when they examined the uptake of two different strains of *C. jejuni* by chicken peritoneal macrophages. *Campylobacter jejuni* was readily internalized by chicken macrophages within a 30 minute incubation period, with increased internalization when serum or macrophages from previously colonized hosts were added to the assay. Day et al. (2000) demonstrated a significant uptake of a clinical isolate of *C. jejuni* M129 in mouse and porcine peritoneal macrophages, as well as a mouse macrophage cell line (J774), independent of serum or complement. Thus, uptake is strain and host dependent, and probably increases in the presence of immune serum.

Extended survival of *C. jejuni* in host macrophages may contribute to the severity of the disease, duration of clinical signs, and recrudescence. However, results of such studies have been inconsistent, and survival may reflect strain, assay, and host-cell differences. Survival assays in activated human peripheral blood monocytes (Wassenaar et al. 1997) demonstrated killing of *C. jejuni* by 90% of these cells within 24 to 48 h, with *C. jejuni* surviving in 10% of the monocytes. A high passage clinical isolate and a poultry isolate were killed by chicken peritoneal macrophages within 6 h (Myszewski and Stern 1991). Findings of Joens et al. (2001) were similar; two of five poultry isolates internalized by a mouse macrophage cell line (J774.A1) were inactivated after 24 h, and the remaining three isolates were killed within 72 h (Pitts 2001). Other studies have demonstrated survival of *C. jejuni* in macrophages. Kiehlbauch et al. (1985) recovered *C. jejuni* from three phagocytic cell types over a 6 day period and demonstrated intraphagosomal replication. *Campylobacter jejuni* clinical isolate M129 survived for 72 h in porcine and murine peritoneal macrophages and in the J774.A1 macrophage cell line, although there was a noticeable reduction in numbers recovered from each cell type at 72 h postinoculation (Day et al. 2000). Joens (2002) confirmed the above findings

by demonstrating survival of clinical isolates 81-176 and F38011 in a J774.A1 macrophage cell line over a 72 h period. These findings support the view that strain differences, rather than the host macrophage, are the critical factors in *C. jejuni* intracellular survival, and that isolates obtained from clinical cases appear to survive at a higher rate than environmental or poultry isolates.

Many bacteria circumvent antigen processing by altering the intracellular trafficking of the phagolysosome, preventing acidification or inhibiting the effects of phagolysosome toxic radicals. These strategies allow bacteria to occupy a modified phagosome and create a niche that provides nutrients for continued existence and shelter from the host immune system. Reactive oxygen species such as superoxide and hydrogen peroxide from the respiratory burst of the phagolysosome can be extremely toxic to bacteria, but bacteria have defensive mechanisms that can circumvent these effects. *Campylobacter* superoxide dismutase (from *sod*B) and catalase (from *kat*A) might be expected to facilitate survival of *C. jejuni* in spite of the occurrence of the respiratory burst. However, survival of a *sod*B mutant of strain 81-176 was no different from that of the parent strain (Joens et al. 2001). In contrast, a *kat*A mutant was susceptible to killing by J774.A1 cells, while the parent strain (*C. jejuni* M129) remained viable 72 h after internalization. Viability of the mutant was equivalent to that of the parent strain when the respiratory burst or production of nitric oxide were inhibited (Day et al. 1999).

Salmonella interferes with antigen processing and the endocytic pathway by preventing phagolysosome maturation. Pitts (2000, unpublished data) examined the effect of phagolysosome development in J774.A1 cells following engulfment of a virulent clinical or avirulent environmental isolate of *C. jejuni*. Macrophages were infected with the two strains at a multiplicity of infection of ~ 10 bacteria per macrophage, and the phagosome was examined for early and late proteins during a 72 h incubation period. Approximately 80–85% of internalized, virulent *C. jejuni* colocalized with early and late proteins of the endocytic pathway. Ninety percent of the phagolysosomes containing *C. jejuni* underwent normal acidification and were processed through the normal endocytic pathway. Thus, the majority of phagocytosed *C. jejuni* undergo normal processing through the endocytic pathway, but a small fraction of isolates do not colocalize with endocytic proteins and perhaps live intracellularly in modified mononuclear phagocytes. It is still unclear which population survives prolonged incubation.

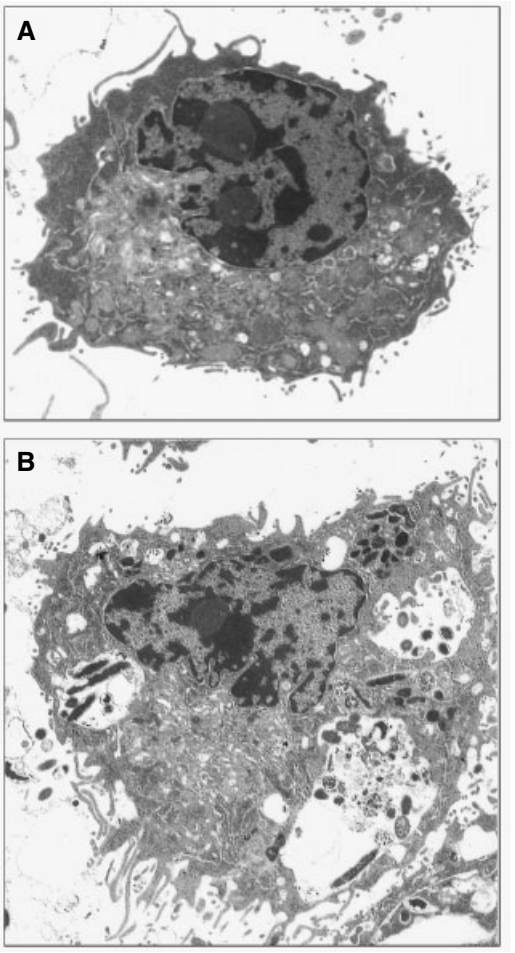


Figure 25.1. (*A*) Uninfected J774.A1 cell; (*B*) J774.A1 cell infected with *C. jejuni.*

Invasive bacteria can induce macrophage death through either apoptosis, necrosis, or a combination of the two mechanisms. Konkel and Mixter (2000) demonstrated that THP-1 monocytes underwent apoptosis as a direct result of infection with *C. jejuni*, rather than as an indirect result of bacterial cytopathic products. Joens (2002, unpublished data) demonstrated both apoptosis and necrosis in human U937 phagocytic cells infected with clinical isolates of *C. jejuni*. In contrast, murine macrophages infected with the same isolates were killed through necrosis in a dose-dependent fashion (fig. 25.1). These results indicate that the mechanism by which the macrophage dies following infection with *C. jejuni*

may be more host dependent than bacterial strain dependent.

Campylobacter upsaliensis, *Campylobacter hyointestinalis*, and *Campylobacter mucosalis*

Campylobacter upsaliensis is catalase negative and is susceptible to both nalidixic acid and cephalothin. The species accounted for 64% of campylobacters isolated from canine stools (Sandstedt et al. 1983). *C. upsaliensis* has been isolated from a dog with chronic diarrhea (Blaser and Pei 1993) but also from healthy dogs and cats (Konkel et al. 1997). It is difficult to associate this organism with disease, as two-thirds of companion and laboratory cats carry *C. upsaliensis* (Moreno et al. 1993) and the carrier rate in dogs is similar.

C. upsaliensis has been isolated from human patients with gastroenteritis, of whom 92% had diarrhea and 14% reported vomiting (Goossens et al. 1990). Moreover, *C. upsaliensis* accounted for 20% of *Campylobacter* isolates among Australian (Albert et al. 1992) and South African pediatric patients with diarrhea (Lastovica 1996).

Sylvester et al. (1996) demonstrated binding of *C. upsaliensis* to epithelial cells *in vitro*. A subset of bacterial surface proteins bound to host cell phosphatidylethanolamine. *C. upsaliensis* bound to purified human small intestine mucin in a dose-dependent manner, suggesting that the bacteria adhere to and penetrate the mucous layer in order to bind host cells.

Cytolethal distending toxin (CDT) activity of *C. upsaliensis* was demonstrated in both epithelial cells and T lymphocytes (Mooney et al. 2001). CDT-exposed epithelial cells underwent apoptosis, presumably due to cell cycle arrest. In addition, a *cdtB* homolog, the active subunit of CDT, is encoded by *C. upsaliensis* (Pickett et al. 1996).

Campylobacter hyointestinalis is differentiated from other *Campylobacter* spp. by differences in pulsed-field gel electrophoresis (PFGE) patterns (Salama et al. 1992) and in the sequence of the 16S rRNA gene (Wesley et al. 1991). The organism is susceptible to cephalothin but resistant to nalidixic acid. It is also an H_2S producer when incubated in the presence of hydrogen.

Although *C. hyointestinalis* has been isolated from pigs with enteritis (Gebhart et al. 1985), little is known about its pathogenesis. In one study, greater than two-thirds of porcine-derived isolates produced a detectable cytotoxin (Ohya and Nakazawa 1992). PCR amplification with degenerate primers revealed *cdtB* in *C. hyointestinalis* (Picket et al. 1996), but a nucleotide probe consisting of the *C. jejuni cdtB* gene did not hybridize to *C. hyointestinalis* DNA (Picket et al. 1996).

C. hyointestinalis has been isolated from hamsters, cattle, and monkeys (*Macaca nemestrina*) (Walder et al. 1983; Russell et al. 1992). Similarities in PFGE patterns suggest that a strain causing diarrhea and vomiting in a human originated in a pig (Gorkiewicz et al. 2002).

Campylobacter mucosalis was identified by monospecific immunofluorescence staining in 80% of frozen ileal sections from pigs with proliferative enteritis (Chang et al. 1984); monospecific antisera against *C. hyointestinalis* bound to antigens in 100% of these sections. Large numbers of *C. mucosalis* can be isolated from cases of porcine intestinal adenomatosis (pseudomembranous blockage of the colon) (Lawson and Rowland, 1984).

C. mucosalis requires hydrogen for growth *in vitro* and is catalase negative. Restriction fragment length polymorphisms among *C. mucosalis* isolates suggest the occurrence of strain-to-strain variation (Lin et al. 1991). Amplified fragment length polymorphism (AFLP) fingerprinting discriminates *C. mucosalis* from other *Campylobacter* species (Duim et al. 2001).

HELICOBACTER

Helicobacter spp. infections have been documented in mammals and birds, and mainly infect the stomach, intestine, liver, and gallbladder. Gastric ulcers in food animals (especially in swine) have been linked to the presence of *Helicobacter* spp., but the exact role of the bacterium in lesion development is unclear. Most problems linked to this bacterial genus have been associated with mouse hepatitis.

Helicobacter hepaticus and *Helicobacter bilis*

Helicobacter hepaticus has been clearly documented as a mouse pathogen, and infections by this organism and *H. bilis* are common in research colonies and commercial breeding facilities. Initial lesions of *H. hepaticus* infection consist of focal hepatic necrosis and focal, subacute, nonsuppurative hepatic inflammation. Lesions progress to involve more lobes of the liver and become chronic, with bile duct hyperplasia. Eventually, the lesions become preneoplastic hepatocellular foci and hepatocellular tumors (Ward et al. 1994; Fox et al. 1996).

Helicobacter bilis colonizes liver, gallbladder, and intestine. It can be isolated readily from bile and has been associated with multifocal hepatitis. It does not seem to be involved in hyperplastic hepatitis (Fox et al. 1995).

HELICOBACTER PYLORI

Helicobacter pylori infections cause chronic inflammation of the stomach in humans, leading to chronic atrophic gastritis, intestinal metaplasia, and gastric cancer. *H. pylori* must attach to gastric epithelial cells to remain resident, and attachment and persistence are accomplished through the expression of an antigen-binding adhesin (Rad et al. 2002; Mizushima et al. 2001), phospholipase A, flagellins (Ottemann and Lowenthal 2002), and urease (Eaton et al. 2002). Damage to the gastric epithelium is associated with delivery of virulence factors by a type IV secretion system. One of those factors, VacA cytotoxin, acts as a functional autotransporter (Fisher et al. 2001) to ensure the availability of urea for *H. pylori* utilization. VacA cytotoxin also has a role in induction of apoptosis (Kuck et al. 2001), involving mitochondrial targeting and cytochrome c release in AG8 cells. The toxin may also have a role in inducing inflammation. Supajatura et al. (2002) reported that binding of VacA to mast cells *in vitro* resulted in release of tissue-damaging cytokines.

The type IV secretion machinery of *H. pylori* is also used for delivery of CagA proteins to host cells. CagA becomes tyrosine phosphorylated and induces changes in the tyrosine phosphorylation state of host-cell proteins. These changes cause abnormal growth of gastric epithelial cells and promote the development of gastric cancer (Ferber 2001).

REFERENCES

Albert, M. J., W. Tee, A. Leach, V. Asche, and J. L. Penner. 1992. Comparison of a blood-free medium and a filtration technique for the isolation of *Campylobacter* spp. from diarrhoeal stools of hospitalised patients in central Australia. J. Med. Microbiol. 37:176–179.

Babakhani, F. K., G. A. Bradley, and L. A. Joens. 1993. Newborn piglet model for campylobacteriosis. Infect. Immun. 61:3466–75.

Banfi, E., M. Cinco, and G. Zabucchi. 1986. Phagocytosis of *Campylobacter jejuni* and *C. coli* by peritoneal macrophages. J. Gen. Microbiol. 132:2409–12.

Blaser, M. J., and E. C. Gotschlich. 1990. Surface array protein of *Campylobacter fetus*. Cloning and gene structure. J. Biol. Chem. 265:14529–35.

Blaser, M. J., and Z. Pei. 1993. Pathogenesis of *Campylobacter fetus* infections: critical role of high-molecular-weight S-layer proteins in virulence. J. Infect. Dis. 167:372–377.

Blaser, M. J., P. F. Smith, J. E. Repine, and K. A. Joiner. 1988. Pathogenesis of *Campylobacter fetus* infections. Failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. J. Clin. Invest. 81:1434–1444.

Bruce, D., W. Zochowski, and G. A. Fleming. 1980. *Campylobacter* infections in cats and dogs. Vet. Rec. 107:200–201.

Chang, K., H. J. Kurtz, G. E. Ward, and C. J. Gebhart. 1984. Immunofluorescent demonstration of *Campylobacter hyointestinalis* and *Campylobacter sputorum* subsp *mucosalis* in swine intestines with lesions of proliferative enteritis. Am. J. Vet. Res. 45:703–710.

Corbeil, L. B. 1999. Immunization and diagnosis in bovine reproductive tract infections. Adv. Vet. Med. 41:217–239.

Day, W. A., Jr., J. L. Sajecki, T. M. Pitts, and L. A. Joens. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. Infect. Immun. 68:6337–6345.

Diker, K.S., M. Sahal, and N. Aydin. 1988. Ovine abortion associated with *Campylobacter coli*. Vet. Rec. 122:87.

Doyle, M.P., and D.J. Roman. 1982. Prevalence and survival of *Campylobacter jejuni* in unpasteurized milk. Appl. Environ. Microbiol. 44:1154–1158.

Duim, B., P. A. Vandamme, A. Rigter, S. Laevens, J. R. Dijkstra, and J. A. Wagenaar. 2001. Differentiation of *Campylobacter* species by AFLP fingerprinting. Microbiology 147:2729–37.

Dworkin, J., and M. J. Blaser. 1997a. Molecular mechanisms of *Campylobacter fetus* surface layer protein expression. Mol. Microbiol. 26:433–440.

———. 1997b. Nested DNA inversion as a paradigm of programmed gene rearrangement. Proc. Natl. Acad. Sci. USA 94:985–990.

Eaton, K. A., J. V. Gilbert, and E. A. Joyce. 2002. In vivo complementation of ureB restores the ability of *Helicobacter pylori* to colonize. Infect. Immun. 70:771–778.

Everest, P. H., H. Goossens, J. P. Butzler, D. Lloyd, S. Knutton, J. M. Ketley, and P. H. Williams. 1992. Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. J. Med. Microbiol. 37:319–325.

Ferber, D. 2001. Carcinogenic bacteria. Cracking gut bugs' cell-skewing strategy. Science 294:2269.

Fisher, W., R. Buhrdorf, E. Gerland, and R. Haas. 2001. Outer membrane targeting of passenger proteins by the vacuolating cytotoxin autotransporter of *Helicobacter pylori*. Infect. Immun. 69:6769–6775.

Fleming, M. P. 1983. Association of *Campylobacter jejuni* with enteritis in dogs and cats. Vet. Rec. 113:372–374.

Fox, J. G., L. L. Yan, F. E. Dewhirst, B. J. Paster, B. Shanes, J. C. Murphy, A. Hayward, J. C. Belcher, and E. N. Mendes. 1995. *Helicobacter bilis* sp. Nov., a novel *Helicobacter* species isolated from bile, livers, and intestines of aged, inbred mice. J. Clin. Microbiol. 33:445–54.

Fox, J. G., X. Li, L. Yan, R. J. Cahill, R. Hurley, R. Lewis, J. C. Murphy. 1996. Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: a model of *Helicobacter*-induced carcinogenesis. Infect. Immun. 64:1548–1558.

Garcia, M. M., and B. W. Brooks. 1993. *Campylobacter*. In Pathogenesis of bacterial infections in animals, 2nd ed. C. L. Gyles and C. O. Thoen (eds.). Ames: Iowa State University Press, pp. 262–272.

Garcia, M. M., C. L. Lutze-Wallace, A. S. Denes, M. D. Eaglesome, E. Holst, and M. J. Blaser. 1995. Protein shift and antigenic variation in the S-layer of *Campylobacter fetus* subsp. *venerealis* during bovine infection accompanied by genomic rearrangement of *sapA* homologs. J. Bacteriol. 177:1976–1980.

Gebhart, C. J., P. Edmonds, G. E. Ward, H. J. Kurtz, and D. J. Brenner. 1985. "*Campylobacter hyointestinalis*" sp. nov.: a new species of *Campylobacter* found in the intestines of pigs and other animals. J. Clin. Microbiol. 21:715–20.

Goossens, H., B. Pot, L. Vlaes, C. Van den Borre, R. Van den Abbeele, C. Van Naelten, J. Levy, H. Cogniau, P. Marbehant, J. Verhoef, and et al. 1990. Characterization and description of "*Campylobacter upsaliensis*" isolated from human feces. J. Clin. Microbiol. 28:1039–1046.

Gorkiewicz, G., G. Feierl, R. Zechner, and E. L. Zechner. 2002. Transmission of *Campylobacter hyointestinalis* from a pig to a human. J. Clin. Microbiol. 40:2601–2605.

Graham, L. L., and K. L. MacDonald. 1998. The *Campylobacter fetus* S layer is not essential for initial interactions with HEp-2 cells. Can. J. Microbiol. 44:244–250.

Humphrey, T. J., and P. Beckett. 1987. *Campylobacter jejuni* in dairy cows and raw milk. Epidemiol. Infect. 98:263–269.

Humphrey, T. J., and R. J. C. Hart. 1988. *Campylobacter* and *Salmonella* contamination of unpasteurized cow's milk on sale to the public. J. Appl. Bacteriol. 65: 463–467.

Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman, and V. L. Chan. 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol. Microbiol. 39:1225–1236.

Joens, L. A. 2002. Unpublished data.

Kervella, M., J. M. Pagès, Z. Pei, G. Grollier, M.J. Blaser, and J.L. Fauchère. 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect. Immun. 61:3440–3448.

Kiehlbauch, J. A., R. A. Albach, L. L.Baum, and K. P. Chang. 1985. Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. Infect. Immun. 48:446–451.

Konkel, M. E., and L. A. Joens. 1989. Adhesion to and invasion of Hep-2 cells by *Campylobacter* spp. Infect. Immun. 57:2984–2990.

Konkel, M. E., and P. F. Mixter. 2000. Flow cytometric detection of host cell apoptosis induced by bacterial infection. Methods Cell Sci. 22:209–215.

Konkel, M. E., and W. Cieplak, Jr. 1992. Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. Infect. Immun. 60:4945–4949.

Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis. 1999. Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. Mol. Microbiol. 32:691–701.

Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr. 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol. Microbiol. 24:953–63.

Kuck, D., B. Kolmerer, C. Iking-Konert, P. H. Krammer, W. Stremmel, J. Rudi. 2001. Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AG8. Infect. Immun. 69:5080–5087.

Lander, K. P., and K. P. W. Gill. 1980. Experimental infection of the bovine udder with *Campylobacter coli/jejuni*. J. Hyg. 84:421–428.

Lastovica, A. 1996. *Campylobacter/Helicobacter* bacteraemia in Cape Town, South Africa, 1977–1995. In *Campylobacters, Helicobacters,* and related organisms, edited by D. G. Newell, J. M. Ketley, and R. A. Feldman, p.p. 475–479. New York: Plenum Press.

Lawson, G. H. K., and A. C. Rowland. 1984. *Campylobacter sputorum* subsp. *mucosalis*. In *Campylobacter* infections in man and animals. J. P. Butzler (eds.). Boca Raton: CRC Press, Inc., pp. 207–225.

Lin, G. F., C. J. Gebhart, and M. P. Murtaugh. 1991. Southern blot analysis of strain variation in *Campylobacter mucosalis*. Vet. Microbiol. 26:279–89.

Macartney, L., R. R. Al-Mashat, D. J. Taylor, and I. A. McCandlish. 1988. Experimental infection of dogs with *Campylobacter jejuni*. Vet. Rec. 122:245–249.

McCoy, E. C., D. Doyle, L. B. Burda, L. B. Corbeil, and A. J. Winter. 1975. Superficial antigens of *Campylobacter (Vibrio) fetus*: characterization of an antiphagocytic component. Infect. Immun. 11:517–525.

McFaydean, J., and S. Stockman. 1913. *Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizootic Abortion*. Appendix to part III, p.p. 1–29, *Abortion in Sheep*. His Majesty's Stationary Office, London, United Kingdom.

Mizushima, T., T. Sugiyama, Y. Komatsu, J. Ishizuka, M. Kato, M. Asaka. 2001. Clinical relevance of the babA2 genotype of *Helicobacter pylori*. J. Clin. Microbiol. 39:2463–2465.

Mooney, A., M. Clyne, T. Curran, D. Doherty, B. Kilmartin, and B. Bourke. 2001. *Campylobacter upsaliensis* exerts a cytolethal distending toxin effect on HeLa cells and T lymphocytes. Microbiology 147:735–43.

Moreno, G. S., P. L. Griffiths, I. F. Connerton, and R. W. Park. 1993. Occurrence of campylobacters in small domestic and laboratory animals. J. Appl. Bacteriol. 75:49–54.

Morgan, G.P., Chadwick, K.P., Lander, and K.P. W. Gill. 1985. *Campylobacter jejuni* mastitis in a cow: a zoonosis-related incident. Vet. Rec. 116:111.

Myszewski, M. A., and N. J. Stern. 1991. Phagocytosis and intracellular killing of *Campylobacter jejuni* by elicited chicken peritoneal macrophages. Avian Dis. 35:750–5.

Nair, G. B., R. K. Sarkar, S. Chowdhury, and S. C. Pal. 1985. *Campylobacter* infection in domestic dogs. Vet. Rec. 116:237–238.

Newell, D. G., and A. Pearson. 1984. The invasion of epithelial cell lines and the intestinal epithelium of infant mice by *Campylobacter jejuni*. J. Diarrhoeal Dis. Res. 2:19–26.

Newell, D. G., H. Mc Bride, F. Saunders, Y. Dehele, and A. D. Pearson. 1985. The virulence of clinical and environmental isolates of *Campylobacter jejuni*. J. Hyg. Camb. 94:45–54.

Ohya, T., and M. Nakazawa. 1992. Production and some properties of cytotoxins produced by *Campylobacter* species isolated from proliferative enteropathy in swine. J. Vet. Med. Sci. 54:1031–3.

Ottemann, K. M., and A. C. Lowenthal 2002. *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. Infect. Immun. 70:1984–90.

Oyarzabal, O. A., I. V. Wesley, K. M. Harmon, L. Schroeder-Tucker, J. M. Barbaree, L. H. Lauerman, S. Backert, and D. E. Conner. 1997. Specific identification of *Campylobacter fetus* by PCR targeting variable regions of the 16S rRNA. Vet. Microbiol. 58:61–71.

Pei, Z., and M. J. Blaser. 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J. Biol. Chem. 268:18717–18725.

Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin. 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. cdtB gene. Infect. Immun. 64:2070–2078.

Pitts, T. 2001. Intramacrophage survival of *Campylobacter jejuni* and maturation of *Campylobacter jejuni* phagosomes. MS. University of Arizona.

Rad, R., et al. 2002. The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. J. Immunol. 168:3033–3041.

Ruiz-Palacios, G. M., E. Escamilla, and N. Torres. 1981. Experimental *Campylobacter* diarrhea in chickens. Infect. Immun. 34:250–255.

Russell, R. G., J. A. Kiehlbauch, C. J. Gebhart, and L. J. DeTolla. 1992. Uncommon *Campylobacter* species in infant *Macaca nemestrina* monkeys housed in a nursery. J. Clin. Microbiol. 30:3024–3027.

Russell, R. G., M. J. Blaser, J. I. Sarmiento, and J. Fox. 1989. Experimental *Campylobacter jejuni* infection in *Macaca nemestrina*. Infect. Immun. 57:1438–44.

Salama, S. M., H. Tabor, M. Richter, and D. E. Taylor. 1992. Pulsed-field gel electrophoresis for epidemiological studies of *Campylobacter hyointestinalis* isolates. J. Clin. Microbiol. 30:1982–1984.

Sandstedt, K., J. Ursing, and M. K. Walder. 1983. Thermotolerant campylobacter with no or weak catalase activity isolated from dogs. Curr. Microbiol. 8:209–213.

Skirrow, M. B. 1981. *Campylobacter* enteritis in dogs and cats: a "new" zoonosis. Vet. Res. Commun. 5:13–19.

———. 1994. Diseases due to *Campylobacter*, *Helicobacter*, and related bacteria. J. Comp. Pathol. 111:113–149.

Smibert, R. M. 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. 32:673–709.

Supajatura, V., et al. 2002. Cutting edge. VacA, a vacuolating cytotoxin of *Helicobacter pylori*, directly activates mast cells for migration and production of proinflammatory cytokines. J. Immunol. 168:2603–2607.

Sylvester, F. A., D. Philpott, B. Gold, A. Lastovica, and J. F. Forstner. 1996. Adherence to lipids and intestinal mucin by a recently recognized human pathogen, *Campylobacter upsaliensis*. Infect. Immun. 64:4060–4066.

Szymanski, C. M., M. King, M. Haardt, and G. D. Armstrong, 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect. Immun. 63:4295–4300.

Thompson, S. A., and M. J. Blaser. 2000. Pathogenesis of *Campylobacter fetus* infections. In Campylobacter, 2nd ed. I. Nachamkin and M. J. Blaser (eds.). Washington, DC: American Society for Microbiology, pp. 321–347.

van Spreeuwel, J. P., G. C. Duursma, C. J. Meijer, R. Bax, P. C. Rosekrans, and J. Lindeman. 1985. *Campylobacter colitis*: histological immunohistochemical and ultrastructural findings. Gut. 26:945–951.

Walder, M. K., K. Sandstedt, and J. Ursing. 1983. Phenotypic characteristics of thermotolerant *Campylobacter* from human and animal sources. Curr. Microbiol. 9:291–296.

Ward, J. M., J. G. Fox, M. R. Anver, D. C. Haines, C. V. George, M. J. Collins, P. L. Gorelick, K. Nagashima, M. A. Gonda, R. V. Gilden, J. G. Tully, R. J. Russell, R. E. Benveniste, B. J. Paster, F. E. Dewhirst, J. C. Donovan, L. M. Anderson, and J. M. Rice. 1994. Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. J. Natl. Cancer Inst. 86:1222–1227

Wassenaar, T., M. Engelskirchen, S. Park, and A. Lastovica. 1997. Differential uptake and killing potential of *Campylobacter jejuni* by human peripheral monocytes/macrophages. Med. Microbiol. Immunol. 186:139–144.

Wesley, I. V., R. D. Wesley, M. Cardella, F. E. Dewhirst, and B. J. Paster. 1991. Oligodeoxynucleotide probes for *Campylobacter fetus* and *Campylobacter hyointestinalis* based on 16S rRNA sequences. J. Clin. Microbiol. 29:1812–1817.

26
Lawsonia intracellularis

C. J. Gebhart and R. M. C. Guedes

Lawsonia intracellularis is the sole species in the newly formed genus *Lawsonia*. It is the etiologic agent of proliferative enteropathy, an infectious, intestinal hyperplastic disease characterized by thickening of the mucosa of the distal small intestine and proximal colon due to enterocyte proliferation (McOrist and Gebhart 1999) (fig. 26.1A). *Lawsonia intracellularis* is an obligately intracellular, vibroid-shaped bacterium, which is found in the apical cytoplasm of infected enterocytes (fig. 26.1B).

Proliferative enteropathy has been reported in several animal species (Lemarchand et al. 1997; Cooper and Gebhart 1998; Klein et al. 1999; Cooper et al. 1997a) but has been best described in pigs and hamsters. 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 (McOrist and Gebhart 1999; Lawson and Gebhart 2000). 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).

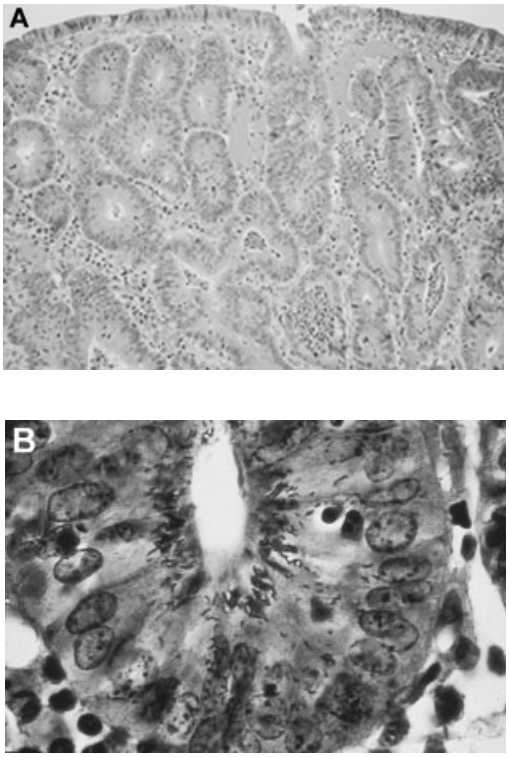


Figure 26.1. Ileal section of a pig experimentally infected with *L. intracellularis*. (A) H & E stain of hyperplastic crypts; (B) Silver stain showing numerous vibrio-shaped bacteria in the apical cytoplasm of the proliferating epithelial cells.

Although the disease in pigs was first reported in 1931 (Biester and Schwarte 1931), it was not until the early 1970s that G. H. K. 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 (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 (Rowland et al. 1973). Roberts et al. (1977) demonstrated that lesion material contained the infectious agent by experimentally infecting pigs with homogenates of affected intestine. It was not until the 1990s that this intracellular bacterium was isolated from hamsters (Stills 1991) and pigs (Lawson et al. 1993) and cultivated *in vitro*. The disease was reproduced with pure cultures (McOrist et al. 1993). 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 *L. INTRACELLULARIS*

Lawsonia intracellularis is a gram-negative, curved or vibroid-shaped rod, which is 1.25–1.75 mm in length and 0.25–0.43 mm 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, an atmosphere of 82.2% nitrogen, 8.8% carbon dioxide, and 8% oxygen, and a temperature of 37°C (Lawson et al. 1993). Others, using similar techniques, cultivated the organism from diseased pigs in Australia (Collins et al. 1996) and the United States (Knittel et al. 1996; Joens et al. 1997; Guedes and Gebhart 2003a) and from a foal in the United States (Cooper 1996).

Since it is not possible to cultivate *L. intracellularis* in conventional bacteriological media, 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 four 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 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 the Proteobacteria (Gebhart et al. 1993) and is taxonomically distinct from other intracellular pathogens (McOrist et al. 1995a; Dale et al. 1998). This intracellular bacterium, previously known as a

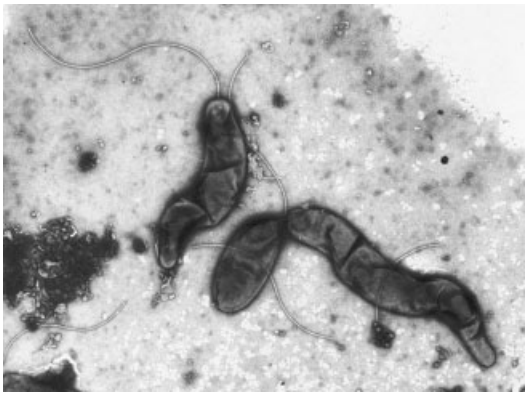


Figure 26.2. *L. intracellularis* cells with a single polar flagellum shown in transmission electron microscopy photograph.

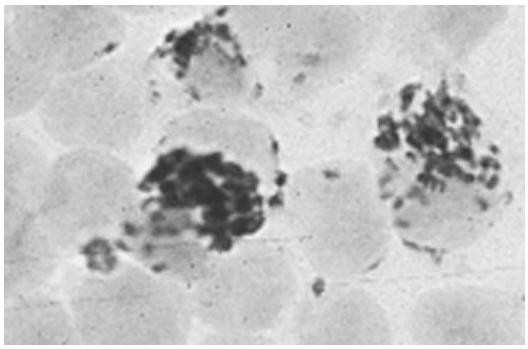


Figure 26.3. Cell culture of intestinal 407 cells infected with *L. intracellularis*. Only a few cells are heavily infected. Immunoperoxidase stain using *L. intracellularis*-specific monoclonal antibody.

Campylobacter-like organism, *Ileal symbiont intracellularis*, and *Ileobacter intracellularis,* was established in a new genus as *Lawsonia intracellularis* (McOrist et al. 1995a). Isolates of intracellular bacteria from lesions of a variety of animal species have >98% 16S rDNA similarity to the pig isolates (Peace et al. 1994; Cooper 1996; Cooper et al. 1997b**;** Cooper and Gebhart 1998). Phenotypic characterization, by immunoblotting of outer-membrane proteins, and more sensitive molecular characterization of the *L. intracellularis* genome demonstrate only minor differences among isolates (AlGhamdi 2003).

SOURCES OF *L. 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 (Rowland and Lawson 1992; Cooper and Gebhart 1998; Frank et al. 1998). *L. intracellularis* infection has been established experimentally in mice deficient in interferon-gamma receptors (Smith et al. 2000) and occurs spontaneously in conventional mice (Abshier et al. 2001). There are no reports of proliferative enteropathy in human beings, but recent reports of the disease in nonhuman primates suggest that human cases will 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. Species-to-species transmission has been documented. Pig isolates infect hamsters (McOrist and Lawson 1987), mice (Smith et al. 2000), and horses (AlGhamdi 2003), and a horse isolate infects hamsters (Cooper 1996).

Feces from infected pigs may be the source of new infections for susceptible animals (McOrist and Gebhart 1999), 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 reisolation of viable organisms from diseased intestines is very difficult, due to the difficulty in extraction, growth, and maintenance of *L. intracellularis in vitro*. Therefore, information about 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 culture-positive animals, which had been stored for up to 2 weeks at 5°C or 15°C (Collins et al. 2000). Pure cultures were fully susceptible to a quaternary ammonium disinfectant (3% cetrimide), less so to 1% povidone-iodine, and not at all to 1% potassium peroxymonosulfate or a 0.33% phenolic mixture.

VIRULENCE FACTORS

The virulence factors of *L. intracellularis* are not as yet well characterized. Its major pathogenic mechanism is infection of and induction of hyperplasia in enterocytes (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* (McCluskey et al. 2002).

Most studies on 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. *In vivo*, proliferating enterocytes show poor major histocompatibility complex class II expression. This loss of antigen-presenting function may provide an immunologically safe environment for the growth of *L. intracellularis* (McOrist et al. 1992, 1995a). Temporary reduction of apoptosis induced by *L. intracellularis* infection might be one

of the mechanisms 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, frequency of apoptotic events in affected and unaffected cases were not compared. Machuca et al. (1999) showed 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 of apoptosis.

Little is known about the genetic basis for 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 remains undetermined. A pilot study determined, in part, the genomic sequence of *L. intracellularis* (Gebhart et al. 2002). This random sequencing approach identified several sequences in *L. intracellularis* that are of interest from a virulence standpoint. Sequences homologous to genes encoding proteins involved in flagellar biosynthesis and assembly were identified. 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 Yop (*Yersinia* outer protein) (Donnenberg 2000) and of LvrV (Bleves and Cornelius 2000), suggesting that *L. intracellularis* is likely to contain a type III secretion system.

PATHOGENESIS

Overview

Comprehensive studies of 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 enterocyte hyperplasia is directly preceded by the presence of the intracellular organism (Jacoby 1978; Johnson and Jacoby 1978; Guedes 2002). *In vivo*, the onset of hyperplasia associated with proliferative enteropathy follows an increase in numbers of intracellular *L. intracellularis* in enterocytes. Likewise, resolution of lesions is closely related to 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 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 (Guedes 2002). An ultrastructural study (Johnson and Jacoby 1978) using hamsters inoculated with homogenate 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 epithelial 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 mucosal 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, b). 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 revealed that *L. intracellularis* in 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's 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 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 (Guedes 2002; Guedes and Gebhart 2003a, b). 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. Conversely, the organism was detected by PCR in one challenged pig on day 35, suggesting that nondetection by immunohistochemistry may be a false negative. *L. intracellularis* antigen was not detected in any organ other than intestine, lymph node, and tonsil. 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. Tonsil does 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 mechanisms involved in 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 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 cell surfaces of cultured (IEC-18) and pig enterocytes (IPEC-J2) was observed 10 minutes after infection. Bacteria were internalized after 3 hours 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 to 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 balloonlike, cytoplasmic protrusions, replete with bacteria that are then released from the cell (McOrist et al. 1995a).

Influence of Environmental Factors

Intestinal flora may modify the ability of *L. intracellularis* to 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), but pigs on widely different diets may be infected (McOrist et al. 1993).

Lesions

Macroscopic lesions of proliferative enteropathy are usually located in the terminal ileum, 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

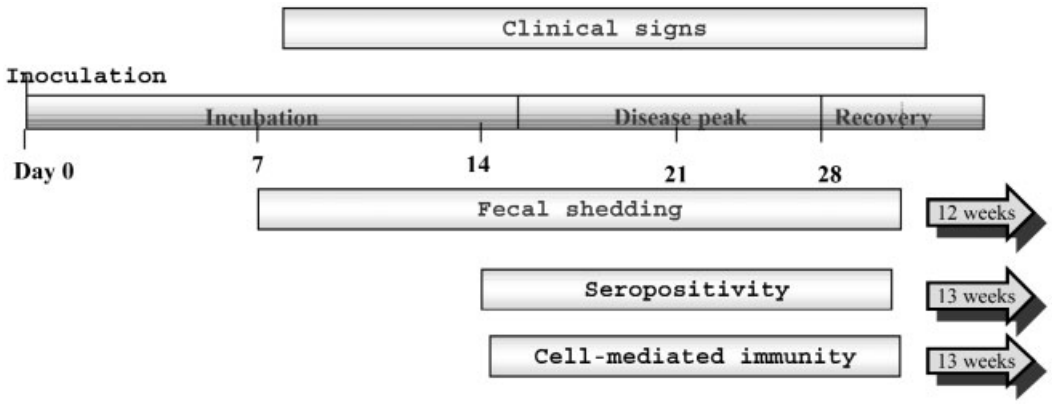


Figure 26.4. Course of proliferative enteropathy in experimentally infected pigs. The onset and duration of clinical signs, fecal shedding, and systemic humoral and cell-mediated immune responses are shown after inoculation of pigs on day 0 with cell-cultured *L. intracellularis*.

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; Jubb et al. 1993). 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 1999). 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 organism 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), and immunoperoxidase stains (McOrist et al. 1987; Jensen et al. 1997). The organism can also be detected by hybridization with DNA probes (Gebhart et al. 1991, 1994).

Immune Response

IgG can first be 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 et al. 2001). Animals challenged a second time, after cessation of fecal shedding, were evaluated clinically and their feces were tested by PCR to detect shedding. Animals previously infected did not shed detectable numbers 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 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 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 infection 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 of 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 large numbers 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.

REFERENCES

Abshier, J. M., C. L. Besh-Williford, C. L. Franklin, and S. P. Russell. 2001. Spontaneous infection of *Lawsonia intracellularis*-like bacteria in the mouse. In *Proceedings of the 82nd Annual Meeting, Conf. Res. Workers Anim. Dis.*, St. Louis, MO.

AlGhamdi, G. 2003. Characterization of proliferative enteropathy in horses. Ph.D. Thesis. University of Minnesota, St. Paul.

Biester, H. E., and L. H. Schwarte. 1931. Intestinal adenoma in swine. Am. J. Pathol. 7:175–185.

Bleves, S., and G. R. Cornelis. 2000. How to survive in the host: the *Yersinia* lesson. Microbes and Infect. 2:1451–1460.

Collins, A. M., I. Swift, and R. P. Monkton. 1996. Replication of Australian porcine isolates of *Ileal symbiont intracellularis* in tissue culture. Vet. Microbiol. 49:249–255.

Collins, A. M., M. V. Dijk, N. Q. Vu, J. Pozo, and R. J. Love. 2001. Immunity to *Lawsonia intracellularis*. In Proceedings of the 28th Allen D. Leman Conference, pp. 115–120.

Collins, A. M., R. J. Love, J. Pozo, S. H. Smith, and S. McOrist. 2000. Studies on the ex vivo survival of *Lawsonia intracellularis*. Swine Health and Production. 8:211–215.

Cooper, D. M. 1996. Proliferative enteritis in the hamster, horse, deer and ostrich: detection and characterization of *Lawsonia intracellularis*. MS thesis, University of Minnesota, St. Paul.

Cooper, D. M., and C. J. Gebhart. 1998. Comparative aspects of proliferative enteritis. J. Am. Vet. Med. Assoc. 212:1446–1451.

Cooper, D. M., D. L. Swanson, and C. J. Gebhart. 1997a. Diagnosis of proliferative enteritis in frozen and formalin fixed, paraffin-embedded tissues from a hamster, horse, deer and ostrich using a *Lawsonia intracellularis*-specific multiplex PCR assay. Vet. Microbiol. 54:47–62.

Cooper, D. M., D. L. Swanson, S. M. Barns, and C. J. Gebhart. 1997b. Comparison of the 16S ribosomal DNA sequence from the intracellular agent of proliferative enteritis in a hamster, deer, and ostrich with the sequence of a porcine isolate of *Lawsonia intracellularis*. Int. J. Syst. Bacteriol. 47:635–639.

Dale, C. J. H., E. K. Moses, C.-C. Ong, C. J. Morrow, M. B. Reed, D. Hasse, and R. A. Strugnell. 1998. Identification and sequencing of the groE operon and flanking genes of *Lawsonia intracellularis*: use in phylogeny. Microbiol. 144:2073–2084.

Donnenberg, M. S. 2000. Pathogenic strategies of enteric bacteria. Nature 406:744–768.

Frank, N., C. E., Fishman, C. J. Gebhart, and M. Levy. 1998. *Lawsonia intracellularis* proliferative enteropathy in a weanling foal. Equine Vet. J. 30:549–552.

Frisk, C. 1976. The etiology and pathogenesis of hamster enteritis. Ph. D. thesis, University of Missouri.

Gebhart, C. J., G. F. Lin, S. McOrist, G. H. K. Lawson, and M. P. Murtaugh. 1991. Cloned DNA probes specific for the intracellular *Campylobacter*-like organism of porcine proliferative enteritis. J. Clin. Microbiol. 29:1011–1015.

Gebhart, C. J., L. L. Li, Q. Zhang, R. M. C. Guedes, L. L. Heron, and V. Kapur. 2002. A genomic sequence survey of *Lawsonia intracellularis* identifies numerous genes of relevance to diagnosis, virulence, and immunoprophylaxis. In Proceedings of the 83rd Annual Meeting, Conf. Res. Workers Anim. Dis., St. Louis, MO.

Gebhart, C. J., S. M. Barns, S. McOrist, G. F. Lin, and G. H. K. Lawson. 1993. *Ileal symbiont intracellularis*, an obligate bacterium of porcine intestine showing a relationship to *Desulfuvibrio* species. Int. J. Syst. Bacteriol. 43:533–538.

Gebhart, C. J., S. McOrist, G. H. K. Lawson, J. E. Collins, and G. E. Ward. 1994. Specific in situ hybridization of the intracellular organism of porcine proliferative enteropathy. Vet. Path. 31:462–467.

Guedes, R. M. C. 2002. Porcine proliferative enteropathy: diagnosis, immune response and pathogenesis. Ph.D. Thesis. University of Minnesota, pp. 213–242.

Guedes, R. M. C., and C. J. Gebhart. 2003a. Onset and duration of fecal shedding, cell-mediated and humoral immune responses in pigs after challenge with a pathogenic isolate or attenuated vaccine strain of *Lawsonia intracellularis*. Vet. Microbiol. 91:135–145.

———. 2003b. Comparison of intestinal mucosa homogenate and pure culture of the homologous *Lawsonia intracellularis* isolate in reproducing proliferative enteropathy in swine. Vet. Microbiol. 93:159–166.

Hannigan, J. 1997. Identification and preliminary characterization of *Lawsonia intracellularis* cytolytic activity. M.Sc. Thesis. University of Edinburgh, Edinburgh.

Jacoby, R. O. 1978. Transmissible ileal hyperplasia of hamsters. Am. J. Pathol. 91:433–444.

Jacoby, R. O., and E. A. Johnson. 1981. Transmissible ileal hyperplasia. In Hamster immune responses in infection and oncologic diseases. J. Stein-Streilein, W. Kuncan, and R. Billingham. New York: Plenum, pp. 267–285.

Jacoby, R. O., G. W. Osbaldiston, and A. W. Jonas. 1975. Experimental transmission of atypical ileal hyperplasia of hamsters. Lab. Anim. Sci. 25:465–473.

Jasni, S., S. McOrist, and G. H. K. Lawson. 1994. Reproduction of proliferative enteritis in hamsters with a pure culture of porcine *Ileal symbiont intracellularis*. Vet. Microbiol. 41:1–9.

Jensen, T. K., K. Moller, T. D. Leser, and S. E. Jorsal. 1997. Comparison of histology, immunohistochemistry and polymerase chain reaction for detection of *Lawsonia intracellularis* in natural porcine proliferative enteropathy. Eur. J. Vet. Pathol. 3:115–123.

Joens, L. A., S. Nibbelink, and R. D. Glock. 1997. Induction of gross and microscopic lesions of porcine proliferative enteritis by *Lawsonia intracellularis*. Am. J. Vet. Res. 58:1125–1131.

Johnson, E. A., and R. O. Jacoby. 1978. Transmissible ileal hyperplasia of hamster. Am. J. Pathol. 91:451–459.

Jubb, K., P. Kennedy, and N. Palmer. 1993. Infectious and parasitic diseases of the gastrointestinal tract. In Pathology of domestic animals. K. Jubb, P. Kennedy, and N. Palmer. San Diego: Academic Press, pp. 229–233.

Klein, E. C., C. J. Gebhart, and G. E. Duhamel. 1999. Fatal outbreaks of proliferative enteritis caused by *Lawsonia intracellularis* in young colony-raised rhesus macaques. J. Med. Primatol. 28:11–18.

Knittel, J. P., D. I. Larson, D. L. Harris, M. B. Roof, and S. McOrist. 1996. United States isolates of *Lawsonia intracellularis* from porcine proliferative enteropathy resemble European isolates. Swine Health and Production. 4:119–122.

Knittel, J. P., D. M. Jordan, K. J. Schwartz, B. H. Janke, M. B. Roof, S. McOrist, and D. L. Harris. 1998. Evaluation of *antemortem* polymerase chain reaction and serologic methods for detection of *Lawsonia intracellularis* exposed pigs. Am. J. Vet. Res. 59:722–726.

Lamm, M. E., J. G. Nedrud, C. S. Kaetzel, and M. B. Mazane. 1995. IgA and mucosal defense. APMIS. 103:241–246.

Lawson, G. H. K., A. C. Rowland, L. Roberts, G. Fraser, and E. McCartney. 1979. Proliferative haemorrhagic enteropathy. Res. Vet. Sci. 27:46–51.

Lawson, G. H. K., and C. J. Gebhart. 2000. Proliferative enteropathy: review. J. Comp. Path. 122:77–100.

Lawson, G. H. K., R. A. Mackie, D. G. E. Smith, and S. McOrist. 1995. Infection of cultured rat enterocytes by Ileal symbiont intracellularis depends on host cell function and actin polymerization. Vet. Microbiol. 45:339–350.

Lawson, G. H. K., S. McOrist, S. Jasni, and R. A. Mackie. 1993. Intracellular bacteria of porcine proliferative enteropathy: cultivation and maintenance *in vitro*. J. Clin. Microbiol. 31:1136–1142.

Lemarchand, T. X., T. N. Tully, S. M. Shane, and D. E. Duncan. 1997. Intracellular *Campylobacter*-like organisms associated with rectal prolapse and proliferative enteroproctitis in emus (*Dromaius novaehollandiae*). Vet. Pathol. 34:152–156.

Lomax, L. G., and R. D. Glock. 1982. Naturally occurring porcine proliferative enteritis: pathologic and bacteriologic findings. Am. J. Vet. Res. 43:1608–1621.

Love, D. N., and R. J. Love. 1979. Pathology of proliferative haemorrhagic enteropathy in pigs. Vet. Pathol. 16:41–48.

Machuca, M. A., M. A. Quiroga, J. Riganti, A. R. Massone, J. R. Idiart, A. D. Armocida, and C. J. Perfumo. 1999. Porcine proliferative enteropathy: Immune labeling of proliferation cell nuclear antigen (PCNA) and apoptosis. In Proceedings of the 9th Encontro Nacional de Patologia Veterinaria. Veterinary School, UFMG, Belo Horizonte, MG-Brazil: FEP-MVZ/UFMG Editora, Jule 26–30, p. 11.

Mapother, M. E., L. A. Joens, and R. D. Glock. 1987. Experimental reproduction of porcine proliferative enteritis. Vet. Rec. 121:533–536.

McCluskey, J., J. Hannigan, J. D. Harris, B. Wren, and D. G. E. Smith. 2002. LsaA, an antigen involved in cell attachment and invasion, is expressed by *Lawsonia intracellularis* during infection *in vitro* and *in vivo*. Infect. Immun. 70:2899–2907.

McOrist, S., and C. J. Gebhart. 1999. Porcine proliferative enteropathies. In Diseases of swine, 8th ed. B. E. Straw, S. D'Allaire, D. Mengeling, and J. Taylor (ed.). Ames: Iowa State University Press, pp. 521–534.

McOrist, S., and G. H. K. Lawson. 1987. Possible relationship of proliferative enteritis in pigs and hamsters. Vet. Microbiol. 15:293–302.

———. 1989. Reproduction of proliferative enteritis in gnotobiotic pigs. Res. Vet. Sci. 46:27–33.

McOrist, S., C. J. Gebhart, R. Boid, and S. M. Barns. 1995a. Characterization of *Lawsonia intracellularis* gen. nov, sp nov, the obligately intracellular bacterium of porcine proliferative enteropathy. Int. J. Syst. Bacteriol. 45:520–525.

McOrist, S., G. H. K. Lawson, A. C. Rowland, and N. MacIntyre. 1989. Early lesions of proliferative enteritis in pigs and hamsters. Vet. Path. 26:260–264.

McOrist, S., L. Roberts, S. Jasni, A. C. Rowland, G. H. K. Lawson, C. J. Gebhart, and B. Bosworth. 1996. Developed and resolving lesions in porcine proliferative enteropathy: possible pathogenic mechanisms. J. Comp. Pathol. 115:35–45.

McOrist, S., N. MacIntyre, C. R. Stokes, and G. H. K. Lawson. 1992. Immunocytological responses in porcine proliferative enteropathies. Infect. Immun. 60:4184–4191.

McOrist, S., R. A. M. Mackie, and G. H. K. Lawson. 1997. In vitro interactions of *Lawsonia intracellularis* with cultured enterocytes. Vet. Microbiol. 54:385–392.

McOrist, S., R. A. M. Mackie, I. Aitken, and G. H. K. Lawson. 1994. Synergism of *ileal symbiont intracellularis* and gut bacteria in the reproduction of porcine proliferative enteropathy. Vet. Rec. 134:331–332.

McOrist, S., R. Boid, G. H. K. Lawson, and I. McConnell. 1987. Monoclonal antibodies to intracellular *Campylobacter*-like organisms of the porcine proliferative enteropathies. Vet. Rec. 121:421–422.

McOrist, S., S. Jasni, R. A. Mackie, H. M. Berschneider, A. L. Rowland, and G. H. K. Lawson. 1995b. Entry of the bacterium *Ileal symbiont intracellularis* into cultured enterocytes and its subsequent release. Res. Vet. Sci. 59:255–260.

McOrist, S., S. Jasni, R. A. Mackie, N. MacIntyre, N. Neef, and G. H. K. Lawson. 1993. Reproduction of porcine proliferative enteropathy with pure culture *of Ileal symbiont intracellularis.* Infect. Immun. 61:4286–4292.

Peace, T. A., K. V. Brock, and H. F. Stills. 1994. Comparative analysis of the 16S rRNA gene sequence of the putative agent of proliferative ileitis of hamsters. Int. J. System. Bacteriol. 44:832–835.

Roberts, L., A. C. Rowland, and G. H. K. Lawson. 1977. Experimental reproduction of porcine intestinal adenomatosis and necrotic enteritis. Vet. Rec. 100:12–13

Rowland, A. C., and G. H. K. Lawson. 1974. Intestinal adenomatosis in the pig: immunofluorescent and electron microscopic studies. Res. Vet. Sci. 17:323–330.

———. 1992. Porcine proliferative enteropathy. In Diseases of swine, 7th ed. A. D. Leman et al. Ames: Iowa State University Press.

Rowland, A. C., G. H. K. Lawson, and A. Maxwell. 1973. Intestinal adenomatosis in the pig: occurrence of a bacterium in affected cells. Nature. 243:417.

Sapico, F. L., D. Reeves, H. M. Wexler, J. Duncan, K. H. Wilson, and S. M. Finegold. 1994. Preliminary study using species-specific oligonucleotide probe for rRNA of *Bilophila wadsworthia*. J. Clin. Microbiol. 32:2510–2513.

Smith, D. G. E., and G. H. K. Lawson. 2001. *Lawsonia intracellularis*: getting inside the pathogenesis of proliferative enteropathy. Vet. Microbiol. 82:331–345.

Smith, D. G. E., S. C. Mitchell, and T. Nash, S. Rhind. 2000. Gamma interferon influences intestinal epithelial hyperplasia caused by *Lawsonia intracellularis* infection in mice. Infect. Immun. 68:6737–6743.

Smith, S. H., and S. McOrist. 1997. Development of persistent intestinal infection and excretion of *Lawsonia intracellularis* by pigs. Res. Vet. Sci. 62:6–10.

Stills, H. F., Jr., 1991. Isolation of an intracellular bacterium from hamsters (*Mesocricetus auratus*) with proliferative ileitis and reproduction of the disease with a pure culture. Infect. Immun. 59:3227–3236.

Ward, G. E., and N. L. Winkelman. 1990. Recognizing the three forms of proliferative enteritis in swine. Vet. Med. 85:197–203.

Winkelman, N. L. 1996. Ileitis: an update. Compend. Cont. Educ. Pract. Vet. 19:519–525.

27
Gram-Negative Anaerobes

D. J. Hampson, T. G. Nagaraja, and N. B. Buller

Gram-negative anaerobic bacteria are a large and diverse group of organisms, many of which remain poorly defined. Their taxonomy and nomenclature continue to undergo pronounced reorganization, and new genera and species are regularly described. Most recognized species are part of the normal flora of the mouth or of the intestinal, upper respiratory, urinary, or genital tracts. A small number of well-defined species are important pathogens of animals, and these primarily belong to the genera *Bacteroides, Brachyspira*, *Dichelobacter*, *Fusobacterium*, *Porphyromonas,* and *Prevotella*. These and other anaerobic species often are present in lesions in mixed cultures, where they may act synergistically in disease production.

GENERAL BACTERIAL VIRULENCE FACTORS

The ability of pathogenic anaerobic bacteria to create an anaerobic microenvironment (low redox potential) or to tolerate oxygen exposure is prerequisite to their ability to establish infection. An anaerobic microenvironment may be created by the action of certain virulence factors (e.g., endotoxin, hemolysin, platelet aggregation factor) or because of synergy with facultative bacteria. Oxygen tolerance allows anaerobes to survive in infected tissues until conditions are conducive for multiplication and invasion. Many pathogenic anaerobes can grow at low oxygen tensions, with superoxide dismutase, catalase, and NADH oxidase protecting against the toxic effects of oxygen.

The precise mechanisms by which gram-negative anaerobes cause disease are generally ill defined. However, adherence, colonization, invasion, and tissue destruction are facilitated by a variety of structures (e.g., capsules, pili, agglutinins, adhesins, LPS, and outer-membrane proteins), exotoxins (e.g., enterotoxin, hemolysin, and leukotoxin), and extracellular enzymes (e.g., neuraminidase, proteases, DNases, lipases). Additionally, fermentation products such as lactic acid, butyric acid, and ammonia, which create the characteristic putrid odor of anaerobic infections, also have inflammatory and cytotoxic effects (Botta et al. 1994).

Suppurative infections caused by these organisms are often polymicrobial, potentially involving both anaerobic and facultative bacteria. The association of certain organisms may generate a synergy resulting in enhanced infectivity. For example, the minimum infective dose of *Fusobacterium necrophorum* in experimental infections in mice was greatly reduced when the anaerobe was mixed with various facultative bacteria (Smith et al. 1991). Mechanisms involved may be the supply of energy substrate, essential growth factors, or production of a low redox potential. The frequent association of *F. necrophorum* and *Arcanobacterium pyogenes* provides a classic example of such synergistic interaction (table 27.1).

BACTEROIDES

The genus *Bacteroides* currently includes several species of gram-negative rods, but only *B. fragilis* and *B. thetaiotaomicron* are common and clinically important. *Bacteroides fragilis* constitutes about 1 to 2% of the normal colonic population in humans, but it is the most common anaerobe isolated from human clinical samples. It is associated with abscesses (mainly intra-abdominal), soft tissue infections, and bacteremias.

Table 27.1. Synergistic Interactions Between *Fusobacterium necrophorum* and *Arcanobacterium pyogenes*

Factor	*F. necrophorum*	*A. pyogenes*	Interaction
Oxygen relationship	Anaerobic	Facultative	*A. pyogenes* creates anaerobic condition by utilizing oxygen
Toxin production	Leukotoxin	Hemolysin	Hemolysin lyses erythrocytes to provide iron to *F. necrophorum;* also helps to create anaerobiosis. Leukotoxin provides protection to *A. pyogenes* against phagocytosis.
Substrate/Product	Lactate utilizer	Lactate producer	*A. pyogenes* produces lactate as a product that is utilized by *F. necrophorum* as an energy substrate.

Virulence Factors

Bacteroides species, particularly *B. fragilis,* contain surface features, including pili, capsule, and LPS, and produce toxins, such as hemolysin and enterotoxin, and several enzymes including proteases, collagenase, fibrinolysin, neuraminidase, heparinase, chondroitin sulfatase, and glucuronidase, which contribute to adhesion, invasion, and tissue destruction (Duerden 1994). *B. fragilis* contains a complex capsular polysaccharide, which 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 of polysaccharides A and B contain both positively and negatively charged groups that may confer a selective advantage for survival in the colon, and the zwitterionic charge motif is a critical feature for abscess formation (Tzianobos et al. 1993).

Strains of *B. fragilis* cause diarrhea in lambs, calves, piglets, foals, infant rabbits, and children. *B. fragilis* enterotoxin, fragilysin, is a 20-kDa heatlabile 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, the toxic properties of which result from its proteolytic activity (Obiso et al. 1995). 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).

FUSOBACTERIUM

The genus *Fusobacterium* includes 13 species of gram-negative rods with tapering or fusiform ends that produce large amounts of butyric acid. Of these, only *Fusobacterium necrophorum* is a major animal pathogen (Jang and Hirsh 1994). *Fusobacterium nucleatum* and *F. russi* are members of the normal oral flora of dogs and cats and are frequently isolated from dog- and cat-bite wounds (Citron 2002). *Fusobacterium equinum* is part of the normal oral flora of horses, and is isolated from oral-associated and respiratory diseases of horses.

Fusobacterium necrophorum

Fusobacterium necrophorum is a normal inhabitant of the mouth and gastrointestinal and urogenital tracts, and is also present in soil. *Fusobacterium necrophorum* is classified into subspecies *necrophorum* and *funduliforme*, formerly biotypes/biovars A

and B. The subspecies have different morphology, growth patterns, and biochemical and biological characteristics (Nagraja and Chengappa 1998).

Virulence Factors

Several toxins and enzymes play a role in the pathogenesis of *F. necrophorum* infections. These include leukotoxin, endotoxin, hemolysin, hemagglutinin, capsule, adhesins or pili, platelet aggregation factor, dermonecrotic toxin, and extracellular enzymes including proteases, lipases, and DNases (table 27.2). Of these, leukotoxin and, to a certain extent, endotoxin are the major virulence factors (Tan et al. 1996). For example, non-leukotoxin-producing strains do not induce foot abscesses in cattle (Emery et al. 1985), and cattle experimentally vaccinated with *F. necrophorum* leukotoxoid are protected against abscess formation in the liver (Nagaraja and Chengappa 1998). Leukotoxin is a high-molecular-weight (336 kDa) protein that is toxic to ruminant leukocytes, macrophages, hepatocytes, and rumen epithelial cells (Tan et al. 1996; Narayanan et al. 2002). 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 either of the presence of high-affinity receptors or of increased numbers of receptors on the surface of ruminant leukocytes. At high concentrations, leukotoxin causes primary cell necrosis. Leukotoxin presents several potentially important pathogenic mechanisms, including modulation of the host immune system by its toxicity, cellular activation of leukocytes, and apoptosis-mediated

Table 27.2. Virulence Factors of *Fusobacterium necrophorum*

Factor	Characteristics	Mechanism of action	Role in pathogenesis
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	Necrotic effect and induces disseminated intravascular coagulation	Creates anaerobic micro-environment conducive for anaerobic growth
Hemolysin	Extracellular but cell associated, protein	Lyses erythrocytes of various animals	Aids in acquiring iron, which is stimulatory to bacterial growth, from the host; creates anaerobic micro-environment
Hemagglutinin	Possibly protein, cell-wall associated	Agglutinates erythrocytes of various animals	Mediates attachment to rumen epithelial cells and hepatocytes
Adhesins (pili)	Extracellular, possibly protein, not well documented	Attaches to eukaryotic cell surface	Helps in colonization of rumen epithelium
Dermonecrotic toxin	Cell-wall associated, not characterized	Causes necrosis in the epithelium	Aids in penetration of the rumen epithelium
Platelet aggregation factor	Cell associated, not characterized	Aggregates bovine platelets, thrombocytopenia, induces disseminated intravascular coagulation	Creates anaerobic micro-environment conducive for growth; fibrin deposition may protect the bacteria
Proteases	Extracellular, protein	Breaks down cell protein	Facilitates penetration of rumen epithelium, skin, etc.

killing of phagocytes and immune effector cells (Narayanan et al. 2002).

The leukotoxin open reading frame (*lktA*) comprises 9,726 base pairs, and encodes a 3,241 amino acid protein with an overall molecular mass of 336 kDa (Narayanan et al. 2001). The protein may represent a new class of bacterial leukotoxins since it has no sequence similarity to other known leukotoxins and is also unusual in that it lacks cysteine. The leukotoxin operon consists of at least three open reading frames, *lkt A, B, C,* with the *lktA* region coding for the leukotoxin gene, and *lktB* and *lktC* being upstream and downstream of *lktA*, respectively. The *lktB* gene consists of 551 codons yielding a protein with a molecular weight of 62 kDa, which has the best sequence similarity to the HxuB, heme-hemopexin utilization protein of *Haemophilus influenzae.* The *lktC* gene encodes a predicted protein of 146 amino acids with a molecular weight of 17 kDa and the protein does not show significant sequence similarity to proteins currently in the NCBI databases. The activity of the LktA protein expressed in *E. coli* indicates that the other proteins encoded in the putative 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*.

Diseases

Fusobacterium necrophorum causes a variety of necrotic infections in animals including necrotic stomatitis of calves, lambs, and pigs, footrot in cattle and sheep, gangrenous dermatitis in horses and mules, pulmonary and hepatic abscesses in cattle and pigs, and jaw abscesses in wild ruminants and marsupials (Tan et al. 1996). *Fusobacterium equinum* is a frequent component of certain respiratory infections of horses commonly associated with transportation, including necrotizing pneumonia and pleurisy (Racklyeft and Love 2000). 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." 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 and 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 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 *A. pyogenes* in the 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. The disease is characterized by necrosis of the mucous membrane 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 adjacent underlying soft tissues of the feet. Fecal excretion of *F. necrophorum* and pasture contamination by manure 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 1998). 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, therefore the importance of *F. necrophorum* antibodies in immunity 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 because it is considered the primary virulence factor involved in the production of liver abscesses, antibodies directed against it may correlate with protection. Cell-free culture supernatant of

a high leukotoxin producing strain of *F. necrophorum* elicited a high leukotoxin antibody titer when injected into steers, and provided significant protection from liver abscesses (Nagaraja and Chengappa 1998). A next step will be to develop a recombinant leukotoxin vaccine to protect cattle.

PREVOTELLA AND *PORPHYROMONAS*

These genera are composed mainly of saccharolytic (*Prevotella*) and asaccharolytic (*Porphyromonas*), pigmented and nonpigmented species previously classified as *Bacteroides.* The genus *Prevotella* currently includes 8 species (Jousimies-Somer and Summanen 2002), whereas *Porphyromonas* includes 12 pigmented and 1 nonpigmented species, 9 of which are of animal origin. The strains isolated from the gingival sulcus of various animals, which are distinct from related human strains of *Porphyromonas gingivalis,* are now included under a new species *P. gulae.*

VIRULENCE FACTORS

Black-pigmented anaerobes produce a variety of virulence factors. These include those that protect the organisms 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) (Finegold et al. 1993).

DISEASES

Prevotella and *Porphyromonas* species have a prominent role in oral and bite-wound infections. Periodontal disease is the most common oral disease of adult animals, and includes gingivitis, periodontitis, and periodontal abscesses initiated by bacteria in the dental plaque. Periodontal diseases affect a wide range of animals, including dogs and cats, sheep, cattle, and captive and free-roaming wild animals (Bird et al. 2002). *Prevotella* and *Porphyromonas* are frequently isolated from infected dog- and cat-bite wounds in humans (Talan et al. 1999). *Prevotella melaninogenica* is readily cultured from footrot or foot abscesses of cattle, and may work in synergy with *F. necrophorum* to produce the infection (Clark et al. 1985).

DICHELOBACTER

Dichelobacter (*Bacteroides*) *nodosus* is the major infectious agent of ovine footrot, a disease primarily affecting sheep and goats. Lesion severity ranges from mild to severe, as progressive degeneration of the soft horn of the hoof occurs. Clinical expression is influenced by host, environmental, and pathogen factors. Footrot causes significant economic losses.

D. nodosus is a large gram-negative rod, 3 to 10 μm in length with straight sides and enlarged ends. After 48 hours growth on trypticase arginine serine agar in an anaerobic atmosphere, colonies are characteristically slow growing, flat, and transparent with a spreading margin.

Infected sheep and goats are the main reservoir of the organism. Virulent isolates are rarely carried by cattle, and they are not a significant source of infection. The organism also has been isolated from deer and pigs. Animals can become infected from environment sources, including transport trucks contaminated with infected soil and feces. *D. nodosus* does not survive on pasture for more than two weeks, a fact that allows identification of uninfected pastures for use in control programs. Infection may be latent in the hoof, therefore animals that are infected but apparently healthy pose a potential threat of reinfection (Depiazzi et al. 1998).

Footrot is a seasonal disease and occurs in areas of warm, temperate (Mediterranean) climates in late spring to early summer. Transmission occurs when the temperature is above 10°C and rainfall exceeds 500 mm per annum. Coarse and long pasture helps initiate the disease through hoof damage, assisting skin penetration. An isolate may induce a severe lesion in the hoof in an area conducive to the expression of footrot, yet produce a benign or mild lesion under drier or colder conditions (Depiazzi et al. 1998).

VIRULENCE FACTORS

D. nodosus produces several potential virulence factors including outer-membrane proteins, LPS, extracellular proteases, and type IV pili. Virulent isolates produce a heat-stable protease, whereas benign strains produce a heat-labile protease. Protease activity is detected in the gelatin gel test where virulent isolates retain enzyme activity against gelatin after heating to 68°C for 16 minutes. Virulent and benign isolates also usually have distinctive zymogram profiles of the protease isoenzymes (Depiazzi et al. 1991; Palmer 1993).

Type IV pili have a highly conserved terminal amino acid region and an N-methylphenylalanine

residue at the N-terminal end. They are responsible for extracellular protein secretion, and are involved in twitching motility, antigenic and phase variation, and adhesion (Strom and Lory 1993). The type IV pili cause K-type agglutination, and because of the highly variable region are responsible for the 9 recognized serogroups (A–I), or 18 serotypes (Claxton 1989). Virulent isolates tend to be more heavily piliated than benign isolates. Inactivation of the type IV pili gene *fim*A by allelic exchange produced mutants that did not exhibit twitching motility, had lower levels of extracellular protease, and did not cause infection when inoculated into sheep hoof (Kennan et al. 2001).

Regions of the genome that appear to be significant in relation to virulence include the fimbrial genes (*fim*A), basic protease (*bpr*V), acidic protease (*apr*V5), a virulence-related locus (*vrl*), and virulence-associated proteins (*vap*). The *vap* and *vrl* initially were suggested to relate to virulence because they occurred more frequently in virulent strains than in benign strains. However, protein expression studies on these gene regions did not appear to relate to known phenotypic virulence factors. The virulence-associated regions are scattered throughout the genome, suggesting that virulence is an evolving trait and not the result of a single event, although horizontal exchange of DNA appears to be common (Billington et al. 1996; Fontaine and Rood 1997; Billington et al. 1999). Conclusive proof that these regions are directly involved in virulence is still to be obtained.

Pathogenesis

Ovine footrot has a spectrum of clinical expression from severe through intermediate to benign. Because of the lameness caused by severe or virulent lesions, sheep show inappetence and are unable to feed, with a resulting loss of body condition, wool growth, and wool quality. Benign lesions usually show spontaneous recovery within a few weeks. In the hoof, *D. nodosus* penetrates the epidermis and may predominate where separation of the soft horn occurs, however, a tissue response often does not occur. The inflammation and necrosis is thought to result from a secondary invader, *F. necrophorum* (Egerton et al. 1969). Polymorphonuclear cells in the superficial layers of the epithelium together with activated macrophages are capable of phagocytosing opsonized *D. nodosus* cells *in vitro*. IgG_2 enhances phagocytosis. However, phagocytosis may have little effect in the overall lesion resolution due to the progressive nature of a severe lesion (Emery et al. 1984).

The lesion usually develops from the interdigital skin, involves both digits, and often affects more than one foot. Initially an interdigital dermatitis occurs that progresses to underrunning of the horn, and eventual separation of the horn from the underlying tissues. There is usually moist, necrotic, foul-smelling material on the interdigital skin and hyperkeratosis at the skin-horn junction. In advanced lesions, the deeper laminae of the hoof can become necrotic causing the hoof wall to separate from the underlying tissues.

Although *D. nodosus* is the primary transmissible agent of footrot, it is rarely the predominant organism in the hoof. Other organisms include spirochetes, *Fusobacterium* species, *A. pyogenes*, and *Bacteroides* species. *F. necrophorum*, in particular, seems to be involved in the early invasion in the hoof, thus allowing *D. nodosus* to multiply in the epidermis and invade the epidermal matrix beyond the skin-horn junction. *F. necrophorum* causes tissue inflammation, whereas *D. nodosus* is involved in the separation of the skin from the horn, through the action of its proteases (Egerton et al. 1969).

Immunity

Although bacterial invasion in the hoof lesion is restricted to the avascular epidermis (*stratum spinosum*), when sheep are challenged with different antigenic fractions from *D. nodosus,* a weak immune response is elicited. Pilus antigens generate a greater immune response than do cell-envelope antigens, LPS, or extracellular protease antigens. The primary and anamnestic humoral immune response, as detected by antibody level, is highest when lesions are severe and the infection prolonged (Whittington and Nicholls 1995).

Based on the pilus antigen, there are nine serogroups (A–I) of *D. nodosus,* and protection is serogroup specific. Infection with multiple serogroups occurs within a flock and within a hoof (Claxton 1989). The immune response to infection varies among breeds of sheep and within an infected flock. Coarse-wooled British breeds tend to be more resistant than Merino sheep. However, under experimental conditions, all breeds can show severe lesions when the organism is applied directly to the hoof (Escayg et al. 1997). Natural resistance manifests as mild lesions, delayed infection, and self-curing lesions. Natural resistance is thought to have a genetic basis that is paternally inherited, and early

studies suggest a relationship between alleles in the ovine major histocompatibility complex (MHC) class II region and resistance (Escayg et al. 1997). An involvement in the SY6 and SY1b class I ovine lymphocyte antigens also suggests a contribution toward disease resistance by producing a greater antibody response after vaccination (Outteridge et al. 1989).

TREPONEMA

Anaerobic treponemes colonize the mouth and intestinal and genital tracts. Recently, spirochetes related to human oral treponemes have been associated with bovine papillomatous digital dermatitis, an economically significant condition in dairy cows (Walker et al. 1995). Affected feet have variable presentation from moist, painful strawberry-like lesions of the skin, to chronic, raised, hairy wartlike lesions. Lesions mainly occur at the rear of the hoof between the bulbs of the heel, and lead to lameness. Currently, little is known about the epidemiology or pathogenesis of the infection.

A disease called severe virulent ovine footrot has been described in the United Kingdom, again associated with treponemes related to those found in cattle with digital dermatitis (Collinghan et al. 2000). Lesions begin around the coronary band, the feet are red and swollen, and the disease is aggressive. *D. nodosus* is not isolated from the lesion in these cases, nor is it seen in microscopic examination. Further work is needed to clarify the pathogenicity of these organisms and the status of the disease as separate from classical ovine footrot.

BRACHYSPIRA

The genus *Brachyspira* (formerly *Serpulina*) contains seven named species of anaerobic intestinal spirochetes that colonize the large intestines of a variety of animal species. Most species are 6–8 μm long and 0.3–0.4 μm wide, with 2–4 loose coils. Pathogenic species include *Brachyspira alvinipulli*, *B. hyodysenteriae*, *B. intermedia*, *B. pilosicoli*, and possibly *B. aalborgi*. Other species are considered commensal. *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, while *B. alvinipulli* appears to be uncommon. *B. aalborgi* infects humans and nonhuman primates. The pathogenicity of these organisms has not been studied in detail.

BRACHYSPIRA HYODYSENTERIAE

B. hyodysenteriae is the primary etiological agent of swine dysentery (SD), a severe mucohemorrhagic colitis typically affecting weaner/grower/finisher pigs. The spirochete also causes a typhlitis in Rheas. SD occurs worldwide and causes considerable economic loss because of poor growth rates and occasional mortality. Recovered carrier pigs and pens and trucks contaminated with infected feces are the main source of infection. Mice may transmit the infection within a piggery.

Virulence Factors

Several potential virulence factors have been described, including chemotaxis, motility, adherence, hemolysin production, and lipooligosaccharide. Colonization by *B. hyodysenteriae* is enhanced by the presence of other anaerobic species, and these may contribute to lesion formation. Consistent with this, diets that result in limited fermentation in the large intestine reduce both colonization and disease (Siba et al. 1996). The spirochete is chemotactic toward mucin (Milner and Sellwood 1994). A corkscrew motility is generated through the activity of the periplasmic flagella, and the importance of the motility is emphasized by the fact that FlaA and FlaB mutants created through allelic exchange show both reduced motility and reduced colonization in pigs (Kennedy et al. 1997). NADH oxidase activity also appears to be important in colonization of the mucosal surface, since the enzyme consumes oxygen and hence reduces potential oxygen toxicity. Consistent with this, NADH oxidase-negative mutants of *B. hyodysenteriae* show reduced colonization (Stanton et al. 1999). Several putative hemolysin genes of *B. hyodysenteriae* have been described (*tlyA, tlyB,* and *tlyC*), and hemolysin-negative mutants of *B. hyodysenteriae* have reduced virulence in pigs (Hyatt et al. 1994). Recently, a distinct gene (*hlyA*) encoding a beta-hemolytic protein with marked similarities to the native *B. hyodysenteriae* hemolysin has been identified (Hsu et al. 2001). It appears that one or more of the previously described *tly* "hemolysin" genes may have had regulatory functions, rather than encoding hemolysins. The lipooligosaccharides (LOS) on the surface of *B. hyodysenteriae* have biological activity, and may contribute to lesion formation. Studies with inbred mouse strains have shown that their response to LOS from *B. hyodysenteriae* is correlated with their susceptibility to infection (Nibbelink and Wannemuehler 1991).

Pathogenicity

SD can take between 5 days to several weeks to develop following experimental exposure to *B. hyodysenteriae.* Once established, the spirochete colonizes mucus deep within the crypts of Lieberkühn in 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 deeper into the tissue. The main lesions are epithelial necrosis and erosion, likely resulting from the cytotoxic action of the pore-forming hemolysin. The eroded and subsequently bleeding surface becomes invaded by other anaerobic bacteria and the protozoan *Balantidium coli*, with the formation of extensive fibrinous plaques attached to and overlying the lesions in chronic cases. Failure to absorb water and electrolytes contributes to diarrhea. Some pigs may die in the acute phase of the disease, possibly due to endotoxic shock.

Immunity

Immunity develops after infection but tends to be LOS serogroup specific (Joens et al. 1979). Recovered pigs have an increased percentage of CD8αα cells, which may be involved in recovery and protection from SD (Waters et al. 2000). Infected pigs show an IgA response in the colon, but this does not necessarily correlate with protection (Rees et al. 1989). The spirochete has a set of variable surface protein (Vsp) genes that may contribute to antigenic variation and host immune system avoidance (McCaman et al. 1999). A 30 kDa outer membrane lipoprotein, which is expressed *in vivo* and which is recognized by immune serum, also has been described (Lee et al. 2000).

BRACHYSPIRA PILOSICOLI

B. pilosicoli colonizes the large intestine of a range of species, notably pigs, chickens, dogs, and humans. It is a cause of mild colitis and diarrhea, particularly in weaner and grower pigs, but it 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."

Virulence Factors and Pathogenicity

Few specific virulence factors have been identified. An outer membrane serine protease has been described (Muniappa and Duhamel 1997), and 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).

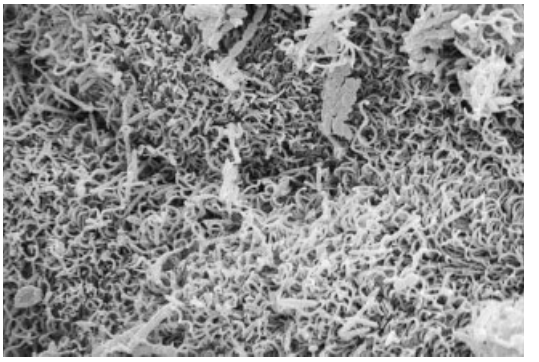


Figure 27.1. Dense mats of *Brachyspira pilosicoli* cells attached by one cell end to the cecal epithelium of a chicken, covering the epithelial surface and physically interfering with absorption (scanning electron micrograph).

Colonization is 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" (fig. 27.1). A similar polar attachment is seen in humans and in nonhuman primates colonized by *B. aalborgi*. 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 spirochetemia. 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 is 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).

OVERALL CONCLUSIONS AND FUTURE WORK

Detailed knowledge of the virulence factors and the pathogenesis of infections with most gram-negative anaerobes has lagged behind that for many other bacterial species. In the near future, considerable impetus in understanding these infections will arise as a result of whole genomic sequencing now being undertaken (e.g., for *D. nodosus*). The availability of this sequence data will improve identification of potential virulence factors, and will encourage development of genetic manipulation, such as site-directed mutagenesis, to investigate the function of putative virulence genes. With the use of oligonucleotide arrays and proteomic analyses, study of expression and interactions of such virulence factors *in vivo* will also be greatly enhanced.

REFERENCES

Billington, S. J., A. S. Huggins, P. A. Johanesen, P. K. Crellin, J. K. Cheung, M. E. Katz, C. L. Wright, V. Haring, and J. I. Rood. 1999. Complete nucleotide sequence of the 27-kilobase virulence related locus (*vrl*) of *Dichelobacter nodosus*: evidence for extrachromosomal origin. Infect. Immun. 67:1277–1286.

Billington, S. J., J. Johnston, and J. Rood, J. 1996. Virulence regions and virulence factors of the ovine footrot pathogen, *Dichelobacter nodosus.* FEMS Microbiol. Lett. 145:147–156.

Bird, P. S., S. C. Huynh, D. Davis, D. N. Love, L. L. Blackall, and G. J. Seymour. 2002. Oral diseases in animals: the Australian perspective. Isolation and characterization of black-pigmented bacteria from the oral cavity of marsupials. Anaerobe 8:79–87.

Botta, G., A. Arzese, R. Minisini, and G. Train. 1994. Role of structural and extracellular virulence factors in gram-negative anaerobic bacteria. Clin. Infect. Dis. 18:S260–S264.

Citron, D. M. 2002. Update on the taxonomy and clinical aspects of the genus *Fusobacterium.* Clin. Infect. Dis. 35:S22–27.

Clark, B. L., D. J. Stewart, and D. L. Emery. 1985. The role of *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* in the aetiology of interdigital necrobacillosis in cattle. Aust. Vet. J. 62:47–49.

Claxton, P. D. 1989. Antigenic classification of *Bacteroides nodosus*. In Footrot and foot abscess of ruminants. J. R. Egerton, W. K. Yong, and G. G. Riffkin (eds.). Boca Raton, FL: CRC Press, pp. 155–165.

Collighan, R. J., R. A. Naylor, P. K. Martin, B. A. Cooley, N. B. Buller, and M. J. Woodward. 2000. A spirochete isolated from a case of severe virulent ovine foot disease is closely related to a Treponeme isolated from human periodontitis and bovine digital dermatitis. Vet. Microbiol. 74:249–257.

Coyne, M. J., W. Kalka-Moll, A. O. Tzianobos, D. L. Kasper, and L. E. Comstock. 2000. *Bacteroides fragilis* NCTC9343 produces at least three distinct capsular polysaccharides: cloning, characterization, and reassignment of polysaccharide B and C biosynthesis loci. Infect. Immun. 68:6176–6181.

Depiazzi, L. J., R. B. Richards, J. Henderson, J. I. Rood, M. A. Palmer, and W. J. Penhale. 1991. Characterisation of virulent and benign strains of *Bacteroides nodosus.* Vet. Microbiol. 26:151–160.

Depiazzi, L. J., W. D. Roberts, C. D. Hawkins, M. A. Palmer, D. R. Pitman, N. C. McQuade, P. D. Jelinek, D. J. Devereaux, and R. P. Rippon. 1998. Severity and persistence of footrot in Merino sheep experimentally infected with a protease thermostable strain of *Dichelobacter nodosus* at five sites. Aust. Vet. J. 76:32–38.

Duerden, B. I. 1994. Virulence factors in anaerobes. Clin. Infect. Dis. 18:S253–S259.

Egerton, J. R., D, S. Roberts, and I. M. Parsonson. 1969. The aetiology and pathogenesis of ovine footrot. I. A histological study of the bacterial invasion. J. Comp. Pathol. 79:209–215.

Emery, D. L., D. J. Stewart, and B. L. Clark. 1984. The comparative susceptibility of five breeds of sheep to foot-rot. Aust. Vet. J. 61:85–88.

Emery, D. L., J. A. Vaughan, B. L. Clark, J. H. Dufty, and D. J. Stewart. 1985. Cultural characteristics and virulence of strains of *Fusobacterium necrophorum* isolated from the feet of cattle and sheep. Aust. Vet. J. 62:43–46.

Escayg, A. P., J. G. H. Hickford, and D. W. Bullock. 1997. Association between alleles of the ovine major histocompatibility complex and resistance to footrot. Res. Vet. Sci. 63:283–287.

Finegold, S. M., C. A. Strong, M. McTeague, and M. Marina. 1993. The importance of black-pigmented gram-negative anaerobes in human infections. FEMS Immun. Med. Microbiol. 6:77–82.

Fontaine, S. L., and J. I. Rood. 1997. Physical and genetic map of the chromosome of *Dichelobacter nodosus* strain A198. Gene 184:291–298.

Hampson, D. J., I. D. Robertson, T. La, S. L. Oxberry, and D. W. Pethick. 2000. Influences of diet and vaccination on colonisation of pigs with the intestinal

spirochaete *Brachyspira (Serpulina) pilosicoli.* Vet. Microbiol. 73:75–84.

Hartland, E. L., A. S. J. Mikosza, R. Robins-Browne, R. and D. J. Hampson. 1998. Examination of *Serpulina pilosicoli* for attachment and invasion determinants of Enterobacteria. FEMS Microbiol. Lett. 165:59–63.

Hopwood, D. E., D. W. Pethick, and D. J. Hampson. 2002. Increasing the viscosity of the intestinal contents stimulates proliferation of enterotoxigenic *Escherichia coli* and *Brachyspira pilosicoli* in weaner pigs. Brit. J. Nutr. 88:523–532.

Hsu, T., D. L. Hutto, F. C. Minion, R. L. Zuerner, and M. J. Wannemuehler. 2001. Cloning of a beta-hemolysin gene of *Brachyspira (Serpulina) hyodysenteriae* and its expression in *Escherichia coli*. Infect. Immun. 69:706–711.

Hyatt, D. R., A. A. ter Huurne, B. A. M. van der Zeijst, and L. A. Joens. 1994. Reduced virulence of *Serpulina hyodysenteriae* hemolysin-negative mutants in pigs and their potential to protect pigs against challenge with a virulent strain. Infect. Immun. 62:2244–2248.

Jang, S. S., and D. C. Hirsch. 1994. Characterization, distribution and microbiological associations of *Fusobacterium* spp. in clinical specimens of animal origin. J. Clin. Microbiol. 32:384–387.

Joens, L. A., D. L. Harris, and D. H. Baum. 1979. Immunity to swine dysentery in recovered pigs. Am. J. Vet. Res. 40:1352–1354

Jousimies-Somer, H., and P. Summanen. 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding Spirochetes). Clin. Infect. Dis. 35:S17–21.

Kennan, R., O. Dhungyel, R. Whittington, J. Egerton, and J. I. Rood. 2001. The type IV fimbrial subunit gene (*fimA*) of *Dichelobacter nodosus* is essential for virulence, protease secretion, and natural competence. J. Bacteriol. 183:4451–4458.

Kennedy, M. J., E. L. Rosey, and R. J. Yancey, Jr. 1997. Characterization of flaA- and flaB-mutants of *Serpulina hyodysenteriae*: both flagellin subunits, FlaA and FlaB, are necessary for full motility and intestinal colonization. FEMS Microbiol. Lett. 153:119–128.

Kling, J. J., R. L. Wright, J. S. Moncrief, and T. D. Wilkins. 1997. Cloning and characterization of the gene for the metalloprotease enterotoxin of *Bacteroides fragilis.* FEMS Microbiol. Lett. 146:279–284.

Lee, B. J., T. La, A. S. J. Mikosza, and D. J. Hampson. 2000. Identification of the gene encoding BmbB, a 30kDa outer envelope lipoprotein of *Brachyspira* (*Serpulina*) *hyodysenteriae,* and immunogenicity of recombinant Bmpb in mice and pigs. Vet. Microbiol. 76:245–257.

McCaman, M. T., K. Auer, W. Foley, and J. D. Gabe. 1999. Sequence characterization of two new members of a multi-gene family in *Serpulina hyodysenteriae* (B204) with homology to a 39 kDa surface exposed protein: vspC and D. Vet. Microbiol. 31:273–283.

Milner, J. A., and R. Sellwood. 1994. Chemotactic response to mucin by *Serpulina hyodysenteriae* and other porcine spirochetes: potential role in intestinal colonization. Infect. Immun. 62:4095–4099.

Muniappa, N., and G. E. Duhamel. 1997. Outer-membrane associated serine protease of intestinal spirochetes. FEMS Microbiol. Lett. 154:159–164.

Nagaraja, T. G. 1998. Necrobacillosis associated with *Fusobacterium necrophorum.* In Current veterinary therapy 4-food animal practice. J. L. Howard and R. A. Smith (eds.). Philadelphia: W. B. Saunders, pp. 400–402.

Nagaraja, T. G., and M. M. Chengappa. 1998. Liver abscesses in feedlot cattle: a review. J. Anim. Sci. 76:287–298.

Narayanan S., G. C. Stewart, M. M. Chengappa, L. Willard, W. Shuman, M. Wilkerson, and T. G. Nagaraja. 2002. *Fusobacterium necrophorum* leukotoxin induces activation and apoptosis of bovine leukocytes. Infect. Immun. 70:4609–4620.

Narayanan S., T. G. Nagaraja, M. M. Chengappa, and G. C. Stewart. 2001. Cloning, sequencing and expression of the leukotoxin gene from *Fusobacterium necrophorum*. Infect. Immun. 69:5447–5455.

Nibbelink, S. K., and M. J. Wannemuehler. 1991. Susceptibility of inbred mouse strains to infection with *Serpula* (*Treponema*) *hyodysenteriae*. Infect. Immun. 59:3111–3118.

Obiso, R. J., D. M. Lyerly, R. L. Van Tassell, and T. D. Wilkins. 1995. Proteolytic activity of the *Bacteroides fragilis* enterotoxin causes fluid secretion and intestinal damage in vivo. Infect. Immun. 63:3820–3826.

Obiso, R. J., D. R. Bevan, and T. D. Wilkins. 1997. Molecular modeling and analysis of fragilysin, the *Bacteroides fragilis* toxin. Clin. Infect. Dis. 25:S153–155.

Outteridge, P. M., D. J. Stewart, T. M. Skerman, J. H. Dufty, J. R. Egerton, G. Ferrier, and D. J. Marshall. 1989. A positive association between resistance to ovine footrot and particular lymphocyte antigen types. Aust. Vet. J. 66:175–179.

Palmer, M. A. 1993. A gelatin test to detect activity and stability of proteases produced by *Dichelobacter (Bacteroides) nodosus.* Vet. Microbiol. 36:113–122.

Racklyeft, D. J., and D. N. Love. 2000. Bacterial infection of the lower respiratory tract in 34 horses. Aust. Vet. J. 78:549–559.

Rees, A. S., R. J. Lysons, C. R. Stokes, and F. J. Bourne. 1989. Antibody production by the pig colon during infection with *Treponema hyodysenteriae.* Res. Vet. Sci. 47:263–269.

Siba, P. M., D. W. Pethick, and D. J. Hampson. 1996. Pigs experimentally infected with *Serpulina hyodysenteriae* can be protected from developing swine dysentery by feeding them a highly digestible diet. Epidemiol. Infect. 116:207–216.

Smith, G. R., S. A. Barton, and L. M. Wallace. 1991. Further observations on enhancement of the infectivity of *Fusobacterium necrophorum* by other bacteria. Epidemiol. Infect. 106:305–310.

Stanton, T. B., E. L. Rosey, M. J. Kennedy, N. S. Jensen, and B. T. Bosworth. 1999. Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete *Brachyspira (Serpulina) hyodysenteriae*, etiologic agent of swine dysentery. Appl. Environ. Microbiol. 65:5028–5034.

Stephens, C. P., and D. J. Hampson. 2001. Intestinal spirochaete infections in chickens: a review of disease associations, epidemiology and control. Anim. Health Res. Rev. 2:101–110.

Strom, M. S., and S. Lory. 1993. Structure-function and biogenesis of type IV pili. Ann. Rev. Microbiol. 47:565–596.

Talan, D. A., D. M. Citron, F. M. Abrahamian, J. G. Moran, and E. J. Goldstein. 1999. Bacteriologic analysis of infected dog and cat bites. N. Engl. J. Med. 340:85–92.

Tan, Z. L., T. G. Nagaraja, and M. M. Chengappa. 1996. *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. Vet. Res. Commun. 20:113–140.

Tzianobos, A. O., A. O. Onderdonk, B. Rosner, R. L. Cisneros, and D. L. Kasper. 1993. Structural features of polysaccharides that induce intraabdominal abscesses. Science 262:416–419.

Walker, R. L., D. H. Read, K. J. Loretz, and R. W. Nordhausen. 1995. Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. Vet. Microbiol. 47:343–355.

Waters W. R., R. Hontecillas, R. E. Sacco, F. A. Zuckermann, K. R. Harkins, J. Bassaganya-Riera, and M. J. Wannemuehler. J. 2000. Antigen-specific proliferation of porcine CD8αα cells to an extracellular bacterial pathogen. Immunol. 101:333–341.

Whittington, R. J., and P. J. Nicholls. 1995. Effects of the severity and duration of lesions on the primary and anamnestic humoral responses of sheep to *Dichelobacter nodosus* and observations of natural resistance to footrot. Res. Vet. Sci. 59:128–135.

28
Leptospira

B. Adler and A. de la Peña-Moctezuma

INTRODUCTION AND HISTORICAL PERSPECTIVES

Leptospira comprises a genus of helical bacteria that can exist either as free-living saprophytes or as pathogens of a very diverse range of animal hosts, either as the cause of acute leptospirosis or associated with host tissues, especially the renal proximal tubules, on a long-term basis, which may last for the life of the animal. Two additional species previously included in the genus, *L. parva* and *L. illini*, have been reclassified as the genera *Turneria* and *Leptonema,* respectively (Hovind-Hougen 1979; Hookey et al. 1993; Ellis 1995). These genera make up the family Leptospiraceae within the order Spirochaetales, which also contains the families Spirochaetaceae and Serpulinaceae.

Descriptions of human disease consistent with leptospirosis have been known since the earliest available records, from which it seems clear that at least some of the reports of febrile jaundice were due to leptospirosis. Leptospirosis in animals is less well documented, but it is apparent that animal infection must have been the source of human infections from the time that humans have cohabited with both wild and domesticated animals. Leptospires were first isolated in pure culture by Japanese workers in 1914, although it is clear that the first visual observations of *Leptospira* were made a few years earlier by Stimson in silver-stained sections of human kidney from a patient thought to be suffering from yellow fever.

Disease Patterns

Acute leptospirosis is similar in all animal species and in its most severe form is manifested by listlessness, loss of appetite, fever, ruffled fur, red eyes, and sometimes diarrhea. Frank hemorrhages and/or jaundice may occur. Movement is often accompanied by a characteristic arching of the back, indicative of acute nephritis. In milk-producing cattle, agalactia may also occur. Congenital infection of fetuses *in utero* follows a similar course, leading to abortion if the fetus dies. The abortion products may be hemorrhagic and/or jaundiced, and they may contain large numbers of leptospires, thereby constituting a danger to animal handlers and a source of infection for other animals. Less-severe infections, ranging down to asymptomatic, occur frequently.

Chronic persistence of leptospirosis in animals is the central feature of the infection cycle, leading to infection of new animal (or human) hosts. Animals that recover from acute leptospirosis may become carriers, with leptospires remaining in the renal tubules and shed in the urine for periods of days to years. Leptospires may also persist in other organs, notably the genital tract, the brain, and the eye. In horses, especially, recurrent, autoimmune uveitis is well documented (Lucchesi et al. 2002).

CHARACTERISTICS OF THE ORGANISM

Cellular Morphology

Leptospires are thin, helical, motile organisms, ranging from 10 to more than 20 μm in length. Because they are very thin, dark-field, phase contrast, or electron microscopy is required to observe them (fig. 28.1). The detailed structure of the leptospiral cell resembles closely that of a typical gram-negative bacterial cell. There is an outer

membrane, external to an inner membrane and peptidoglycan, wound in a helical shape. Leptospires are usually hooked at one or both ends and exhibit rapid, darting motility in liquid media, as well as both flexing motion and constant rotational movement. Straight unhooked variants occur, which are less motile; the straight forms are sometimes avirulent, but the molecular basis for this is unknown. Each leptospire has two flagella, one arising at each end from an insertion through the inner membrane/peptidoglycan complex and lying in the periplasmic space between the peptidoglycan and the outer membrane. The two polar, periplasmic, single flagella are characteristic and unique distinguishing features of *Leptospira*.

The flagella are similar in general structure to the flagella of other gram-negative bacteria, with a hooked proximal end and disk rotor insertion structures. The central core is made up of the flagellar subunit protein, FlaB. The *flaB* gene may exist in multicopy in some species, but the significance for flagellar structure or antigenic specificity is unclear. The central core filament is surrounded by a sheath. Because of the periplasmic nature of leptospiral flagella, their role in motility and in the retention of the helical cell morphology has been controversial (Li et al. 2000), but the inactivation of the *flaB* gene in *L. biflexa* (Picardeau et al. 2001) demonstrated unequivocally the essential role of the flagella in motility, but not in helical cell morphology. The *flaB* mutants were nonmotile and devoid of flagella, and lacked hooked ends, but they retained their helical shape.

The leptospiral outer membrane contains proteins, lipids, lipoproteins, and LPS. The concentration of outer-membrane proteins is about 100-fold less than the numbers seen in *E. coli* (Haake et al. 1991). In contrast, the leptospiral membrane contains abundant numbers of lipoproteins, some of which are exposed on the cell surface and may be

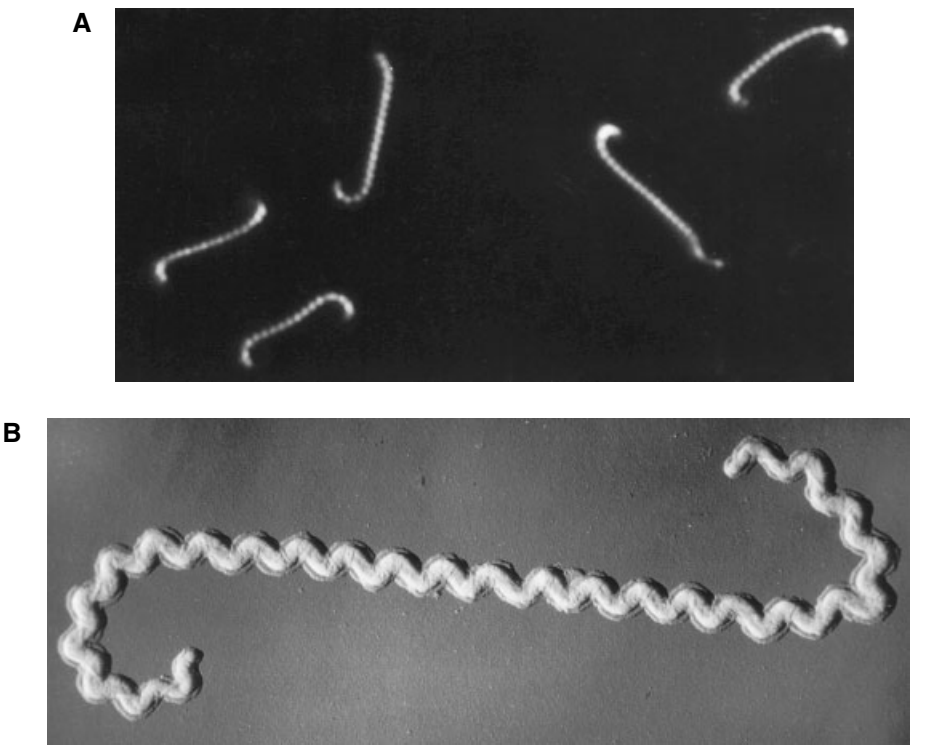


Figure 28.1 (*A*) *Leptospira* sp., showing helical morphology and hooked ends (dark-field photomicrograph, magnification ca. 1,300X); (*B*) *Leptospira* sp. showing characteristic helical shape, periplasmic flagella, and outer membrane (shadowed electron micrograph, magnification ca. 30,000X).

involved in pathogenesis and immunity (Haake 2000; Cullen et al. 2002).

The leptospiral peptidoglycan layer is likewise similar to that of typical gram-negative bacteria, lying external to, but associated more intimately with, the inner membrane. A role has been suggested for peptidoglycan in stimulating phagocytosis and production of the cytokine TNF-α by human monocytes (Cinco et al. 1996). Penicillin-binding proteins have been shown to be associated with the inner membrane/peptidoglycan complex (Brenot et al. 2001).

Nutrition and Metabolism

Leptospires have simple but unusual nutritional requirements. The only major carbon and energy sources are long-chain fatty acids (usually 12 to 18 carbon), from which energy is derived by β-oxidation; sugars are not fermented and cannot be used as carbon sources. Leptospires can shorten or desaturate these essential long-chain fatty acids and incorporate them, but cannot synthesize them *de novo*. Paradoxically, the essential fatty acids required for nutrition and energy metabolism are also toxic, so that they must be presented to the leptospires in the presence of a detoxifier that adsorbs them and releases them slowly in suitable low concentrations. The most commonly used detoxicant is bovine serum albumin, with fatty acids presented in the form of a sorbitol complex (Tween). Glycerol enhances the growth of some leptospires, while pyruvate assists the growth of some strains from small inocula. Ammonium ions, provided either as ammonium salts or by the deamination of amino acids, are the only recognized nitrogen source. Cyanocobalamin (vitamin B_{12}), thiamin (vitamin B_1), and for some strains, biotin, are required. Phosphate is essential, as are calcium, magnesium, and iron, optimally as Fe^{+++} or heme compounds such as hemoglobin or hematin.

Taxonomy and Nomenclature

The genus is defined as *Leptospira* Noguchi (1917), type species *Leptospira interrogans* (Stimson 1907), type strain Serovar Icterohaemorrhagiae, Ictero No. I (ATCC 43782). However, its taxonomy has been confused and controversial. At various times over the past 60 years, the genus has contained only 2 species or almost 200 species. During the period 1915 to 1950, scores of strains were isolated from a wide range of animal species as well as from humans. Although clearly different with respect to epidemiology and disease pattern, most strains were identical morphologically and with no other phenotypic markers available, antigenic differences in agglutinating (LPS) antigens were used as the basis for identification and classification. The serovar became the basic taxon, with each serovar accorded species status (e.g., *L. hardjo*, *L. icterohaemorrhagiae*, *L. pomona* etc.). Antigenically related serovars were placed together in serogroups. From a taxonomic viewpoint, this scheme was clearly incorrect and so was replaced by one in which all pathogenic leptospires were designated the single species *L. interrogans* (also known as the *L. interrogans* complex) followed by the serovar name. All saprophytic serovars were placed in the species *L. biflexa* (the *L. biflexa* complex).

The recent application of defined molecular methods has clarified the taxonomic status of the genus. Several species were thereby identified (Yasuda et al. 1987); currently 12 species of *Leptospira* are recognized (Brenner et al. 1999), with at least four as yet unnamed additional species (table 28.1). See Faine et al. (1999) for a complete listing of the more than 230 serovars currently recognized. A significant outcome of the genetic classification scheme was the finding that many antigenically related serovars, or even subtypes of the same serovar, may belong to different species. For example, the antigenically indistinguishable subtypes of Hardjo (Hardjobovis and Hardjoprajitno) are classified in *L. borgpetersenii* and *L. interrogans,* respectively.

SOURCES OF THE BACTERIUM

Leptospira is arguably one of the most ubiquitous bacterial genera worldwide, both in terms of geographical occurrence and host animal association. The key feature of the epidemiology of leptospirosis is the renal carrier state, in which leptospires colonize the proximal renal tubules of carrier animals, from where they are excreted in the urine, leading to either direct or indirect infection of other animals and humans (fig. 28.2). Virtually every species of mammal can be infected and subsequently become a urinary shedder for time periods from days (e.g., humans) to lifelong (rodents). Animal species that become long-term carriers (usually asymptomatic) are referred to as maintenance hosts for those serovars (e.g., rodents/Icterohaemorrhagiae, cattle/Hardjo). Accidental host associations usually lead to acute leptospirosis (e.g., cattle/Pomona, humans/all serovars). However, this association is not absolute, and under certain circumstances most serovars can infect many host species. See Faine et al.

Table 28.1. Species Recognized Within the Genus *Leptospira*

Species	Pathogenesis	G + C content (%)
L. alexanderi	Pathogenic	38.0
L. borgpetersenii		39.8
L. interrogans		34.9
L. kirschneri		ND[b]
L. noguchii		36.5
L. santarosai		40.5
L. weilii		40.5
Unnamed species 1[a]		39.8
Unnamed species 3[a]		43.4
Unnamed species 4[a]		38.9
Unnamed species 5[a]		37.9
L. fainei	Indeterminate	ND[b]
L. inadai		42.6
L. biflexa	Saprophytic	33.5
L. meyeri		35.2
L. wolbachii		37.2

[a] Brenner et al. (1999).
[b] Not determined.

(1999) for a comprehensive reference list of animal species and serovar associations. In reality, almost all human infections are acquired directly or indirectly from either rodents or domestic animals (cattle, pigs, dogs).

BACTERIAL VIRULENCE FACTORS

The specific mechanisms by which leptospires cause host-tissue damage and disease remain inadequately understood. Reports of so-called pathogenic mechanisms almost all fail to identify the leptospiral component(s) responsible. For example, virulent leptospires adhered to mouse renal tubular epithelial cells *in vitro* (Ballard et al. 1986), while adhesion to cultured fibroblasts was enhanced by antibody (Vinh et al. 1984). However, in neither case were the leptospiral adhesins identified. Virulent leptospires resist the bactericidal action of complement and neutrophils in nonimmune hosts (Wang et al. 1984), but are rapidly killed by either mechanism in the presence of specific antibody (Farrelly et al. 1987). There is no unequivocal evidence of a role for secreted toxins in *Leptospira*. On the contrary, endothelial damage was associated with the presence of leptospiral cells in the kidneys of experimentally infected guinea pigs (de Brito et al. 1992). 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 after just a single *in vitro* subculture. *Leptospira* is not considered to be an intracellular pathogen. Nevertheless, the organisms must penetrate host epithelial and endothelial cell barriers for both hematogenous spread and localization in target organs such as liver and kidneys. Not surprisingly, virulent leptospires were able to transcytose a polarized MDCK cell monolayer (Barocchi et al. 2002) more efficiently than avirulent ones. It is interesting that this was achieved without any apparent cytoskeletal perturbation. A cytotoxic, glycolipoprotein fraction was shown to inhibit host ATPase (Younes-Ibrahim et al. 1995), with the activity ascribed to the presence of long-chain fatty acids (Burth et al. 1997). The molecular basis for reported chemotaxis toward hemoglobin (Yuri et al. 1993) was likewise undefined. 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). However, again the variant was not genetically defined.

Hemolytic activity has been reported in a number of leptospiral serovars. Subsequently, genes encod-

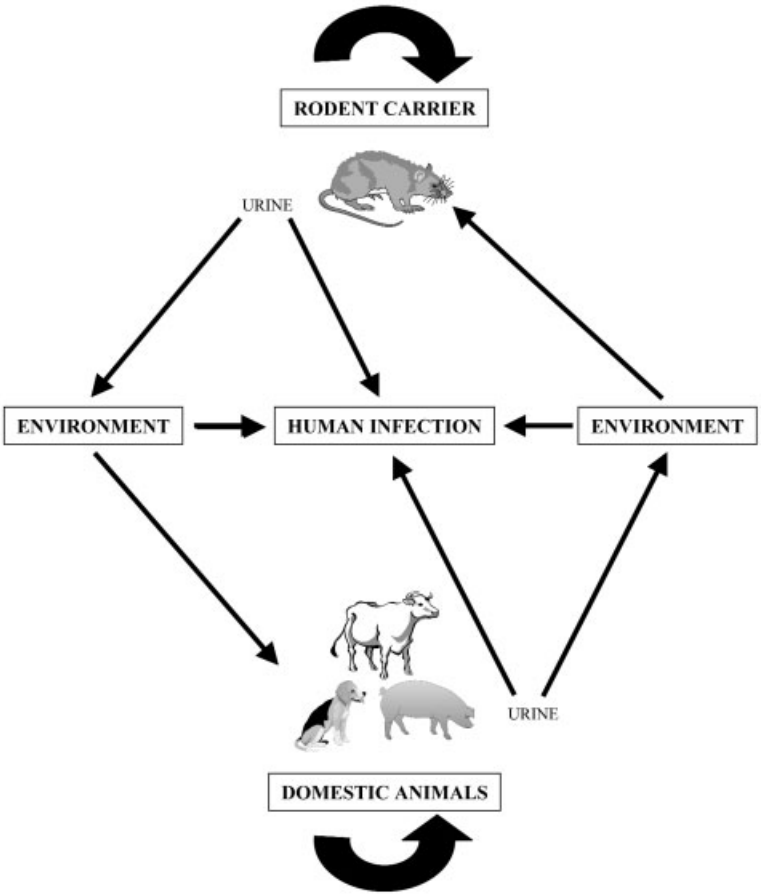


Figure 28.2. Epidemiology of *Leptospira* in rodents, domestic animals, and humans. Cycles are maintained in wild and domestic animals via infected urine and vertical transmission. Humans become infected through direct or environmental contact with infected animal tissues.

ing sphingomyelinases that are either cell associated and/or extracellular have been identified in different *Leptospira* spp. A cell-associated sphingomyelinase C enzyme encoded by *sphA* was identified in Hardjobovis (Segers et al. 1990). Similar sequences were present in all pathogenic strains tested, but not in nonpathogenic serovars (Segers et al. 1992). On the other hand, the enzyme activity appeared to be extracellular in Canicola and Pomona. On the basis of hybridization and cloning experiments, at least seven other *sphA*-like genes were detected among the pathogenic species of *Leptospira*. A further sphingomyelinase, SphH, identified in Lai, was postulated to be a pore-forming hemolysin (Lee et al. 2000). SphA and SphB did not elicit a protective immune response in hamsters. The significance in pathogenesis or immunity of the other Sph enzymes remains undetermined.

Leptospiral LPS is the major surface component of leptospires and contributes to the pathology associated with disease. The structure of LPS remains unknown, but chemical analysis of leptospiral LPS showed the presence of common hexoses, amino hexoses and pentoses, and some sugars found more rarely as LPS components, such as xylose and arabinose (Vinh et al. 1986b, 1989). Methylated and O-acetylated sugars have also been reported (Yanagihara et al. 1983). The detection of KDO in the

LPS of Copenhageni and Hardjo indicates a composition similar to that of typical gram-negative LPS.

Despite the structural, biochemical, and immunological similarity of leptospiral LPS to gram-negative bacterial LPS, it is at least tenfold less toxic for animals or cells. Nevertheless, leptospiral LPS can activate macrophages and act as a B-cell mitogen (Isogai et al. 1990). Activation of macrophages occurs via the CD14 and TLR2 receptors, rather than via TLR4 as seen for the LPS of gram-negative bacteria (Werts et al. 2001). Notably, TLR2 is commonly used as a receptor for gram-positive bacteria and for other non-LPS microbial components.

PATHOGENESIS

Overview

Infections have been described in all types of vertebrates. The classical description of leptospirosis in humans corresponds to so-called Weil's disease, an acute febrile illness characterized by jaundice, hemorrhages, pulmonary distress, splenomegaly, nephritis, and death (Faine 1998). The disease may be less severe with influenza-like signs. In animals, signs are also highly variable, ranging from insignificant to life threatening and representing a risk to public health (Faine et al. 1999). Hardjo, Grippotyphosa, and Pomona are more commonly involved in abortion, infertility, febrile jaundice, agalactia, hemoglobinuria, and premature birth in cattle and less frequently in sheep, goats, and other ungulates (Carroll and Campbell 1987; Feresu 1987; Faine 1998). In piggeries, abortions and stillbirths are the more common signs of infection, with Autumnalis, Bratislava, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona the more commonly involved (Chappel et al. 1989; Paz-Soldan et al. 1991; Potts et al. 1995). Traditionally, leptospirosis in dogs has been related to Canicola and Icterohaemorrhagiae. However, other serovars such as Australis, Grippotyphosa, Pomona, and Tarassovi have also been reported (Myburgh et al. 1993; Harkin and Gartrell 1996; Weekes et al. 1997). Dogs may present with either acute hemorrhagic or with chronic icteric and uremic (Stuttgart disease) syndromes (Adin and Cowgill 2000). Cats very rarely suffer from clinical leptospirosis; cases might be confined to animals with an immunodeficient status (Agunloye and Nash 1996). Horses may present a clinical picture similar to cattle, with stillbirths and abortions. A form of chronic recurrent uveitis called "periodic ophthalmia" has been described in horses associated mainly with Pomona (Donahue et al. 1995; Faber et al. 2000). Many wild animals act as reservoirs for *Leptospira*, although clinical forms of leptospirosis are rarely observed. The most common clinical form of leptospirosis in wild mammals is abortion. Outbreaks have been reported in sea lions (Dierauf et al. 1985), and isolated cases of leptospirosis in rhinoceroses in captivity have been identified (Jessup et al. 1992).

Wild or domestic animals, especially rodents, cattle, pigs, and dogs may become carriers. Pathogenic leptospires live in the proximal renal tubules of carriers (Faine et al. 1999). They may persist there for periods of weeks to years and in some host-serovar associations, often for the lifetime of the animal, particularly in rodents. From the kidneys, leptospires are excreted in the urine and may contaminate soil, water, or food. Animals may excrete leptospires intermittently or regularly. For example, excretion rates in cattle vary from animal to animal and from time to time, from very few to 10^8 ml^{-1} of urine (Thiermann 1982; Ellis et al. 1986). Such animals are an important source of infection, not only for other cows but also for dairy farm workers and other people and animals (Waitkins 1986). Humans usually do not shed leptospires for long periods (Faine 1998).

Environmental Survival of Leptospires

Once excreted in urine, leptospires may survive for periods of days or weeks if conditions in the environment are favorable. Such conditions include moderately alkaline soils, moderate temperatures, and humid conditions (Elder 1986; Leonard et al. 1992). Survival of leptospires in water has been identified as the source of infection for rice harvesters (Padre et al. 1988), banana plantation workers (Smythe et al. 1997), and outdoor water sports athletes (Morgan et al. 2002). Outbreaks of leptospirosis in humans and animals are commonly associated with floods (Kinde et al. 1996; Trevejo et al. 1998).

Interactions Between Leptospires and the Host

Leptospires enter a susceptible host directly from urine or indirectly from contaminated water, soil, or mud. Leptospires penetrate through small and sometimes invisible abrasions in the skin or through the host mucosal surfaces such as the conjunctiva. A

very small number of leptospires (from 1 to 10) can cause a fatal infection in susceptible animals (Faine 1998). Once inside the body, leptospires spread rapidly; they can be found in the bloodstream minutes after subcutaneous, intraperitoneal, or intramuscular inoculation. Leptospiremia can last until approximately 10 days after the onset of symptoms. Motility probably facilitates spread in tissues. Small-vessel vasculitis is the characteristic lesion found in leptospirosis, leading to fluid and cell leakage and eventually to severe haemorrhage. Tissue lesions are characterized by the occurrence of major cellular damage in the presence of few microorganisms, suggesting the involvement of toxic factors from either the spirochete or the host, associated with bacterial-cellular adherence phenomena (de Brito et al. 1979). Considering the wide distribution of fibronectin within mammalian host tissues, Merien et al. (2000) suggested a role for a 36 kDa fibrinectin-binding protein in attachment to host cells. Cytotoxic activity of leptospiral cellular components was demonstrated (Cinco et al. 1980). A preparation of peptidoglycan of Copenhageni was able to induce the release of TNF-α from peripheral blood mononuclear cells (Cinco et al. 1996). As in classical bacterial sepsis, plasma cytokine levels are elevated in severe leptospirosis. In the acute phase, TNF-α levels rise and are associated with the severity of the disease (Estavoyer et al. 1991; Tajiki and Salomao 1996). The glycolipoprotein (GLP) complex exerts toxic effects through its lipid portion, leading to cell membrane perforation, leakage of cell contents, and cell death (Vinh et al. 1986a). In rabbit renal tubule epithelial cells, GLP inhibits sodium-potassium ATPase (Na,K-ATPase) pump activity in a dose-dependent manner (Younes-Ibrahim et al. 1995). In experimental models with guinea pigs injected with pathogenic leptospires, GLP can be detected in the damaged tissues adhering to endothelial cells and to epithelial membranes, accompanying other antigen deposits resulting from bacterial destruction by the immune system (Alves et al. 1991). Cellular activation was induced by GLP as measured by a significative rise in TNF-α and IL-10. Expression of surface activation markers such as CD69 on T lymphocytes and monocytes and HLA-DR (d-related human leukocyte antigens) expression on monocytes was induced by GLP from *L. interrogans* but not from *L. biflexa* (Diament et al. 2002). Pathogenic leptospires are able to persist in some anatomically localized and immunologically privileged sites after antibody and phagocytes have cleared leptospires from all other sites. The most significant site of persistence is the renal tissue. Leptospires appear 2–4 weeks after acute infection, in the apical surface of the proximal renal tubules. Microscopic lesions and clinical signs appear 3–10 days after experimental infection in guinea pigs or hamsters. Chronic nephritis and uremia are sequels to infection in some animals (Faine 1998; Plank and Dean 2000).

Lesions

The host inflammatory response to renal tubular infection results in interstitial nephritis characterized by a mixed cellular infiltrate consisting of lymphocytes, monocytes, plasma cells, and occasional neutrophils. Fever recurs and the patient deteriorates rapidly as renal failure leads to uremia and oliguria; jaundice may appear clinically or subclinically, and hemorrhages develop rapidly throughout the body. The spleen and liver may be enlarged, and myocarditis and meningitis may develop (Faine et al. 1999).

Immunoblotting and immunohistochemistry studies involving the hamster model of leptospirosis found that the lipoproteins LipL32 and LipL41 are expressed by organisms within the proximal renal tubule, while LipL36 expression is down-regulated during infection (Haake et al. 1998; Barnett et al. 1999; Haake et al. 2000). However, the role of these outer-membrane components in pathogenesis is unknown.

Immunity

Clearly, leptospiral LPS is a major protective antigen. In an immunologically competent host, LPS antigens are recognized and processed to stimulate the development of specific IgM (Jost et al. 1989; Vinh et al. 1994). The IgM opsonizes the leptospires so that phagocytes engulf them in the reticuloendothelial organs (liver, spleen, lungs, and lymph nodes) resulting in rapid clearance of circulating leptospires from the bloodstream. Leptospires are able to persist in some anatomically localized and immunologically privileged sites after antibody and phagocytes have cleared leptospires from all other sites (Faine 1998).

Antibodies against leptospires are produced early after infection and maximum titers are achieved in 2–3 weeks. The nature of the immunoglobulin and its duration in the circulation differ according to animal species. Leptospires may be killed directly by complement or opsonized by specific immunoglobulins

directed at epitopes of the LPS, appearing as spherical degeneration forms inside macrophages and granulocytes. The specific immune protective epitopes of leptospires are complexes of oligosaccharides including phosphorylated sugars and aminosugars in the LPS side chains (Jost et al. 1989; Midwinter et al. 1994). Monoclonal antibody against a polysaccharide epitope of leptospiral LPS protected guinea pigs and hamsters against leptospirosis (Jost et al. 1986; Schoone et al. 1989). In cattle, IgM is usually detectable within 10 days of infection, most commonly within a week but sometimes delayed. In some individuals, IgG never appears and in others it is the first response to be detected.

The immune response to leptospires is almost entirely B-cell mediated, both in the initial infection and in the immediate response to reinfection (Adler and Faine 1977; Adler et al. 1980). Resistance to reinfection appears to depend on antibodies directed against serovar- or serogroup-reactive antigens. When detected, subsequent infections usually occur with a different serovar. It is estimated that specific IgG persists after a single episode of infection for 0.5–20 years or longer. Antibody against LPS is an important component of the protective immune response in some animal models (Jost et al. 1989). In contrast, cattle with high antibody titers to LPS were not protected against experimental challenge. This suggests that either other immune mechanisms or antibody to other leptospiral components are required to develop protective immunity in this species (Bolin et al. 1989a, 1989b; Zuerner et al. 1991). A protective killed vaccine against Hardjo induced strong antigen-specific proliferative responses by peripheral blood mononuclear cells. Interferon gamma (IFN-γ) was produced by up to a third of the mononuclear cells that in turn were shown to be of the γδT and the $CD4^+$ types (Naiman et al. 2001). These results indicated that the protective Hardjo vaccine induced a Th1, cell-mediated immune response, since $CD4^+$ T cells from vaccinated cattle produced IFN-γ in response to stimulation by leptospiral antigen, in contrast to the paradigm that protective immunity is primarily humoral (Naiman et al. 2001).

Guerreiro et al. (2001) confirmed that antibodies directed against LPS are predominantly IgM, while antibodies to proteins were mainly IgG. In addition, seven proteins—p76, p62, p48, p45, p41, p37, and p32—were identified as targets of the humoral response during natural infection by patient sera (Guerreiro et al. 2001). In that study, it was determined that the major outer-membrane lipoprotein LipL32, or Hap1 (Branger et al. 2001) is the immunodominant protein recognized by the humoral response during natural infection, at least in humans. Similar definitive studies have not been performed in animals. Immunohistochemistry demonstrated intense LipL32 reactivity in the kidneys of hamsters infected with Grippotyphosa, consistent with its expression *in vivo* (Haake et al. 2000). More recently, cross-protection against challenge with a heterologous serovar in gerbils was obtained following vaccination with Adenovirus expressing recombinant Hap1 (LipL32; Haake et al. 2000), whereas a similar OmpL1 construct failed to protect the animals (Branger et al. 2001).

The lipoprotein LipL41 is also recognized by antibodies produced during leptospiral infection. Synergistic immunoprotection of hamsters challenged with Grippotyphosa was reported with OmpL1 in conjunction with LipL41 expressed in *E. coli* as membrane-associated proteins. This constituted the first report of protection using recombinant proteins of *Leptospira* (Haake et al. 1999). Neither OmpL1 nor LipL41 were protective when administered individually.

Although statistically significant, the degree of protection induced by either Hap1 (LipL32) or OmpL1/LipL41 was marginal and disappointing. Hap1-vaccinated gerbils had a survival rate of 87% compared with 50% in unimmunized controls, whereas 71% of the OmpL1/LipL41-vaccinated hamsters survived compared with 20% in the control group. These protection rates compare unfavorably with the 100% protection that can be readily achieved in experimental animals by immunization with LPS. However, LPS-based immunity is restricted to antigenically related serovars. Future research must therefore be aimed at the identification and characterization of additional protein antigens with potential as vaccine candidates.

CONCLUSIONS

The major obstacle in gaining an understanding of leptospiral pathogenesis at the molecular level has been the lack of a genetic exchange system for pathogenic leptospires. Early reports (Ritchie and Ellinghausen 1966) of bacteriophage-like particles in leptospires were never confirmed, but Saint Girons et al. (1990) isolated three bacteriophages whose replication was limited to the saprophyte *L. biflexa*. One of these phages, LE1, was shown to

replicate as a plasmid in *L. biflexa* and was used as the basis for the first *L. biflexa–E. coli* plasmid shuttle vector (Saint Girons et al. 2000). Unfortunately, no such system has yet been reported for the pathogenic leptospires. There are only two reports of gene inactivation by recombination-mediated allelic replacement in *Leptospira*. *L. biflexa* mutants with an inactivated *flaB* gene, encoding the flagellar subunit protein, were nonmotile and lacked flagella and hooked ends but retained their helical shape (Picardeau et al. 2001). Inactivation of *recA* in *L. biflexa* resulted in reduced growth rate and altered nucleoid morphology (Kameni et al. 2002). Despite concerted efforts by a number of research groups, allelic replacement in pathogenic leptospires has not yet been reported.

At the time of writing, four *Leptospira* genome sequencing projects are in progress, but none has yet been published. However, the impending release of genome sequences for serovars Lai, Copenhageni, and Hardjobovis (two strains) will provide exciting opportunities for advancement of the elucidation of the mechanisms of pathogenesis, the identification of potential vaccine candidate antigens, and the understanding of the fundamental biology of *Leptospira*.

REFERENCES

Adin, C. A., and L. D. Cowgill. 2000. Treatment and outcome of dogs with leptospirosis: 36 cases (1990–1998). J. Am. Vet. Med. Assoc. 216:371–375.

Adler, B., and S. Faine. 1977. Host immunological mechanisms in the resistance of mice to leptospiral infections. Infect Immun. 17:67–72.

Adler, B., S. Faine, H. K. Muller, and D. E. Green. 1980. Maturation of humoral immune response determines the susceptibility of guinea-pigs to leptospirosis. Pathology 12: 529–538.

Agunloye, C. A., and A. S. Nash. 1996. Investigation of possible leptospiral infection in cats in Scotland. J. Small Anim. Pract. 37:126–129.

Alves, V. A., L. C. Gayotto, P. H. Yasuda, A. Wakamatsu, C. T. Kanamura, and T. de Brito. 1991. Leptospiral antigens *L. interrogans* serogroup ictero-haemorrhagiae in the kidney of experimentally infected guinea-pigs and their relation to the pathogenesis of renal injury. Exp. Pathol. 42:81–93.

Ballard, S. A., M. Williams, B. Adler, and S. Faine. 1986. Interactions of virulent and avirulent leptospires with primary cultures of renal epithelial cells. J. Med. Microbiol. 21:59–67.

Barnett, J., D. Barnett, C. Bolin, T. Summers, E. Wagar, N. Cheville, R. Hartskeerl, and D. Haake. 1999. Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. Infect. Immun. 67:853–861.

Barocchi, M. A., A. I. Ko, M. G. Reis, K. L. McDonald, and L. W. Riley. 2002. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. Infect. Immun. 70:6926–6932.

Bolin, C. A., A. B. Thiermann, A. L. Handsaker, and J. W. Foley. 1989a. Effect of vaccination with a pentavalent leptospiral vaccine on *Leptospira interrogans* serovar hardjo type hardjo-bovis infection of pregnant cattle. Am. J. Vet. Res. 50:161–165.

Bolin, C. A., R. L. Zuerner, and G. Trueba. 1989b. Effect of vaccination with a pentavalent leptospiral vaccine containing *Leptospira interrogans* serovar hardjo type harjo-bovis on type hardjo-bovis infection of cattle. Am. J. Vet. Res. 50:2004–2008.

Branger, C., C. Sonrier, B. Chatrenet, B. Klonjkowski, N. Ruvoen-Clouet, A. Aubert, G. Andre-Fontaine, and M. Eloit. 2001. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect. Immun. 69:6831–6838.

Brenner, D. J., A. F. Kaufmann, K. R. Sulzer, A. G. Steigerwalt, F. C. Rogers, and R. S. Weyant. 1999. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. Int. J. Syst. Bacteriol. 49:839–858.

Brenot, A., D. Trott, I. Saint Girons, and R. Zuerner. 2001. Penicillin-binding proteins in *Leptospira interrogans*. Antimicrob. Agents Chemother. 45:870–877.

Burth, P., M. Younes-Ibrahim, F. H. Goncalez, E. R. Costa, and M. V. Faria. 1997. Purification and characterization of a Na+, K+ ATPase inhibitor found in an endotoxin of *Leptospira interrogans*. Infect. Immun. 65:1557–1560.

Carroll, A. G. and R. S. F. Campbell. 1987. Reproductive and leptospiral studies on beef cattle in central Queensland. Aust. Vet. J. 64:1–5.

Chappel, R. J., B. Adler, R. T. Jones, L. J. Mead, B. D. Millar, and R. W. Prime. 1989. The prevalence of antileptospiral antibodies in the sera of pigs slaughtered at Victorian abattoirs. Aust. Microbiol. 10:347.

Cinco, M., E. Banfi, A. Furlani, and V. Scarcia. 1980. Cytotoxic activity of supernatant extracts of virulent and saprophytic leptospires. Zblatt. Bakt. Mikro. Hyg. Abt. I. Orig. A 248:260–267.

Cinco, M., E. Vecile, R. Murgia, P. Dobrina, and A. Dobrina. 1996. *Leptospira interrogans* and *Leptospira* peptidoglycans induce the release of tumor necrosis factor alpha from human monocytes. FEMS Microbiol. Lett. 138:211–214.

Cullen, P. A., S. J. Cordwell, D. M. Bulach, D. A. Haake, and B. Adler. 2002. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infect. Immun. 70:2311–2318.

de Brito, T., G. M. Böhm, and P. H. Yasuda. 1979. Vascular damage in acute experimental leptospirosis of the guinea-pig. J. Pathol. 128:177–182.

de Brito, T., M. J. Prado, V. A. Negreiros, A. L. Nicastri, E. E. Sakata, P. H. Yasuda, R. T. Santos, and A. Va. 1992. Detection of leptospiral antigen (*L. interrogans* serovar copenhageni serogroup Icterohaemorrhagiae) by immunoelectron microscopy in the liver and kidney of experimentally infected guinea-pigs. Int. J. Exp. Pathol. 73:633–642.

Diament, D., M. K. Brunialti, E. C. Romero, E. G. Kallas, and R. Salomao, R. 2002. Peripheral blood mononuclear cell activation induced by *Leptospira interrogans* glycolipoprotein. Infect. Immun. 70:1677–1683.

Dierauf, L. A., D. J. Vandenbroek, J. Roletto, M. Koski, L. Amaya, and L. J. Gage. 1985. An epizootic of leptospirosis in Californian sea lions (*Zalophus californianus*). J. Am. Vet. Med. Assoc. 187:1145–1148.

Donahue, J. M., B. J. Smith, K. B. Poonacha, J. K. Donahoe, and C. L. Rigsby. 1995. Prevalence and serovars of leptospira involved in equine abortions in central Kentucky during the 1991–1993 foaling seasons. J. Vet. Diagn. Invest. 7:87–91.

Elder, J. K. 1986. The influence of environmental factors on the survival of zoonotic bacterial pathogens with special reference to leptospirae. Aust. Microbiol. 7:323–324.

Ellis, W. A. 1995. International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira*. Minutes of the meetings, 1 and 2 July 1994, Prague, Czech Republic. Int. J. Syst. Bacteriol. 45:872–874.

Ellis, W. A., J. J. O'Brien, S. D. Neill, and D. G. Bryson. 1986. Bovine leptospirosis: experimental serovar hardjo infection. Vet. Microbiol. 11:23–299.

Estavoyer, J. M., E. Racadot, G. Couetdic, J. Leroy, and L. Grosperrin. 1991. Tumor necrosis factor in patients with leptospirosis. Rev. Infect. Dis. 13:1245–1246.

Faber, N. A., M. Crawford, R. B. LeFebvre, N. C. Buyukmihci, J. E. Madigan, and N. H. Willits. 2000. Detection of *Leptospira* spp. in the aqueous humor of horses with naturally acquired recurrent uveitis. J. Clin. Microbiol. 38:2731–2733.

Faine, S. 1998. Leptospirosis. In Topley and Wilson's microbiology and microbial infections. W. J. Hausler and M. Sussman (eds.). London: Arnold, pp. 849–869.

Faine, S., B. Adler, C. Bolin, and P. Perolat. 1999. *Leptospira* and Leptospirosis, 2nd. ed. Melbourne, Australia: MedSci.

Farrelly, H. E., B. Adler, and S. Faine. 1987. Opsonic monoclonal antibodies against lipopolysaccharide antigens of *Leptospira interrogans* serovar hardjo. J. Med. Microbiol. 23:1–7.

Feresu, S. B. 1987. Serological survey of leptospiral antibodies in cattle in Zimbabwe. Trop. Anim. Health Prod. 19:209–214.

Guerreiro, H., J. Croda, B. Flannery, M. Mazel, J. Matsunaga, M. Galvao Reis, P. N. Levett, A. Ko, and D. Haake. 2001. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect. Immun. 69:4958–4968.

Haake, D. A., C. Martinich, T. A. Summers, E. S. Shang, J. D. Pruetz, A. M. McCoy, M. K. Mazel, and C. A. Bolin. 1998. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect. Immun. 66:1579–1587.

Haake, D. A., E. M. Walker, D. R. Blanco, C. A. Bolin, J. N. Miller, and M. A. Lovett. 1991. Changes in the surface of *Leptospira interrogans* serovar grippotyphosa during *in vitro* cultivation. Infect. Immun. 59:1131–1140.

Haake, D. A., G. Chao, R. L. Zuerner, J. K. Barnett, D. Barnett, M. Mazel, J. Matsunaga, P. N. Levett, and C. A. Bolin. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68:2276–2285.

Haake, D. A., M. K. Mazel, A. M. McCoy, F. Milward, G. Chao, J. Matsunaga, and E. A. Wagar. 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect. Immun. 67:6572–6582.

Harkin, K. R., and C. L. Gartrell. 1996. Canine leptospirosis in New Jersey and Michigan: 17 cases (1990–1995). J. Am. Anim. Hosp. Assoc. 32:495–501.

Hookey, J. V., J. Bryden, and L. Gatehouse. 1993. The use of 16S rDNA sequence analysis to investigate the phylogeny of *Leptospiraceae* and related spirochaetes. J. Gen. Microbiol. 139:2585–2590.

Hovind-Hougen, K. 1979. *Leptospiraceae*, a new family to include *Leptospira* Noguchi 1917 and

Leptonema, gen. nov. Int. J. Syst. Bacteriol. 29:245–251.

Isogai, E., H. Isogai, N. Fujii, and K. Oguma. 1990. Biological effects of leptospiral lipopolysaccharide on mouse B, T and NK cells. Jpn. J. Vet. Sci. 52:923–930.

Jessup, D., R. Miller, C. Bolin, M. Kock, and P. Morkel. 1992. Retrospective evaluation of leptospirosis in free-ranging and captive black rhinoceroses (*Diceros bicornis*) by microscopic agglutination titres and fluorescent antibody testing. J. Zoo. Wild. Med. 401–408.

Jost, B. H., B. Adler, and S. Faine. 1989. Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J. Med. Microbiol. 29:115–120.

Jost, B. H., B. Adler, T. Vinh, and S. Faine. 1986. A monoclonal antibody reacting with a determinant on leptospiral lipolysaccharide protects guinea pigs against leptospirosis. J. Med. Microbiol. 22:269–275.

Kameni, A.-P. T., E. Couture-Tosi, I. Saint-Girons, and M. Picardeau. 2002. Inactivation of the spirochete *recA* gene results in a mutant with low viability and irregular nucleoid morphology. J. Bacteriol. 184:452–539.

Kinde, H., S. K. Hietala, C. A. Bolin, and J. T. Dowe. 1996. Leptospiral abortion in horses following a flooding incident. Equine Vet. J. 28:327–330.

Lee, S. H., K. A. Kim, Y. G. Park, I. W. Seong, M. J. Kim, and Y. J. Lee. 2000. Identification and partial characterization of a novel hemolysin from *Leptospira interrogans* serovar lai. Gene 254:19–28.

Leonard, F., P. J. Quinn, and W. A. Ellis. 1992. Possible effect of pH on the survival of leptospires in cattle urine. Vet. Rec. 131:53–4.

Li, C., A. Motaleb, M. Sal, S. F. Goldstein, and N. W. Charon. 2000. Spirochete periplasmic flagella and motility. J. Mol. Microbiol. Biotech. 2:345–354.

Lucchesi, P. M., A. E. Parma, and G. H. Arroyo. 2002. Serovar distribution of a DNA sequence involved in the antigenic relationship between *Leptospira* and equine cornea. BMC Microbiol. 2:3.

Merien, F., G. Baranton, and P. Perolat. 1997. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. Infect. Immun. 65:729–738.

Merien, F., J. Truccolo, G. Baranton, and P. Perolat. 2000. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar icterohaemorrhagiae. FEMS Microbiol. Lett. 185:17–22.

Midwinter, A., T. Vinh, S. Faine, and B. Adler. 1994. Characterization of an antigenic oligosaccharide from *Leptospira interrogans* serovar pomona and its role in immunity. Infect. Immun. 62:5477–5482.

Morgan, J., S. L. Bornstein, A. M. Karpati, M. Bruce, C. A. Bolin, C. C. Austin, C. W. Woods, J. Lingappa, C. Langkop, B. Davis, B., D. R. Graham, M. Proctor, D. A. Ashford, M. Bajani, S. L. Bragg, K. Shutt, B. A. Perkins, and J. W. Tappero. 2002. Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. Clin. Infect. Dis. 34:1593–1599.

Myburgh, J. G., S. J. Posnett, and J. V. Lawrence. 1993. Serological survey for canine leptospirosis in the Pretoria area. J. S. Afr. Vet. Assoc. 64:37–38.

Naiman, B. M., D. Alt, C. A. Bolin, R. G. Zuerner, and C. L. Baldwin. 2001. Protective killed *Leptospira borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and gammadelta T lymphocytes. Infect. Immun. 69:7550–7558.

Padre, L. P., G. Watt, M. L. Tuazon, R. Gray, and L. W. Laughlin. 1988. A serologic survey of rice-field leptospirosis in central Luzon, Philippines. SE Asian J. Trop. Med. Public Hlth. 19:197–199.

Paz-Soldan, S. V., M. T. Dianderas, and R. S. Windsor. 1991. *Leptospira interrogans* serovar canicola: a causal agent of sow abortions in Arequipa, Peru. Trop. Anim. Health Prod. 23:233–240.

Picardeau, M., A. Brenot, and I. Saint Girons. 2001. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa flaB* results in non-motile mutants deficient in endoflagella. Mol. Microbiol. 40:189–199.

Plank, R., and D. Dean. 2000. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. Microbes Infect. 2:1265–1276.

Potts, A. D., C. Lotter, and J. T. Robinson. 1995. Serological prevalence of leptospiral antibodies in pigs in South Africa. Onderstepoort J. Vet. Res. 62:281–284.

Ritchie, A. E., and H. C. Ellinghausen. 1966. Bacteriophage-like entities associated with a leptospire. 27th Ann. Proc. EMSA , not numbered.

Saint Girons, I., D. Margarita, P. Amouriaux, and G. Baranton. 1990. First isolation of bacteriophage for a spirochaete: potential genetic tools for *Leptospira*. Res. Microbiol. 141:1131–1138.

Saint Girons, I., P. Bourhy, C. Ottone, M. Picardeau, D. Yelton, D., R. W. Hendrix, P. Glaser, and N. Charon. 2000. The LE1 bacteriophage replicates as a plasmid within *Leptospira biflexa*: construction of

an *L. biflexa-Escherichia coli* shuttle vector. J. Bacteriol. 182:5700–5705.

Schoone, G. J., C. O. R. Everard, H. Korver, D. G. Carrington, V. A. Inniss, J. Baulu, and W. J. Terpstra. 1989. An immunoprotective monoclonal antibody directed against *Leptospira interrogans* serovar copenhageni. J. Gen. Microbiol. 135:73–78.

Segers, R. P., A. van der Drift, A. de Nijs, P. Corcione, B. A. van der Zeijst, and W. Gaastra. 1990. Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar hardjo. Infect. Immun. 58:2177–2185.

Segers, R. P., J. A. van Gestel, G. J. van Eys, B. A. van der Zeijst, and W. Gaastra. 1992. Presence of putative sphingomyelinase genes among members of the family *Leptospiraceae*. Infect. Immun. 60:1707–1710.

Smythe, L., M. Dohnt, M. Norris, M. Symonds, and J. Scott. 1997. Review of leptospirosis notifications in Queensland 1985 to 1996. Commun. Dis. Intell. 21:17–20.

Tajiki, H., and R. Salomao. 1996. Association of plasma levels of tumor necrosis factor alpha with severity of disease and mortality among patients with leptospirosis. Clin. Infect. Dis. 23:1177–1178.

Thiermann, A. 1982. Experimental leptospiral infections in pregnant cattle with organisms of the Hebdomadis serogroup. Am. J. Vet. Res. 43:780–784.

Trevejo, R. T., P. R. J. G. Rigau, D. A. Ashford, E. M. McClure, C. Jarquin-Gonzalez, J. J. Amador, J. O. de los Reyes, A. Gonzalez, S. R. Zaki, W. J. Shieh, R. G. McLean, R. S. Nasci, R. S. Weyant, C. A. Bolin, S. L. Bragg, S. L., B. A. Perkins, and R. A. Spiegel. 1998. Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995. J. Infect. Dis 178:1457–1463.

Vinh, T., B. Adler, and S. Faine. 1986a. Glycolipoprotein cytotoxin from *Leptospira interrogans* serovar copenhageni. J. Gen. Microbiol. 132:111–123.

———. 1986b. Ultrastructure and chemical composition of lipopolysaccharide extracted from *Leptospira interrogans* serovar copenhageni. J. Gen. Microbiol. 132:103–109.

Vinh, T., M-H. Shi, B. Adler, and S. Faine. 1989. Characterization and taxonomic significance of lipopolysaccharides of *Leptospira interrogans* serovar hardjo. J. Gen. Microbiol. 135:2663–2673.

Vinh, T., S. Faine, and B. Adler, B. 1984. Adhesion of leptospires to mouse fibroblasts (L929) and its enhancement by specific antibody. J. Med. Microbiol. 18:73–85.

Vinh, T., S. Faine, C. J. Handley, and B. Adler. 1994. Immunochemical studies of opsonic epitopes of the lipopolysaccharide of *Leptospira interrogans* serovar hardjo. FEMS Immunol. Med. Microbiol. 8:99–107.

Waitkins, S. A. 1986. Leptospirosis as an occupational disease. Brit. J. Indust. Med. 43:721–725.

Wang, B., J. Sullivan, G. W. Sullivan, and G. L. Mandell. 1984. Interaction of leptospires with human polymorphonuclear neutrophils. Infect. Immun. 44:459–464.

Weekes, C. C., C. O. Everard, and P. N. Levett. 1997. Seroepidemiology of canine leptospirosis on the island of Barbados. Vet. Microbiol. 57:215–22.

Werts, C., R. I. Tapping, J. C. Mathison, T. H. Chuang, V. Kravchenko, I. Saint Girons, D. A. Haake, P. Godowski, F. Hayashi, A. Ozinsky, D. M. Underhill, C. J. Kirschning, H. Wagner, H., A. Aderem, P. S. Tobias, and R. J. Ulevitch. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nature Immunol 2:346–352.

Yanagihara, Y., K. Kamisango, K. Takeda, I. Mifuchi, and I. Azuma. 1983. Identification of 4-o-methylmannose in cell wall polysachharide of *Leptospira*. Microbiol. Immunol. 27:711–715.

Yasuda, P. H., A. G. Steigerwalt, K. R. Sulzer, A. F. Kaufmann, F. Rogers, and D. J. Brenner. 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. Int. J. Syst. Bacteriol. 37:407–415.

Younes-Ibrahim, M., P. Burth, M. V. Faria, B. Buffin-Meyer, S. Marsy, C. Barlet-Bas, L. Cheval, and A. Doucet. 1995. Inhibition of Na,K-ATPase by an endotoxin extracted from *Leptospira interrogans*: a possible mechanism for the physiopathology of leptospirosis. Compt. Rend. Acad. Sci. Serie III, Sci. Vie. 318:619–625.

Yuri, K., Y. Takamoto, M. Okada, T. Hiramune, N. Kikuchi, and R. Yanagawa. 1993. Chemotaxis of leptospires to hemoglobin in relation to virulence. Infect. Immun. 61:2270–2272.

Zuerner, R. L., W. Knudtson, C. A. Bolin, and G. Trueba. 1991. Characterization of outer membrane and secreted proteins of *Leptospira interrogans* serovar pomona. Microb. Path. 10:311–322.

29
Mycoplasma

K. L. Whithear and G. F. Browning

Mycoplasmas are unique, cell-wall-less prokaryotes that are the smallest cells (down to approximately 300 nm diameter) and have 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. 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 *Haemobartonella* spp. and *Eperythrozoon* spp., formerly classified as rickettsia, are now considered to be members of the genus *Mycoplasma* (Neimark et al. 2001). Phylogenetically, Mollicutes are related to gram-positive bacteria with low genomic G + C mol % content (*Clostridium*, *Lactobacillus*, *Bacillus*, and *Streptococcus*) (Maniloff 1992).

CHARACTERISTICS OF THE ORGANISM

The small cell and genome sizes of mycoplasmas reflect fastidious requirements for growth, so that a highly enriched medium is needed to cultivate them in the laboratory. Osmotic stability under physiological conditions is maintained by incorporation in the cell membrane of cholesterol (unique among the prokaryotes), which, in all genera except *Acholeplasma*, must be supplied preformed. In nature, mycoplasmas are obligate parasites that are well adapted to survive on moist mucosal surfaces of their vertebrate hosts.

Although sharing common features, mycoplasmas are nevertheless 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, whereas 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. Intercurrent factors including coinfection with other agents and adverse environmental influences can play an important role in precipitating disease.

Whereas animal mycoplasmas are generally regarded as extracellular parasites, it is increasingly being recognized that at least some species may have the capacity to invade nonphagocytic cells.

SOURCES OF THE BACTERIUM

Mycoplasmas are usually considered to be relatively host specific. Although infection may occur in more than one host species, they tend to persist in a primary host, to which disease is often confined. Distribution 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 of carrier animals, while others have been found in unusual sites such as, for

example, members of the *Mycoplasma mycoides* cluster in the ears of goats.

Pathogenic mycoplasmas may be transmitted in respiratory aerosols, in milk, and in reproductive tract secretions of infected animals. They may be egg transmitted 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. 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

The adhesins have been characterized in only a limited number of mycoplasmas, and, whereas there are homologues of the characterized adhesins in some other mycoplasmas, it is clear that there may be different molecules and structures involved in adhesion in different species.

Attachment Organelle

The terminal attachment organelle is a specialized polar structure, composed of at least nine proteins, found in some mycoplasmas (Balish and Krause 2002). It is best characterized in the human pathogen *M. pneumoniae*, but a homologue is found in *M. gallisepticum* (fig. 29.1) and may be present in some other mycoplasmas (Goh et al. 1998; Hnatow et al. 1998; Papazisi et al. 2002b). The primary receptors are sialoglycoconjugates.

In *M. pneumoniae,* the attachment organelle consists of an elongated, electron-dense core, with a terminal button wider than the core. Six of the proteins associated with these structures appear to be concentrated in the button, while the other three are found in the core and are believed to be structural accessory proteins (Balish and Krause 2002).

The major adhesin molecule associated with this organelle is a large integral membrane protein called P1 in *M. pneumoniae* and GapA in *M. gallisepticum* (Goh et al. 1998). This protein is clustered at the tip of the attachment organelle and this clustering appears to be essential for adhesion and, in *M. pneumoniae*, for hemagglutination and hemadsorption. Antibody against GapA can inhibit adhesion (Goh et al. 1998). An additional two proteins, P40 and P90, cleaved from a 130 kDa protein

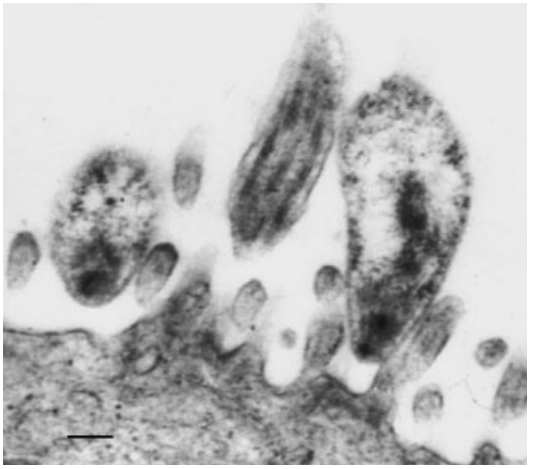


Figure 29.1. *M. gallisepticum* attaching to ciliated tracheal epithelial cells via terminal attachment organelle. Bar = 100 nm (transmission electron micrograph).

that derives from ORF6, immediately downstream of the gene that encodes P1, are also clustered at the tip of the attachment organelle and are closely associated with P1 (Balish and Krause 2002). They are also essential for adhesion, and they seem to function cooperatively with P1 in adhesion. The homologue of the ORF6 gene in *M. gallisepticum* is *crmA,* and its products are also essential for adhesion and virulence (Papazisi et al. 2002b)**.** A 47 to 49 kDa peripheral membrane protein, known as P65 because of its apparent molecular weight in electrophoretic analyses, also clusters at the tip of the attachment organelle and appears to be involved in adhesion, although its precise role has not been established (Balish and Krause 2002). P30 is an integral membrane protein localized at the tip of the attachment organelle. Its homologue in *M. gallisepticum* has been called Mgc2 (Hnatow et al. 1998). Antibody against Mgc2 is able to inhibit attachment *in vitro*, implicating it as a second adhesin.

The three accessory proteins, Hmw1, Hmw2, and Hmw3, are necessary for assembly of the core and, thus, the typical elongated morphology of the organism. Hmw3 is contained within the core, while Hmw1 and Hmw2 seem to be peripheral membrane proteins located around the core (Balish and Krause 2002). It is possible that other cytoskeletal proteins are also associated with the formation of the attachment organelle.

VlhA Hemagglutinin

In addition to the attachment organelle, *M. gallisepticum* contains a lipoprotein, known as VlhA (variable lipoprotein and hemagglutinin) or Pmga (in

earlier publications), that functions as its major hemagglutinin (Markham et al. 1994, 1998, 1999). This 70 kDa lipoprotein, distributed over the entire surface of the organism, binds to sialoglycoconjugates. The only known homologues of this hemagglutinin are found in two other avian mycoplasmas, *M. imitans*, a pathogen of game birds closely related phylogenically to *M. gallisepticum*, and *M. synoviae*, which is very distantly related phylogenically (Noormohammadi et al. 1998; Markham et al. 1999), but it has not been found in the apathogenic avian *Mycoplasma* species.

In most strains of *M. synoviae,* the *vlhA* gene product is posttranslationally cleaved into two proteins approximately equivalent in size, an amino terminal lipoprotein and a carboxyl terminal hemagglutinin, both of which remain localized in the membrane (Noormohammadi et al. 1997, 1998). Antibody against the hemagglutinin, but not the lipoprotein, inhibits hemagglutination (Noormohammadi et al. 1997).

Ciliary Adhesin of *M. hyopneumoniae*

The adhesin of the porcine pathogen *M. hyopneumoniae* is a 97 kDa peripheral membrane protein that binds specifically to three distinct sulphated glycolipids on the host-cell membrane of porcine tracheal cilia (Hsu et al. 1997). The region responsible for attachment to the cilial membrane is a repeated sequence of AAKP(V,E), with eight repeats required for binding (Minion et al. 2000). While adhesion can be inhibited by antibody against this repeated sequence, variation between strains in their capacity to adhere cannot be attributed to variation in the ciliary adhesin, suggesting involvement of other proteins in adherence. Binding by the ciliary adhesin is inhibited by heparin sulphate, dextran sulphate, mucin, fucoidan, and chondroitin sulphate B (Zhang et al. 1994).

LppS, a lipoprotein adhesin of *M. conjunctivae*, the cause of ovine and caprine infectious keratoconjunctivitis, has significant sequence similarity to the ciliary adhesin of *M. hyopneumoniae* but lacks the repeat sequences responsible for attachment. Antibody against this lipoprotein inhibits binding to lamb synovial cells *in vitro*, but the receptors on these cells have not been characterized (Belloy et al. 2003).

Cytadhesin of *M. agalactiae*

The P40 cytadhesin of *M. agalactiae* is a lipoprotein that mediates adherence to lamb synovial cells *in vitro* (Fleury et al. 2002). The receptor has not been defined, but binding of a homologue in the human pathogen *M. hominis*, P50, is inhibited by dextran sulphate, implicating sulphatides as the moieties to which this adhesin binds (Kitzerow et al. 1999).

Other Putative Adhesins

Some of the repeated peptide sequences in the variably expressed Vsp lipoproteins of *M. bovis* have been implicated as adhesins in studies using embryonic bovine lung cells (Sachse et al. 2000). Several additional proteins have been implicated as adhesins. PvpA of *M. gallisepticum* is a cell surface lipoprotein with a proline-rich carboxyl terminal region like proteins that are found in the terminal organelle (Boguslavsky et al. 2000). Proteins of 64 and 41 kDa in *M. hyopneumoniae* have been shown to be involved in hemagglutination of turkey erythrocytes (Young et al. 1989).

Capsules

Polysaccharide capsules have been demonstrated in many *Mycoplasma* species. However, in contrast to many other bacterial pathogens, production of a capsule does not appear to influence phagocytosis by neutrophils or macrophages. The capsular galactan of *M. mycoides* subsp. *mycoides* has been shown to have toxic effects when injected intravenously, causing apnea, increased pulmonary blood pressure, decreased systemic blood pressure, pulmonary hemorrhage and edema, and thrombosis in calves (Buttery et al. 1976). In addition, inoculation of purified galactan potentiates infection with *M. mycoides* subsp. *mycoides*, particularly enhancing dissemination in the circulation (Cottew 1979). It has been suggested that the capsular galactan may also promote deposition of fibrin around chronic lung lesions.

Hydrogen Peroxide Production

The only products of mycoplasmas established to cause direct cell damage are hydrogen peroxide and its superoxide derivatives. It appears to be generated as a product of the flavin-terminated electron transport chain common to most mycoplasmas. It is responsible both for the hemolytic activity of mycoplasmas *in vitro* and for their ciliostatic effect in tracheal organ cultures (Niang et al. 1998). Its action requires very close proximity to host cells and is inhibited by host catalase. Recent studies of less-virulent strains of *M. mycoides* subsp. *mycoides* have suggested that the reduced virulence of these strains may be associated with a decreased capacity to generate hydrogen peroxide (Vilei and Frey 2001).

With the possible exception of an experimentally induced nervous disease in mice and rats associated with *M. neurolyticum*, exotoxins do not appear to be important in the pathogenesis of mycoplasmal disease.

Superantigens

Superantigens are proteins capable of nonspecifically linking particular V beta chains of T-cell receptors and MHC class II molecules on antigen-presenting cells. This linkage, independent of antigen, results in clonal expansion of particular T-cell subsets, differentiation of B cells and polyclonal stimulation of antibody production, and activation of macrophages. The arthritogenic pathogen of mice and rats, *M. arthritidis*, produces a superantigen known as MAM (*M. arthritidis* mitogen), which is released by senescent cells (Atkin et al. 1994). Susceptibility of mice to acute death after intravenous inoculation, and dermal necrosis after subcutaneous inoculation, correlates with susceptibility to MAM (Cole et al. 1971). Superantigens have not been identified in other mycoplasmas.

Lipoproteins

The lipoproteins of mycoplasmas have a distinctive structure. Whereas in most bacteria acyl residues are attached to both the thiol and the amino groups of a terminal cysteine residue, mycoplasmas lack the enzyme for acylation of the amino group, and thus their lipoproteins are only acylated at the thiol group. This difference in structure results in a vastly increased stimulatory effect on macrophages (Muhlradt et al. 1998; Kaufmann et al. 1999). The high potency of these unusual lipoproteins for macrophage activation, similar to that of lipopolysaccharide (Galanos et al. 2000), contributes to the immunopathology associated with mycoplasma infections (Luhrmann et al. 2002). The lipoproteins are also among the most immunodominant antigens in mycoplasmas.

PATHOGENESIS

From a pathogenetic point of view, 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 localized extension of infection is of primary importance. In the second category, mycoplasmemia may occasionally occur, but it 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 29.2. Where other manifestations of disease are regularly recognized, these are also included in the tables.

Invasive Infections

Invasive *Mycoplasma* spp. 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, tendosynovitis, or arthritis. Sometimes more generalized infection and even acute septicemia may occur. Septicemic diseases are acute with fever and often death. Infections leading to polyserositis/arthritis tend to become persistent and are accompanied by more chronic inflammatory processes.

Septicemia and Multiple Organ System Diseases

M. mycoides subsp. *capri* (Thiaucourt et al. 2000) has the pathogenic potential to cause septicemia, principally in young goats. This may occur when host immunity is low or as a sequel to primary disease at another site (conjunctivitis, pneumonia, or mastitis). Clinical, pathologic, and clinicopathologic signs in kids are typical of acute septicemic disease (Ruffin 2001). Mortality is high, with postmortem changes including fibrinopurulent polyarthritis, embolic pneumonia, and thromboembolic lesions in various organs indicating a generalized intravascular coagulation crisis (Rosendal 1996).

Disease associated with *M. 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 been identified as the cause of a fatal multisystemic disease including fibrinous polyserositis, arthritis, and necrotizing pneumonia in captive American alligators (*Alligator mississippiensis*) in Florida, USA (Brown et al. 2001).

Polyserositis/Arthritis/Synovitis Syndromes

In pigs, *M. hyorhinis* colonizes the upper respiratory tract and *M. hyosynoviae* the tonsil without apparent harmful effect, although *M. hyorhinis* is a common secondary opportunistic pathogen in preexisting pneumonia. However, in young pigs

around weaning age, *M. hyorhinis* may 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 pleurisy, pericarditis, and peritonitis are present, and in chronic cases, fibrous adhesions are prominent. Affected joints initially contain serosanguineous synovial fluid and in the chronic stage the synovial membranes show nonsuppurative proliferative changes. Virulent strains of *M. hyosynoviae* cause arthritis in older growing pigs following some stress such as movement or vaccination. Joint lesions are similar but milder than those caused by *M. hyorhinis*. Heavy swine breeds are more susceptible.

M. synoviae is an inhabitant of the respiratory airways 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 tendosynovitis and arthritis. Exudate that is initially clear, then turbid, and later caseous is a prominent feature.

Several *Mycoplasma* spp. have been associated with arthritis and polyarthritis in cattle (table 29.1) particularly in young cattle soon after arrival at a feedlot. With *M. bovis*, arthritis often follows a primary pneumonia. It 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).

A polyarthritis and subacute pneumonia syndrome caused by *M. crocodylus* (47) has been described in farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe (Mohan et al. 1995).

Hemotrophic Infections

The hemotrophic mycoplasmas (and *Haemobartonella* and *Eperythrozoon* spp. of uncertain taxonomic affiliation) are epicellular parasites of red cells. There are a number of described species, each specific for a particular host. Only *M. haemofelis* comb. nov., nom. nov. (formerly *Haemobartonella felis*) in cats and *Eperythrozoon ovis* in lambs are regularly associated with clinically apparent hemolytic anemia. Anemia results 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 includes biting arthropods. Vertical transmission may also occur.

Localized Infections

Respiratory Tract

There are two main manifestations of mycoplasmal respiratory disease in mammalian species. One involves bronchitis, bronchiolitis, and pneumonia as features, occurring 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, for which there are two specific well-recognized conditions; contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP). Pleuritis in the horse is occasionally associated with *M. felis*. In avian species, mycoplasmal respiratory disease occurs as sinusitis, tracheitis, and air sacculitis.

Pneumonia. A persistent airway infection is a feature of many mycoplasma pneumonias. The *Mycoplasma* spp. most commonly involved are listed in table 29.2. Early stages of mycoplasmal disease 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 feature of most chronic mycoplasma pneumonias is proliferation of bronchiolar lymphoid tissue (cuffing pneumonia). Bronchiectasis is a feature of the disease caused by *M. pulmonis* in rats and mice. *M. cynos* in dogs causes a mild bronchointerstitial pneumonia and may contribute to the kennel cough complex. *M. bovis* is more aggressive than other *Mycoplasma* spp. associated with pneumonia in calves and produces lesions that include foci of necrosis. *M. hyopneumoniae* is of global economic importance as the cause of swine enzootic pneumonia and as a key component in porcine respiratory disease complex.

Pneumonic lesions typically occur in ventral parts of the apical and cardiac lobes of the lung, consistent with gravitational effects in the deposition of infectious aerosols and drainage of inflammatory products. Pneumonic lesions caused by *Mycoplasma* spp. alone are generally mild and, in the case of *M. ovipneumoniae* (nonprogressive pneumonia) in lambs and *M. dispar* in calves, are typically subclinical. However, they may predispose to secondary bacterial infections and more severe pneumonic lesions. Complex respiratory diseases with the mycoplasma as a key primary component

Table 29.1. Mycoplasma Diseases of Animals Characterized by Invasive Bloodborne Infection

Primary disease	Host(s)	Species	Other manifestations
Septicemia	Goats, sheep	[1]*M. mycoides* subsp. *capri*	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*	Air sacculitis
Arthritis/ polyarthritis	Cattle	*M. bovis* *Mycoplasma* sp. Bovine Group 7	Mastitis, pneumonia
		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	[2]*M. haemofelis comb. nov., nom. nov.*	

Source: Table modified from Rosendal (1996).
[1]Includes former subspecies *M. mycoides* subsp. *mycoides* large colony (LC) type and *M. mycoides* subsp. *capri.*
[2]Formerly *Haemobartonella felis.*

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 (SC) type and *M. capricolum* subsp. *capripneumoniae*, respectively. Both diseases are characterized by fibrinonecrotic pneumonia, serofibrinous pleuritis, and accumulations of serosanguineous fluid. Lung lesions may become sequestrated in a fibrous capsule. Organisms may remain viable in these lesions for long periods. Animals with sequestrated lesions are potential shedders of the organism (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 of organism. Strains currently occurring 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 respiratory airways in chickens and turkeys and some other 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 lungs and air sacs. Clinical signs observed may include tracheal rales, nasal discharge, and coughing. Histologic lesions in the respiratory passages (fig. 29.2) include marked thickening of the mucous membranes through hypertrophy and hyperplasia of the epithelium, edema, and subepithelial infiltration with lymphoid cells and heterophils. Deciliation and other degenerative changes of epithelial cells are also apparent, and the luminal surface is covered with a layer of exudate containing heterophils and necrotic cellular debris. When air sacculitis is severe, it is characterized by increased thickness of the air sacs with heterophil and mononuclear cell infiltration, fibrin exudation, and epithelial cell degeneration and hyperplasia. Gross swelling of the paranasal sinuses and ocular discharge are common in the turkey. Production may be compromised with reduced feed consumption, egg production, and weight gains. A similar respiratory syndrome is seen in chickens and turkeys with *M. synoviae*. *M. meleagridis* infection is confined to turkeys, where it

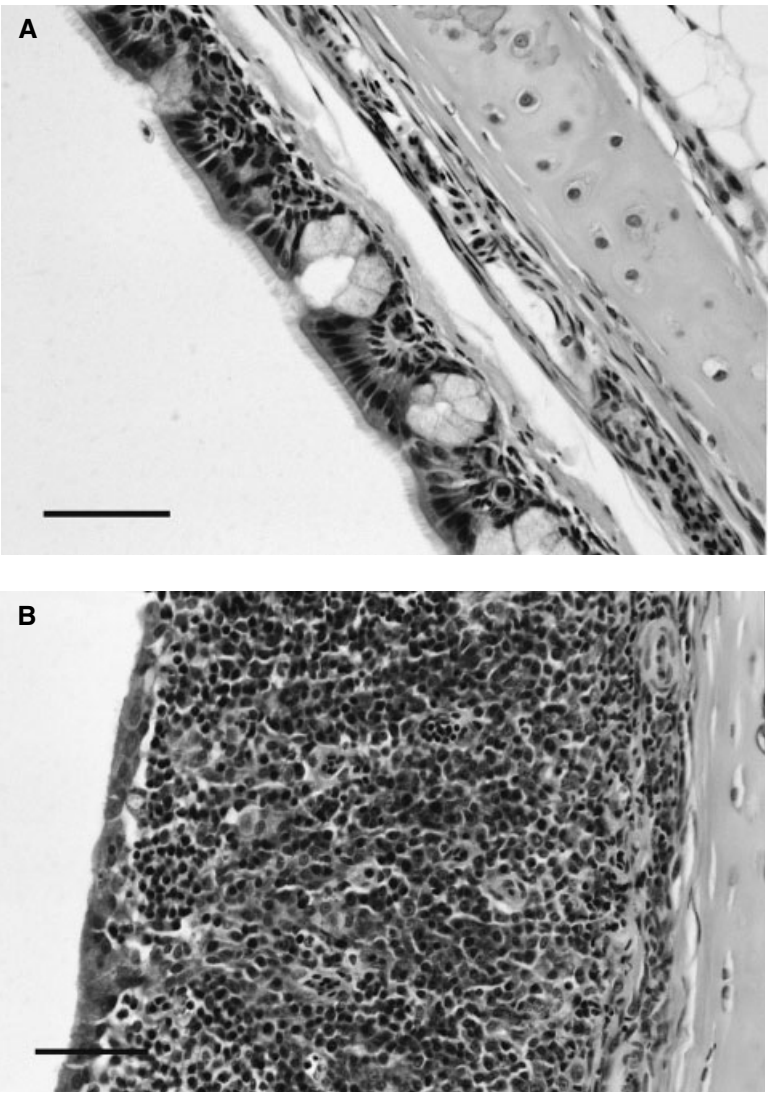


Figure 29.2. Sections of tracheas of 10-week-old chickens (*A*) uninfected and (*B*) infected 2 weeks previously with virulent *M. gallisepticum*. (*A*) shows normal ciliated epithelium. In (*B*) there is prominent thickening caused by infiltration of the lamina propria with macrophages, lymphocytes, plasma cells, and some heterophils. Metaplasia and loss of cilia of epithelial cells are also evident. Bar = 50 μm. (Micrographs courtesy of Dr. Amir Hadjinoormohammadi)

causes mild air sacculitis in turkey poults and has been incriminated in an osteodystrophic condition of tarsal bones. All of the pathogenic avian mycoplasmas can be transmitted horizontally via infectious aerosols or vertically via the egg.

Conjunctivitis

Mycoplasmas have been 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 is a feature of recent outbreaks of disease in songbirds in eastern North America caused by *M. gallisepticum* (Fischer et al. 1997). In cattle, *M. bovoculi* causes mild conjunctivitis but predisposes to more severe infection with *Moraxella bovis*. *M. conjunctivae* causes transient conjunctivitis and keratitis in sheep, goats, and

chamois. *M. felis* is reported as a cause of conjunctivitis in cats, but it can also be isolated from the eyes of clinically normal cats. *M. neurolyticum* also occurs in the conjunctivae of clinically normal and diseased mice. The organism is of interest in that it appears to be the only mycoplasma known that produces an exotoxin. When inoculated intravenously into mice, it elicits a neurologic syndrome and rapid death. The syndrome is termed "rolling disease" because the mice roll around their long axis. *M. 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. 2001).

Reproductive Tract Disease

Reproductive tract disease in animals caused by mycoplasmas is relatively uncommon despite their frequent isolation from that site. In birds, 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. Granular vulvovaginitis has been described in cattle (*Ureaplasma diversum* and *M. 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 most common and most severe. 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 as well as act as a source of infection for other cows.

Contagious agalactia is a syndrome in lactating sheep and goats caused by *M. agalactiae*. It involves primarily a severe mastitis, but the organism is invasive and not infrequently may enter the bloodstream to cause arthritis, pneumonia, and septicemia. It is also associated with keratoconjunctivitis.

Gill Erythrodermatitis in Fish

M. mobile has been isolated from freshwater fish with gill erythrodermatitis, and the disease has been reproduced with the organism (Brown 2002).

INTERACTIONS

Adherence and Motility

The initial step in interactions between mycoplasmas and their hosts is adherence. This has been best studied in the respiratory pathogens. The presence of multiple adhesins in the most-studied pathogens suggests that adherence is a multistep process. It is probable that initial attachment is effected by one adhesin, with subsequent closer adherence 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. Gliding motility, which proceeds in the direction of the terminal organelle (Miyata et al. 2000), is likely to facilitate penetration of mucous layers and insertion of the attachment organelle between the cilia. The organisms adhere with the terminal organelle closely attached to the membranes of the tracheal epithelial cells at the base of the cilia (fig. 29.1). While most of the other mycoplasmas pathogenic for animals do not possess an attachment organelle, they are capable of gliding motility, and it is probable that this promotes attachment to epithelial surfaces.

M. hyopneumoniae, which does not have an attachment organelle, adheres to the tips of the cilia of the respiratory epithelial cells, rather than at the base of the cilia (Blanchard et al. 1992).

Ciliostasis

Within 48 hours of attachment to respiratory epithelia, mycoplasmas induce ciliostasis (DeBey and Ross 1994). Close attachment to the epithelial membrane is essential for this effect, implicating production of reactive oxygen intermediates and the subsequent oxidative damage to the epithelial cells and their membranes, or alternatively utilization of crucial nutrients, such as arginine (Niang et al. 1998). Recent studies have found that 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) and suggest that this may be involved in the damage to cilia. Ciliostasis reduces the efficacy of respiratory clearance mechanisms and predisposes the respiratory tract to infection with other pathogens.

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
Pleuropneumonia	Cattle CBPP	*M. mycoides* subsp. *mycoides* SC type	Arthritis in calves
	Goats CCPP	*M. capricolum* subsp. *capripneumoniae*	
Pleuritis	Horses	*M. felis*	
Air sacculitis	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, vulvovaginitis
		M. bovigenitalium	Vulvovaginitis
		M. californicum	
		M. canadense	
		M. alkalescens	Arthritis
	Sheep, goats	*M. agalactiae*	Arthritis, septicemia, keratoconjunctivitis, vulvovaginitis
		M. capricolum subsp. capricolum	As for *M. agalactiae*
		M. mycoides subsp. *capri*	As for *M. agalactiae*
	Goats	*M. putrefaciens*	
Erythrodermatitis	Freshwater fish	*M. mobile*	Necrotizing gill lesions

Source: Table modified from Rosendal (1996).

Antigenic Variation

A common feature of many pathogenic mycoplasmas is the capacity for antigenic and phase variation of cell-surface proteins. The proteins involved differ between the species, as do the mechanisms used to generate variation, but in the most dramatic example over one hundred thousand variants of the same protein may be able to be generated. It is probable that in some pathogens this variation facilitates immune evasion, although in others it may enable expression of alternative functions, such as adherence to different cell types.

VlhA in *M. gallisepticum*

M. gallisepticum possesses between 30 and 70 variant *vlhA* genes, each of which is translationally competent (Baseggio et al. 1996). 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. The control of expression 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. 1998). 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 two weeks after infection, an increasing proportion of the population is capable of expressing a different member of the gene family. This suggests that the adhesin may be used only in the early stages of adherence and 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.

VlhA in *M. synoviae*

In *M. synoviae*, although the structural gene for VlhA is clearly homologous to those in *M. gallisepticum*, antigenic variation is achieved by distinctly different means. There is only a single, full-length gene, but many partial copies of different extents of the carboxyl end of the gene. 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 one hundred thousand 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.

Vlp in *M. hyorhinis*

The *vlp* gene family encodes variant cell-surface lipoproteins that are the immunodominant antigens of *M. hyorhinis*. Expression of the seven or more different 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 (Citti and Wise 1995). Multiple members of the family appear to be transcribed and translated concurrently, suggesting that the purpose for this phase variation is not likely to be immune evasion.

Vsp in *M. bovis* and *Vsa* in *M. pulmonis*

M. bovis contains at least 13 genes encoding variants of the immunodominant lipoprotein Vsp. Only one *vsp* gene appears to be transcribed at a time as there is only a single promoter sequence contained within the family (Lysnyansky et al. 1999). 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 the promoter sequence adjacent to different genes (Lysnyansky et al. 2001). A homologous gene family is found in *M. agalactiae*, the products of which are called Vpma, and the same mechanism appears to control antigenic variation in this species (Goh et al. 1998; Glew et al. 2000b).

In *M. pulmonis,* site-specific inversion is also used to control transcription of a family of at least 11 *vsa* genes (Bhugra et al. 1995). In this family all but one member lack the sequences encoding the promoter and the amino terminal end of the coding sequence. Site-specific inversions bring this single copy expression region into apposition with different family members.

Since the mechanism employed to generate variation in these two gene families results in expression of a single member at a time, it is possible that it may be involved in immune evasion, although this has not been established definitively.

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 has not been established, in *M. hyorhinis* longer variants of Vlp increase resistance to growth-inhibitory antibody (Citti et al. 1997).

Phase Variation

A number of lipoproteins of different mycoplasmas encoded by single copy genes have also been shown to be subject to high frequency phase variation in expression. This has been observed in the *MAA2* gene of *M. arthritidis* (Washburn et al. 1998), the *pvpA* gene of *M. gallisepticum* (Boguslavsky et al. 2000), the *vmm* gene of *M. mycoides* subsp. *mycoides* SC (Persson et al. 2002), 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 *M. hominis,* this variation has been shown to result 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

Several *Mycoplasma* species have been shown to 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). Although persistence in the invaded cell for at least 48 hours has been demonstrated, it is not yet certain whether intracellular replication can occur. If prolonged intracellular survival and/or replication do occur, this may help account for the chronicity of mycoplasmosis. Even in the absence of prolonged survival, intracellular invasion may facilitate penetration of the mucosal barrier.

The presence of a single bounding membrane in mycoplasmas permits direct interactions between their cell membrane and that of eukaryotic cells. Some *Mycoplasma* 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 immunological responses.

Lymphoid Responses

A major feature of mycoplasma infections is intense lymphoid proliferation. Studies in *M. gallisepticum* have shown that the lymphocytes infiltrating the mucosa of the trachea in the first week after infection are likely to be natural killer cells (CD8+ TCR-) (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 in the third week after infection that large numbers of B cells, in follicular arrangements, are seen. 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 pro-inflammatory chemokines. In the later stages, when B-cell proliferation is dominant, antigenic variation in the lipoproteins may cause chronic lymphoid stimulation.

Mycoplasma lipoproteins also 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 arthritis caused by mycoplasmas (Novak et al. 1995; Piec et al. 1999).

Studies on *M. pulmonis* infections have shown that CD8+ T cells play a significant role in the control of the immunopathology associated with disease, since depletion of these cells increases the severity of the 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 had no influence on the number of organisms in the lung.

Tissue Necrosis

The acute phase of disease caused by *M. mycoides* subsp. *mycoides* SC 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 effected by a diffusible toxin, possibly capsular galactan, which causes tissue necrosis.

Microbial Interactions

Synergistic interactions with other infectious agents are common in many mycoplasma diseases, particularly respiratory diseases. Interactions of mycoplasmas with viral agents may result in more severe manifestation of the viral disease or of the mycoplasmal disease. As an example of the former, *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 system virus (Thacker et al. 1999), thereby exacerbating pneumonic lesions and contributing significantly to the porcine respiratory disease complex. 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.*

Mycoplasmal colonization of the epithelial surface can also be facilitated by prior damage caused by viral infection. For example, infectious bronchitis virus or Newcastle disease virus infections in chickens predispose to more severe infections with *M. gallisepticum* or *M. synoviae.* Damage to the respiratory tract caused by *M. gallisepticum* also appears to predispose chickens to secondary infection with certain strains of *Escherichia coli,* resulting in much more severe disease. Mixed infections of *M. gallisepticum* and *Haemophilus paragallinarum* are also more severe than when either agent is acting alone.

M. bovoculi causes mild conjunctivitis but predisposes to more severe infection with *Moraxella bovis* and thus forms part of the etiologic complex of infectious bovine keratoconjunctivitis.

Influence of Environmental Factors

Environmental factors have a significant influence on many mycoplasmal diseases. Of particular importance has been the intensification of animal production. This form of production provides the sort of conditions most favorable for mycoplasma disease. High stocking densities favor transmission from animal to animal. The aim of intensive husbandry methods is to push the productive performance of the animal to the maximum practical limits. Any disease, no matter how mild, which affects this performance (lower efficiency of feed conversion, longer growing period) is economically important. Intensification is thus a double-edged sword, since it has the potential to be a highly efficient method of production but the margin for management error is small. Otherwise mild mycoplasmal infections can have a dramatic effect when environmental standards are suboptimal. For example, swine enzootic pneumonia is an ongoing problem in herds infected with *M. hyopneumoniae*. However, it varies substantially in severity 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. Economic loss becomes increasingly more apparent as severity (extent of lesions) of the disease increases. 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. The disease is mainly a problem in very large herds.

Environmental stress and pollutants such as ammonia and nitrites increase susceptibility to mycoplasma disease. This may be through a detrimental effect on the mucosal lining or through impaired macrophage function and activity of NK cells.

PROTECTIVE IMMUNITY

Natural Immunity

Innate immunity is important in determining the outcome of the initial interaction between pathogenic mycoplasmas and their hosts by helping to confine the organisms to their natural ecological niches as mucosal parasites of the upper respiratory or lower urogenital tracts. The result is that many mycoplasma diseases are subclinical, and the only

evidence of their occurrence may be flock seroconversion.

In the mouse, alveolar macrophages are the most important first line of defense in protecting the lungs against *M. pulmonis*. Activation of alveolar macrophages may be a direct response to interaction with the mycoplasma or may occur through activation of NK cells with consequent release of IFN-gamma (Hickman-Davis 2002). Opsonins including complement are concluded to be important for phagocytosis and killing of mycoplasmas, as are surfactant proteins SP-A and SP-D and reactive free radicals (Hickman-Davis 2002).

M. gallisepticum stimulates release of macrophage inflammatory protein-1 beta (MIP-1 beta) from macrophages, and this is chemotactic for chicken heterophils lymphocytes (Lam 2002). In the early acute stages of experimental infection with virulent *M. gallisepticum* in the chicken, CD8+ TCR- lymphocytes (believed to be the avian homologue of mammalian NK cells) infiltrate the tracheal mucosa and are arranged in follicle-like aggregates (Gaunson et al. 2000). These early innate responses are presumably an attempt by the host to contain the mucosal infection.

Although innate immunity plays a role in resistance, in naturally occurring infections in immunologically naïve animals, it may be insufficient to prevent disease. This is particularly the case in intensive food-animal production systems where large numbers of animals are housed in conditions of close confinement. 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.

Since mycoplasmas are pathogens of mucosal surfaces, local immune mechanisms are likely to be an important line of defense. It has been proposed that IgA may prevent the attachment of mycoplasma to mucosal epithelial cells. Whereas IgA is most probably involved in resistance to mycoplasma infection, enhancing IgA responses alone may not result in additional protection of the upper respiratory tract, and other mechanisms of mucosal immunity could also be needed to increase resistance (Hodge and Simecka 2002). For example, opsonins are important for efficient phagocytic activity and IgA has little opsonic activity. Antibody is important in preventing dissemination of infection to extrapulmonary sites in *M. pulmonis* (Cartner et al. 1998). The precise mechanism(s) of protection against *M. gallisepticum* remain to be determined. There is evidence that antibody is important, including the fact that neonatally bursectomized chickens show increased susceptibility to *M. gallisepticum* infection (Mukherjee et al. 1990). However, there does not appear to be a correlation between levels of serum antibody and protection (Whithear 1996). It has been suggested that local antibodies in the respiratory tract are likely to be more important, although which antibody class(es) is(are) most important remain unclear.

The nature of the histological response in mycoplasma-infected lung tissue (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 mycoplasma disease will prove to be a complex interaction of a range of responses that could differ for individual mycoplasmas.

Artificial Immunity

Some mycoplasma diseases have been successfully controlled by vaccination, notably *M. mycoides* subsp. *mycoides* SC type in cattle, *M. hyopneumoniae* in pigs, and *M. gallisepticum* and *M. synoviae* in poultry.

Louis Willems in the mid–nineteenth century was the first to use artificial immunization against a mycoplasmal disease (Huygelen 1997). By inoculating susceptible cattle in the tip of the tail with serous fluids taken from clinically affected cattle, Willems was able to protect them against subsequent exposure to infection. Since then, various live attenuated strains of *M. mycoides* subsp. *mycoides* SC have been developed 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 to react more severely to subsequent challenge (Gourlay 1975).

Bacterins are commercially available for the control of swine enzootic pneumonia. These vaccines stimulate humoral but not local antibody and not cell-mediated immunity. They are reported to reduce the incidence of lesions but do not prevent

infection. It is interesting that there is no correlation between serum antibody and protection (Etheridge and Lloyd 1982) with *M. hyopneumoniae*. Protection has also been induced experimentally in swine vaccinated with cell-free culture supernatant (Okada et al. 2000). A bacterin has recently been reported to be efficacious against *M. bovis* pneumonia induced by experimental challenge (Nicholas et al. 2002).

Bacterins have also been used against *M. gallisepticum* and *M. synoviae,* but these have been superseded by live attenuated vaccines. For example, strain ts-11 (Whithear et al. 1990) is a highly attenuated temperature-sensitive mutant of *M. gallisepticum* that has been successfully used in many countries to control *M. gallisepticum*. Temperature-sensitive mutant vaccines have also been reported to protect chickens against *M. synoviae* (Morrow et al. 1998) and rats against *M. pulmonis* infection (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 about the identity of protective immunogens and the mechanisms of protective immunity. However, more recently, expression library immunization (Moore et al. 2001) has been used as a means for screening clones expressing recombinant proteins of *M. hyopneumoniae* that may be protective and an *aro*A deletion mutant of *Salmonella typhimurium* has been used as a vector for oral administration of a recombinant *M. hyopneumoniae* vaccine (Fagan et al. 2001).

An experimental recombinant live attenuated *M. gallisepticum* vaccine has been recently constructed by insertion of wild-type *gap*A gene (see Adhesins) into an avirulent strain that had lost the gene following multiple passages in laboratory medium (Papazisi et al. 2002a). The recombinant strain expressed GapA on its surface and vaccinated chickens were protected following challenge with virulent *M. gallisepticum*. An alternative approach for *M. gallisepticum* vaccine development has been to insert the mgc3 gene (see Adhesins above) into a fowlpox virus vector (Yoshida et al. 2000).

Incorporation of immunogenic mycoplasma membrane proteins into immunostimulating complexes (ISCOMs) may be a novel way of presenting mycoplasma antigens to the mucosal immune system. ISCOMS are reported to elicit strong mucosal adjuvant activity following intranasal administration, initiating secretory IgA production in an IL-12-dependent manner, but independently of IL-4 (Hu et al. 2001).

CONCLUSIONS AND FUTURE DIRECTIONS

The pathogenesis of mycoplasmosis continues to be an area of active research, with most emphasis on further understanding of the mechanisms and significance of antigenic variation, the mechanisms involved in motility, the immune responses to infection, and mechanisms involved in adherence. 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. The proteins involved in motility, and how they propel the organisms, are likely to be elucidated in the next few years. 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 increased 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 homologues of those already known.

The genomes of several pathogenic mycoplasmas, including *M. pneumoniae*, *U. urealyticum,* and *M. pulmonis*, have been fully characterized in recent years, and the completion of genomic sequences for *M. gallisepticum*, *M. mycoides* subsp. mycoides SC, and *M. hyopneumoniae* is imminent. There is likely to be an increased emphasis on assigning function to genes of unknown function in these pathogenic mycoplasmas, with a particular emphasis on identifying virulence genes. A number of 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 are not obvious.

The mechanisms involved in the pathogenesis of the first mycoplasma described, *M. mycoides* subsp. *mycoides* SC, require further study, with recent findings suggesting that a deletion of a glycerol-transport operon, and consequent reduced capacity to produce hydrogen peroxide, may be associated with reduced virulence (Vilei and Frey 2001).

Finally, as the taxonomic position of the hemophilic mycoplasmas has only recently been clarified, the molecular pathogenesis of these species has not been studied. Given the atypical habitat of these pathogens, it is likely that details of the pathogenesis will be found to differ significantly from the other mycoplasmas.

REFERENCES

Atkin, C. L., S. Wei, and B. C. Cole. 1994. The *Mycoplasma arthritidis* superantigen MAM: purification and identification of an active peptide. Infect. Immun. 62:5367–5375.

Balish, M. F., and D. C. Krause. 2002. Cytadherence and the cytoskeleton. In Molecular biology and pathogenesis of mycoplasmas. S. Razin and R. Herrmann (eds.). New York: Kluwer Academic/Plenum Publishers, pp. 491–518.

Baseggio, N., M. D. Glew, P. F. Markham, K. G. Whithear, and G. F. Browning. 1996. Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. Microbiology 142:1429–1435.

Behrens, A., M. Heller, H. Kirchhoff, D. Yogev, and R. Rosengarten. 1994. A family of phase- and size-variant membrane surface lipoprotein antigens (Vsps) of *Mycoplasma bovis*. Infect. Immun. 62:5075–5084.

Belloy, L., E. M. Vilei, M. Giacometti, and J. Frey. 2003. Characterization of LppS, an adhesin of *Mycoplasma conjunctivae*. Microbiology 149:185–193.

Bhugra, B., L. L. Voelker, N. Zou, H. Yu, and K. Dybvig. 1995. Mechanism of antigenic variation in *Mycoplasma pulmonis*: interwoven, site-specific DNA inversions. Mol. Microbiol. 18:703–714.

Blanchard, B., M. M. Vena, A. Cavalier, J. Le Lannic, J. Gouranton, and M. Kobisch. 1992. Electron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*. Vet. Microbiol. 30:329–341.

Boguslavsky, S., D. Menaker, I. Lysnyansky, T. Liu, S. Levisohn, R. Rosengarten, M. Garcia, and D. Yogev. 2000. Molecular characterization of the *Mycoplasma gallisepticum* pvpA gene which encodes a putative variable cytadhesin protein. Infect. Immun. 68:3956–3964.

Brown, D. R. 2002. Mycoplasmosis and immunity of fish and reptiles. Front Biosci. 7:1338–1346.

Brown, D. R., J. M. Farley, L. A. Zacher, J. M. Carlton, T. L. Clippinger, J. G. Tully, and M. B. Brown. 2001. *Mycoplasma alligatoris sp. nov*., from American alligators. Int. J. Syst. Evol. Microbiol. 51:419–424.

Brown, M. B., D. R. Brown, P. A. Klein, G. S. McLaughlin, I. M. Schumacher, E. R. Jacobson, H. P. Adams, and J. G. Tully. 2001. *Mycoplasma agassizii sp. nov*., isolated from the upper respiratory tract of the desert tortoise (*Gopherus agassizii*) and the gopher tortoise (*Gopherus polyphemus*). Int. J. Syst. Evol. Microbiol. 51:413–418.

Buttery, S. H., L. C. Lloyd, and D. A. Titchen. 1976. Acute respiratory, circulatory and pathological changes in the calf after intravenous injections of the galactan from *Mycoplasma mycoides* subsp. *mycoides*. J. Med. Microbiol. 9:379–391.

Cartner, S. C., J. R. Lindsey, J. Gibbs-Erwin, G. H. Cassell, and J. W. Simecka. 1998. Roles of innate and adaptive immunity in respiratory mycoplasmosis. Infect. Immun. 66:3485–3491.

Citti, C., and K. S. Wise. 1995. *Mycoplasma hyorhinis* vlp gene transcription: critical role in phase variation and expression of surface lipoproteins. Mol. Microbiol. 18:649–660.

Citti, C., M. F. Kim, and K. S. Wise. 1997. Elongated versions of Vlp surface lipoproteins protect *Mycoplasma hyorhinis* escape variants from growth-inhibiting host antibodies. Infect. Immun. 65:1773–1785.

Citti, C., R. Watson-McKown, M. Droesse, and K. S. Wise. 2000. Gene families encoding phase- and size-variable surface lipoproteins of *Mycoplasma hyorhinis*. J. Bacteriol. 182:1356–1363.

Cole, B. C., J. R. Ward, R. S. Jones, and J. F. Cahill. 1971. Chronic proliferative arthritis of mice induced by *Mycoplasma arthritidis*. I. Induction of disease and histopathological characteristics. Infect. Immun. 4:344–355.

Cottew, G. S. 1979. Pathogenicity of the subspecies mycoides of *Mycoplasma mycoides* for cattle, sheep and goats. Zblatt. Bakt. [Orig A]. 245:164–170.

DeBey, M. C., and R. F. Ross. 1994. Ciliostasis and loss of cilia induced by *Mycoplasma hyopneumoniae* in porcine tracheal organ cultures. Infect. Immun. 62:5312–5318.

Etheridge, J. R., and L. C. Lloyd. 1982. A method for assessing induced resistance to enzootic pneumonia of pigs. Res. Vet. Sci. 33:188–191.

Fagan, P. K., M. J. Walker, J. Chin, G. J. Eamens, and S. P. Djordjevic. 2001. Oral immunization of swine with attenuated Salmonella typhimurium aroA SL3261 expressing a recombinant antigen of *Mycoplasma hyopneumoniae* (NrdF) primes the immune system for a NrdF specific secretory IgA response in the lungs. Microb. Pathog. 30:101–110.

Fischer, J. R., D. E. Stallknecht, P. Luttrell, A. A. Dhondt, and K. A. Converse. 1997. Mycoplasmal conjunctivitis in wild songbirds: the spread of a

new contagious disease in a mobile host population. Emerg. Infect. Dis. 3:69–72.

Fleury, B., D. Bergonier, X. Berthelot, E. Peterhans, J. Frey, and E. M. Vilei. 2002. Characterization of P40, a cytadhesin of *Mycoplasma agalactiae*. Infect. Immun. 70:5612–5621.

Galanos, C., M. Gumenscheimer, P. Muhlradt, E. Jirillo, and M. Freudenberg. 2000. MALP-2, a Mycoplasma lipopeptide with classical endotoxic properties: end of an era of LPS monopoly? J. Endotoxin. Res. 6:471–476.

Gaunson, J. E., C. J. Philip, K. G. Whithear, and G. F. Browning. 2000. Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. Microbiology 146:1223–1229.

Glew, M. D., G. F. Browning, P. F. Markham, and I. D. Walker. 2000a. pMGA phenotypic variation in *Mycoplasma gallisepticum* occurs in vivo and is mediated by trinucleotide repeat length variation. Infect. Immun. 68:6027–6033.

Glew, M. D., L. Papazisi, F. Poumarat, D. Bergonier, R. Rosengarten, and C. Citti. 2000b. Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. Infect. Immun. 68:4539–4548.

Glew, M. D., N. Baseggio, P. F. Markham, G. F. Browning, and I. D. Walker. 1998. Expression of the pMGA genes of *Mycoplasma gallisepticum* is controlled by variation in the GAA trinucleotide repeat lengths within the 5' noncoding regions. Infect. Immun. 66:5833–5841.

Goh, M. S., T. S. Gorton, M. H. Forsyth, K. E. Troy, and S. J. Geary. 1998. Molecular and biochemical analysis of a 105 kDa *Mycoplasma gallisepticum* cytadhesin (GapA). Microbiology 144:2971–2978.

Gourlay, R. N. 1975. Contagious bovine pleuropneumonia-protection following natural infection and vaccination. Dev. Biol. Stand. 28:586–589.

Hickman-Davis, J. M. 2002. Role of innate immunity in respiratory mycoplasma infection. Front Biosci 7:d1347–55.

Hnatow, L. L., C. L. Keeler, Jr., L. L. Tessmer, K. Czymmek, and J. E. Dohms. 1998. Characterization of MGC2, a *Mycoplasma gallisepticum* cytadhesin with homology to the *Mycoplasma pneumoniae* 30-kilodalton protein P30 and Mycoplasma genitalium P32. Infect. Immun. 66:3436–3442.

Hodge, L. M., and J. W. Simecka. 2002. Role of upper and lower respiratory tract immunity in resistance to Mycoplasma respiratory disease. J. Infect. Dis. 186:290–294.

Hsu, T., S. Artiushin, and F. C. Minion. 1997. Cloning and functional analysis of the P97 swine cilium adhesin gene of *Mycoplasma hyopneumoniae*. J. Bacteriol. 179:1317–1323.

Hu, K. F., K. Lovgren-Bengtsson, and B. Morein. 2001. Immunostimulating complexes (ISCOMs) for nasal vaccination. Adv. Drug Deliv. Rev. 51:149–159.

Huygelen, C. 1997. Louis Willems (1822–1907) and the immunization against contagious bovine pleuropneumonia. An evaluation. Verh. K. Acad. Geneeskd. Belg. 59:237–285.

Jones, H. P., L. Tabor, X. Sun, M. D. Woolard, and J. W. Simecka. 2002. Depletion of CD8+ T cells exacerbates CD4+ Th cell-associated inflammatory lesions during murine mycoplasma respiratory disease. J. Immunol. 168:3493–3501.

Kaufmann, A., P. F. Muhlradt, D. Gemsa, and H. Sprenger. 1999. Induction of cytokines and chemokines in human monocytes by *Mycoplasma fermentans*-derived lipoprotein MALP-2. Infect. Immun. 67:6303–6308.

Kitzerow, A., U. Hadding, and B. Henrich. 1999. Cyto-adherence studies of the adhesin P50 of *Mycoplasma hominis*. J. Med. Microbiol. 48:485–493.

Kleven, S. H., O. J. Fletcher, and R. B. Davis. 1975. Influence of strain of *Mycoplasma synoviae* and route of infection on development of synovitis or airsacculitis in broilers. Avian Dis. 19:126–135.

Lai, W. C., M. Bennett, Y. S. Lu, and S. P. Pakes. 1991. Vaccination of Lewis rats with temperature-sensitive mutants of *Mycoplasma pulmonis*: adoptive transfer of immunity by spleen cells but not by sera. Infect. Immun. 59:346–350.

Lam, K. M. 2002. The macrophage inflammatory protein-1beta in the supernatants of *Mycoplasma gallisepticum*-infected chicken leukocytes attracts the migration of chicken heterophils and lymphocytes. Dev. Comp. Immunol. 26:85–93.

Luhrmann, A., U. Deiters, J. Skokowa, M. Hanke, J. E. Gessner, P. F. Muhlradt, R. Pabst, and T. Tschernig. 2002. In vivo effects of a synthetic 2-kilodalton macrophage-activating lipopeptide of *Mycoplasma fermentans* after pulmonary application. Infect. Immun. 70:3785–3792.

Lysnyansky, I., K. Sachse, R. Rosenbusch, S. Levisohn, and D. Yogev. 1999. The *vsp* locus of *Mycoplasma bovis*: gene organization and structural features. J. Bacteriol. 181:5734–5741.

Lysnyansky, I., Y. Ron, and D. Yogev. 2001. Juxtaposition of an active promoter to vsp genes via site-specific DNA inversions generates antigenic variation in *Mycoplasma bovis*. J. Bacteriol. 183:5698–5708.

Maniloff, J. 1992. Phylogeny of mycoplasmas. In Mycoplasmas: molecular biology and pathogenesis. J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (eds.). Washington, DC: American Society for Microbiology, pp. 549–559.

Markham, P. F., M. D. Glew, G. F. Browning, K. G. Whithear, and I. D. Walker. 1998. Expression of two members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is influenced by pMGA-specific antibodies. Infect. Immun. 66:2845–2853.

Markham, P. F., M. D. Glew, J. E. Sykes, T. R. Bowden, T. D. Pollocks, G. F. Browning, K. G. Whithear, and I. D. Walker. 1994. The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*. FEBS Lett. 352:347-352.

Markham, P. F., M. F. Duffy, M. D. Glew, and G. F. Browning. 1999. A gene family in *Mycoplasma imitans* closely related to the pMGA family of *Mycoplasma gallisepticum*. Microbiology 145:2095–2103.

Minion, F. C., C. Adams, and T. Hsu. 2000. R1 region of P97 mediates adherence of *Mycoplasma hyopneumoniae* to swine cilia. Infect. Immun. 68:3056–3060.

Miyata, M., H. Yamamoto, T. Shimizu, A. Uenoyama, C. Citti, and R. Rosengarten. 2000. Gliding mutants of *Mycoplasma mobile*: relationships between motility and cell morphology, cell adhesion and microcolony formation. Microbiology 146:1311–1320.

Mohan, K., C. M. Foggin, P. Muvavarirwa, J. Honywill, and A. Pawandiwa. 1995. Mycoplasma-associated polyarthritis in farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe. Onderstepoort J. Vet. Res. 62:45–49.

Moore, R. J., C. Lenghaus, S. A. Sheedy, and T. J. Doran. 2001. Improved vectors for expression library immunization—application to *Mycoplasma hyopneumoniae* infection in pigs. Vaccine 20:115–120.

Morrow, C. J., J. F. Markham, and K. G. Whithear. 1998. Production of temperature-sensitive clones of *Mycoplasma synoviae* for evaluation as live vaccines. Avian Dis. 42:667–670.

Muhlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung. 1998. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. Infect. Immun. 66:4804–4810.

Mukherjee, F., B. K. Tiwary, R. L. Prasad, G. J. Jha, and M. K. Roy. 1990. Role of T and B cells in immune response to *Mycoplasma gallisepticum* infection in normal and immunocompromised chicken. Indian J. Anim. Sci. 60:1027–1031.

Neimark, H., K. E. Johansson, Y. Rikihisa, and J. G. Tully. 2001. Proposal to transfer some members of the genera Haemobartonella and Eperythrozoon to the genus Mycoplasma with descriptions of *"Candidatus Mycoplasma haemofelis", "Candidatus Mycoplasma haemomuris", "Candidatus Mycoplasma haemosuis"'* and *"Candidatus Mycoplasma wenyonii"*. Int. J. Syst. Evol. Microbiol. 51:891–899.

Niang, M., R. F. Rosenbusch, M. C. DeBey, Y. Niyo, J. J. Andrews, and M. L. Kaeberle. 1998. Field isolates of *Mycoplasma ovipneumoniae* exhibit distinct cytopathic effects in ovine tracheal organ cultures. Zblatt. Vet. Med. A 45:29–40.

Nicholas, R., R. Ayling, and L. Stipkovits. 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. Vaccine 20:3569–3575.

Noormohammadi, A. H., P. F. Markham, A. Kanci, K. G. Whithear, and G. F. Browning. 2000. A novel mechanism for control of antigenic variation in the haemagglutinin gene family of *Mycoplasma synoviae*. Mol. Microbiol. 35:911–923.

Noormohammadi, A. H., P. F. Markham, K. G. Whithear, I. D. Walker, V. A. Gurevich, D. H. Ley, and G. F. Browning. 1997. *Mycoplasma synoviae* has two distinct phase-variable major membrane antigens, one of which is a putative hemagglutinin. Infect. Immun. 65:2542–2547.

Noormohammadi, A. H., P. F. Markham, M. F. Duffy, K. G. Whithear, and G. F. Browning. 1998. Multigene families encoding the major hemagglutinins in phylogenetically distinct mycoplasmas. Infect. Immun. 66:3470–3475.

Novak, J. F., J. D. Hayes, Jr., and J. H. McMaster. 1995. Mycoplasma-mediated bone resorption in bone organ cultures. Microbios 81:241–260.

Okada, M., T. Asai, M. Ono, T. Sakano, and S. Sato. 2000. Protective effect of vaccination with culture supernate of *M. hyopneumoniae* against experimental infection in pigs. J. Vet. Med. B Infect. Dis. Vet. Public Health 47:527–533.

Papazisi, L., L. K. Silbart, S. Frasca, D. Rood, X. Liao, M. Gladd, M. A. Javed, and S. J. Geary. 2002a. A modified live *Mycoplasma gallisepticum* vaccine to protect chickens from respiratory disease. Vaccine 20:3709–3719.

Papazisi, L., S. Frasca, Jr., M. Gladd, X. Liao, D. Yogev, and S. J. Geary. 2002b. GapA and CrmA coexpression is essential for *Mycoplasma gallisepticum* cytadherence and virulence. Infect. Immun. 70:6839–6845.

Park, S. C., S. Yibchok-Anun, H. Cheng, T. F. Young, E. L. Thacker, F. C. Minion, R. F. Ross, and W. H. Hsu. 2002. *Mycoplasma hyopneumoniae* increases intracellular calcium release in porcine ciliated tracheal cells. Infect. Immun. 70:2502–2506.

Persson, A., K. Jacobsson, L. Frykberg, K. E. Johansson, and F. Poumarat. 2002. Variable surface protein Vmm of *Mycoplasma mycoides* subsp. *mycoides* small colony type. J. Bacteriol. 184:3712–3722.

Piec, G., J. Mirkovitch, S. Palacio, P. F. Muhlradt, and R. Felix. 1999. Effect of MALP-2, a lipopeptide from *Mycoplasma fermentans*, on bone resorption in vitro. Infect. Immun. 67:6281–6285.

Rocha, E. P., and A. Blanchard. 2002. Genomic repeats, genome plasticity and the dynamics of mycoplasma evolution. Nucleic Acids Res. 30:2031–2042.

Rosendal, S. 1996. Mycoplasma. In Pathogenesis of bacterial infections of animals, 2nd ed. C. O. Gyles and C. L. Thoen (eds.). Ames: Iowa State University Press, pp. 297–311.

Rottem, S. 2002. Invasion of mycoplasmas into and fusion with host cells. In Molecular biology and pathogenesis of mycoplasmas. S. Razin and R. Herrmann (eds.). New York: Kluwer Academic/Plenum Publishers, pp. 391–402.

Ruffin, D. C. 2001. Mycoplasma infections in small ruminants. Vet. Clin. North Am. Food Anim. Pract. 17:315–332.

Sachse, K., J. H. Helbig, I. Lysnyansky, C. Grajetzki, W. Muller, E. Jacobs, and D. Yogev. 2000. Epitope mapping of immunogenic and adhesive structures in repetitive domains of *Mycoplasma bovis* variable surface lipoproteins. Infect. Immun. 68:680–687.

Thacker, E. L., P. G. Halbur, R. F. Ross, R. Thanawongnuwech, and B. J. Thacker. 1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. J. Clin. Microbiol. 37:620–627.

Thiaucourt, F., S. Lorenzon, A. David, and A. Breard. 2000. Phylogeny of the *Mycoplasma mycoides* cluster as shown by sequencing of a putative membrane protein gene. Vet. Microbiol. 72:251–268.

Vilei, E. M., and J. Frey. 2001. Genetic and biochemical characterization of glycerol uptake in *Mycoplasma mycoides* subsp. *mycoides* SC: its impact on H_2O_2 production and virulence. Clin. Diagn. Lab. Immunol. 8:85–92.

Washburn, L. R., K. E. Weaver, E. J. Weaver, W. Donelan, and S. Al-Sheboul. 1998. Molecular characterization of *Mycoplasma arthritidis* variable surface protein MAA2. Infect. Immun. 66:2576–2586.

Whithear, K. G. 1996. Control of avian mycoplasmoses by vaccination. Rev. Sci. Tech. 15:1527–1553.

Whithear, K. G., K. Soeripto, E. Harrigan, and E. Ghiocas. 1990. Immunogenicity of a temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust. Vet. J. 67:168–174.

Winner, F., R. Rosengarten, and C. Citti. 2000. In vitro cell invasion of *Mycoplasma gallisepticum*. Infect. Immun. 68:4238–4244.

Yoshida, S., A. Fujisawa, Y. Tsuzaki, and S. Saitoh. 2000. Identification and expression of a *Mycoplasma gallisepticum* surface antigen recognized by a monoclonal antibody capable of inhibiting both growth and metabolism. Infect. Immun. 68:3186–3192.

Young, T. F., B. Z. Erickson, R. F. Ross, and Y. Wannemuehler. 1989. Hemagglutination and hemagglutination inhibition of turkey red blood cells with *Mycoplasma hyopneumoniae*. Am. J. Vet. Res. 50:1052–1055.

Zhang, Q., and K. S. Wise. 1997. Localized reversible frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. Mol. Microbiol. 25:859–869.

———. 2001. Coupled phase-variable expression and epitope masking of selective surface lipoproteins increase surface phenotypic diversity in *Mycoplasma hominis*. Infect. Immun. 69:5177–5181.

Zhang, Q., T. F. Young, and R. F. Ross. 1994. Glycolipid receptors for attachment of *Mycoplasma hyopneumoniae* to porcine respiratory ciliated cells. Infect. Immun. 62:4367–4373.

30
Chlamydia

A. A. Andersen

Chlamydiae are among the most successful of pathogens, being more widespread in nature than most pathogenic organisms. They have evolved to the point at which each chlamydial strain is associated with specific diseases in a given host. Our knowledge of their importance as disease agents and the extent of their distribution in nature is increasing rapidly, benefiting from improvements in laboratory and diagnostic techniques.

CLASSIFICATION

The family Chlamydiaceae was recently reclassified into two genera and nine species based on sequence analysis of its 16S and 23S rRNA genes (Everett et al. 1999). The two new genera, *Chlamydia* and *Chlamydophila*, correlate with the former species *C. trachomatis* and *C. psittaci*, respectively. The genus *Chlamydia* includes *C. trachomatis* (human), *C. suis* (swine), and *C. muridarum* (mouse, hamster). The genus *Chlamydophila* includes *C. psittaci* (avian), *C. felis* (cats), *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea pigs), and the former species *Chlamydia pecorum* (sheep, cattle) and *Chlamydia pneumoniae* (human).

The organization into two genera and nine species is logical at the molecular level and is of practical value in its classification of host range and clinical disease. The species show a high degree of correlation with host range, disease syndrome, and virulence, and thus the classification parallels our understanding of the epidemiology of the various chlamydial species and serovars affecting livestock and birds.

HOSTS AND KINDS OF DISEASE

Table 30.1 gives the new classification along with the primary host range and primary diseases. With improved typing of isolates, new species will likely be identified along with a better understanding of the hosts chlamydiae can infect and the diseases they cause. A number of the chlamydial species are known to infect nonhost animals or birds, causing severe disease; however, secondary spread is usually limited.

The terms "chlamydiosis" and "chlamydia(e)" are used as generic terms to refer to members of both genera. However, the new scientific names are used when referring to a specific chlamydial species.

In humans, *C. trachomatis* is the leading cause of preventable blindness and the most common agent of sexually transmitted diseases (Schachter 1999). *C. pneumoniae* is increasingly being recognized as a significant cause of pneumonia and other acute respiratory tract infections in humans and is thought to infect most individuals at some time during life (Grayston 1992). There is increasing evidence that it may be associated with atherosclerosis.

In production agriculture, four of the chlamydial species have been associated with disease. These are *Chlamydophila psittaci, Chlamydophila abortus, Chlamydophila pecorum,* and *Chlamydia suis*.

C. psittaci primarily infects birds. This species includes six known avian serovars and two mammalian serovars, M56 from muskrats and WC from cattle. M56 and WC were each isolated from a single outbreak. The six avian serovars are labeled A through F and each shows host specificity. The hosts with which each serovar has been associated are: A, psittacine birds; B, pigeons; C, ducks and geese; D, turkeys; E, pigeons and ratites; and F, a single isolate from a psittacine bird (Andersen 1991, 1997). What is not known is how many of these birds and mammals are the natural hosts of the serovars.

Table 30.1. Classification of Chlamydiaceae and Primary Hosts and Diseases

Species	Primary host	Diseases
Chlamydia		
C. trachomatis	Humans	Reproductive, conjunctivitis
C. suis	Swine	Enteritis, respiratory, conjunctivitis
C. muridarum	Rodents	Respiratory
Chlamydophila		
C. psittaci	Birds	Respiratory, enteritis
C. abortus	Ruminants	Abortion
C. caviae	Guinea pigs	Conjunctivitis
C. felis	Cats	Respiratory, conjunctivitis
C. pneumoniae	Humans, horses, koalas	Respiratory
C. pecorum	Ruminants, koalas	Respiratory, conjunctivitis, encephalomyelitis, polyarthritis

Chlamydophila abortus primarily infects ruminants, causing late-term abortions or weak young. Most losses occur in sheep, in which it causes epizootic abortion. It can infect cattle, other ruminants, and pigs. Abortions have also been reported in women caring for sheep after lambing. It originally was thought that there was only one serotype; however, there are now indications that additional serotypes may exist.

C. pecorum, a group of diverse strains causing a number of disease syndromes, has been reported in koalas, swine, and most ruminants. *C. pecorum* isolates have been associated with encephalomyelitis, polyarthritis, conjunctivitis, enteritis, pneumonia, and reproductive problems. Cases are often sporadic, although outbreaks involving large numbers in a flock or herd have been reported. It is clear that a number of distinct strains are involved but whether specific strains are correlated with specific disease syndromes is not known.

C. suis has been identified only recently in swine. It has been associated with and isolated from cases of respiratory disease, enteritis, and conjunctivitis. A number of distinct strains have been isolated. Some of these isolates produced severe disease in gnotobiotic pigs (Rogers et al. 1996; Rogers and Andersen 1996). Serological studies indicate that most pigs are infected early in life. The organism can be isolated from normal pigs and from pigs with enteritis and pneumonia. Isolation studies have shown that pigs with enteritis and/or pneumonia are three times more likely than normal pigs to be infected with chlamydia (Nietfeld et al. 1997; Hoelzle et al. 2000). The significance of these isolates and their roles in these diseases is still not understood.

LIFE CYCLE

Chlamydiae are obligate, intracellular parasites that multiply in the cytoplasm of eukaryotic cells, forming membrane-bound cytoplasmic inclusions. They are dependent on the host cell for energy and the majority of their nucleotide-metabolizing enzymes. The life cycle is unique, having a growth cycle consisting of two major developmental forms. The elementary body (EB) is a condensed form, 200–300 nm in diameter, which is suited to survival outside the cell. The reticulate body (RB), ranging in size from 500 to 1,000 nm, is the replicating form found in the cytoplasmic inclusions and predominates throughout most of the developmental cycle. Replication is by binary fission typical of other bacteria, with the exception that chlamydiae rely on the host cell for nutrients. Intermediate forms are usually seen and vary in size from 300 to 500 nm. These often are called dispersing forms or condensing forms, depending on whether they are a transition from an EB to a RB, or vice versa.

For chlamydia to be an effective pathogen it must go through five phases: attachment and penetration of the EB into a susceptible host cell; transition of the metabolically inert EB into the metabolically active RB and avoidance of destruction by the host cell; growth and replication of the RB using cellular components without destroying host cellular func-

tions; maturation of the noninfectious RB into an infectious EB; and release of the EB from the host cell and transfer to new cells. What is known about these stages is covered in a number of reviews (Moulder et al. 1984; Ward 1988).

One of the critical stages in the life cycle is the phase following entry into the host cell. During that period, the EB transforms into the replicative RB form and must escape destruction by the host cell. Chlamydiae enter the host cell in endosomes, in which they remain during the complete replicative cycle. The normal cell processes endosomes containing foreign material by acidification of the endosome and subsequent fusion of the endosome with lysosomes. This digestion and processing of antigen by the lysosomes is also critical for the processing of antigen during the immune response. Research shows that live chlamydiae, but not heat-inactivated chlamydiae, prevent acidification of the endosomes and subsequent phagolysosomal fusion. The mechanism by which the organisms prevent acidification of the endosome is not fully understood.

HOST RESPONSE

The interaction of chlamydiae with the host in the pathogenesis of chlamydial diseases is poorly understood even though the consequences of chlamydial infection can be very severe. In their natural hosts, chlamydial infections usually cause relatively mild diseases characterized by chronic low-level persistent infections with relatively poor immunity. The chlamydial strain is important in the type of disease seen, though there is increasing evidence that the host's immune response in persistent infections contributes significantly to the tissue damage seen.

The molecular mechanism for the increased pathology following reinfection was first elaborated by Watkins et al. (1986). They found that a triton-soluble chlamydial extract would induce conjunctival disease in guinea pigs that had been previously infected with the guinea pig inclusion conjunctivitis strain of chlamydia, but not in naive guinea pigs. Similar results have been demonstrated in primates infected with the trachoma biovar of *C. trachomatis* (Taylor et al. 1987). Recent data suggest that a single 56 kDa protein plays a major part in the immunopathogenesis of chlamydial disease, both in ocular trachoma and in tubal infertility (Patton 1985; Morrison et al. 1989; Wagar et al. 1990). The protein has been determined to be a homolog of the heat-shock protein (hsp) 60 family and shares 48% sequence identity with the human Hu Cha 60 protein (Cerrone et al. 1991). In humans, the response to the hsp 60 protein is variable, and infertility due to tubal occlusion and increased ectopic pregnancies have been associated with higher levels of antibody response to that protein (Wagar et al. 1990). Studies in mice indicate that the immune response to the protein is genetically controlled (Tuffrey 1992; Zhong and Brunham 1992).

The role of cytokines in chlamydial infection has been investigated. It has been shown that interferon (IFN), interleukin (IL)-1, IL-6, and tumor necrosis factor α (TNF-α) are produced in chlamydial infections (Burnham 1999). IFN exerts antichlamydial activity *in vivo* and may be important in controlling early primary chlamydial infections. However, IFN also has been hypothesized to be responsible for inducing a state of latency or persistence in chlamydial infections (Ward 1999; Burnham 1999). This may be a factor in the production of high antibody titers to the heat-shock protein 60 and increased severity of disease. The mechanisms by which gamma interferon inhibits chlamydial replication have been studied *in vitro*. Byrne et al. (1986) showed that gamma interferon inhibited the replication of *C. psittaci* by inducing indoleamine 2,3-dioxygenase, the enzyme that decycles tryptophan to N-formyl-kynurenine. This was confirmed in a later study using interferon-treated macrophages, in which tryptophan degradation appeared to be the general mechanism for the inhibition of *C. psittaci* replication (Carlin et al. 1989). A later study, however, indicated that inhibition of chlamydial growth in murine cells was due to increased production of nitric oxide (Mayer et al. 1993).

Interleukin-1 has been implicated as a mediator of conjunctival inflammation and scarring in ocular trachoma (Rothermel 1989). TNF-α is produced during *C. trachomatis* infections and may have a modest protective role in the host defense. The effect of TNF-α on chlamydiae resembles that of IFN-γ (Moulder 1991).

In mice and guinea pigs, a potent rapid TH1 and delayed-type hypersensitivity (DTH) response after an infection is important for rapid clearance of chlamydial infection and prevention of latent infection (Wang et al. 1999; Igietseme et al. 2000). A suboptimal TH1 and DTH response leads to the establishment of a persistent infection with a low-grade chronic immune response that causes tissue damage. The role of host genetics and stress in suppressing the TH1 response is not known. Also, the

immune response could be modulated through immune evasive mechanisms of chlamydiae such as inhibiting or enhancing cellular apoptosis or down-regulation of the MHC class I and II antigen expression (Zhong et al. 2000).

In humans, infertility and trachoma are the result of scarring following multiple or persistent infections with *C. trachomatis*. A low level of infection maintains a chronic inflammation that results in scarring, which causes inversion of the eyelid in trachoma or the constriction of the fallopian tubes in infertility. Traditionally, this host response has been viewed as antigen-dependent delayed type hypersensitivity or autoimmunity. Research has not completely supported these theories, and it now has been hypothesized that the persistent infections of nonimmune epithelial cells result in the secretion of large amounts of pro-inflammatory cytokines including IL-11, IL6, IL-8, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These then cause the influx of inflammatory neutrophils, T-cells, B-cells, and macrophages that result in cell necrosis and scar formation (Stephens 2003).

Chlamydiae have a type III secretion (TTS) system. The complete repertoire of genes for the complete secretion apparatus has been identified in both *C. trachomatis* and *C. psittaci* (Hsia et al. 1997; Stephens et al. 1998; Kalmon et al. 1999). Hollow spikelike surface appendages that traverse the inclusion membranes had been observed by electron microscopy as early as 1981 (Matsumato 1981). These projections are now thought to be part of the type III secretion system, giving chlamydiae the ability to directly inject their effector proteins into the cytoplasm of the host cell during the early EB stage.

Identifying the stages of the chlamydial development cycle in which the secretion system is active has been problematic. The unique development cycle of chlamydiae include an infectious elementary body (EB) stage that is thought to be metabolically dormant and a reproductive reticulate body (RB) stage. There is now evidence that some proteins characteristic of type III secreted proteins and some inclusion proteins are secreted during the EB stage (Fields and Hackstadt 2000; Subtil et al. 2001; Rockey et al. 2002; Slepenken et al. 2003). This has led to the prediction that these effector proteins as well as the secretion apparatus may be presynthesized and ready to be activated by contact with the host cell.

A recent study by Fields et al. (2003) supports the existence of a secretion system in the EBs. These researchers determined that a number of potential type III secretions had been secreted early; however, type III secretion apparatus proteins were not present until late in the development cycle after the EBs had converted to RBs. They proposed that the genes for the type III system are expressed during the RB stage, that the secretion apparatus is replenished during the RB stage, and that these secretion pores serve the newly formed EBs.

Chlamydial infections both stimulate and inhibit apoptosis. During the early stages of infection of macrophages or epithelial cells, the infected cells have been found to be resistant to proapoptosis stimuli. This would favor the completion of the chlamydial replicative cycle (Fan et al. 1998). In contrast, during the late stages of infection, apoptosis has been found to be enhanced (Ojcius 1998). This may be important for achieving host-cell rupture and the release of the infectious chlamydial elementary bodies. However, when gamma interferon is used to establish a persistent infection, the infected cells are resistant to apoptosis stimulation even during the late infection stages (Dean and Powers 2001; Perfettini et al. 2002).

The effects of apoptosis on the immune response may be very important, as infected macrophages cocultivated with T cells are resistant to proapoptosis stimulus. However, apoptosis is induced in the T cells (Jendro et al. 2000, 2003). This would delay the normal processing of antigen during the immune response.

DISEASES

The severity and type of disease produced by chlamydiae in mammals and birds depends on the strain of chlamydia and on the species of the host. In mammals, chlamydiae have been associated with pneumonia, enteritis, encephalomyelitis, abortion, urogenital infections, polyarthritis, polyserositis, mastitis, hepatitis, and conjunctivitis (Storz 1988). In birds, chlamydiae cause pericarditis, air sacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis (Andersen and Vanrompay 2003). In both mammals and birds, the severity of the disease may vary from clinically inapparent infections to severe systemic infections. Systemic or generalized infections produce fever, anorexia, lethargy, and, occasionally, shock and death. An asymptomatic carrier state or persistent infection is now thought to be a common sequel if the infection is not properly treated.

Chlamydial conjunctivitis occurs in humans, other mammals, and birds, and the chlamydial strains are often associated with other signs of disease. The eye involvement is usually restricted to the conjunctiva and may last 30 to 60 days or longer. The chlamydial strains causing conjunctivitis in humans, cats, guinea pigs, and koalas have also been associated with reproductive tract infections. The conjunctivitis strain in sheep and cattle is associated with polyarthritis. *C. suis* has also been isolated from conjunctivitis, enteritis, and pneumonia in swine, but other signs have not been observed.

Enzootic abortion of ewes (EAE) has been recognized for a century, but the causal agent was not identified as chlamydiae until 1950 (Stamp et al. 1950). The ovine abortion strain produces abortion or weak young in sheep, goats, and cattle; it has been isolated from cases of human abortion. The agent has been implicated in enteritis and other problems in sheep and cattle, but its role in these syndromes is unclear. Many chlamydial isolates have been recovered from the intestinal tract of sheep and goats; some of these are *C. abortus*. However, recent research indicates that most of the intestinal isolates are *C. pecorum* (polyarthritis-conjunctivitis).

Chlamydial polyarthritis of lambs was first identified in Wisconsin in 1957 as a chlamydial infection of the synovial tissues involving most joints of the limbs. It is now recognized in epizootic proportions in most major sheep-raising areas of the world. Affected lambs have varying degrees of stiffness, lameness, and anorexia. Conjunctivitis is often present. A similar disease caused by the same agent occurs in calves. Polyarthritis is caused by strains belonging to the species *C. pecorum.* It appears that polyarthritis and conjunctivitis are caused by the same agent. Encephalomyelitis may also be caused by this agent or by a serologically similar agent. These organisms are often isolated from fecal specimens and may also be involved in enteritis.

Turkeys infected with strains of high virulence can experience mortality rates of 10 to 30% unless early antibiotic treatment is instituted (Andersen and Vanrompay 2003). Typical postmortem findings include vasculitis, pericarditis, splenitis, and lateral nasal adenitis. The virulent turkey strains cause little if any disease in chickens, pigeons, or sparrows; however, cockatiels and parakeets succumb rapidly to infections with these agents. The virulent turkey strain is implicated in most human infections from turkeys. Outbreaks of chlamydiosis characterized by low virulence of the strain and little or no human involvement are usually caused by pigeon strains, and mortality usually is less than 5%.

Chlamydiosis is a common, chronic infection of psittacine birds. Many birds show no clinical signs until they are stressed. These birds often shed chlamydiae intermittently and serve as a source of infection for humans and other birds. Infections cause enteritis, air sacculitis, pneumonitis, and hepatosplenomegaly. Chlamydiosis in pigeons is similar to that seen in psittacine birds; infections are usually chronic with survivors becoming asymptomatic carriers. 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 the most common signs, and mortality can range up to 30%.

PATHOGENESIS

Four specific diseases in mammals and birds will be discussed: (1) placental and fetal infection in ruminants, (2) polyarthritis-polyserositis in ruminants, (3) conjunctivitis (feline pneumonitis) in cats, and (4) chlamydiosis in turkeys. These represent the most thoroughly investigated syndromes caused by chlamydia and are used to illustrate the disease process. Similar diseases occur in other animals and are often caused by distinctly different serovars of chlamydia. It should also be recognized that chlamydia can cause other disease syndromes such as pneumonia, enteritis, meningoencephalitis, and mastitis, and that as better diagnostic techniques are developed, new chlamydial strains and new diseases caused by chlamydiae are being found.

Placental and Fetal Infections in Ruminants

In enzootically infected sheep flocks, abortions occur year after year at a rate of 1 to 5%, but in flocks in which chlamydia has recently been introduced, the abortion rate may reach 30% (Storz 1988). Most abortions occur during the last month of gestation, but some may occur as early as the one-hundredth day. In experimentally infected sheep, fever as high as 40–41°C is seen on day 1 or 2 and lasts for 3 to 5 days (Stamp et al. 1950). In cattle, chlamydial abortions occur in the last trimester and are usually sporadic, but the abortion rate may sometimes reach 20%.

Sheep become infected by ingesting or inhaling *C. abortus.* It has been suggested that infection is

first established in the tonsil (Jones and Anderson 1988), from which it is disseminated by the blood to other organs. The organism then persists in the dam in a latent form 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 being detected after 90 days (Buxton et al. 1990).

The mechanism by which chlamydiae migrate from the maternal side of the placenta to the fetus is still uncertain. After approximately 60 days of gestation, the physiological invasion of the carunculae stroma by chorionic villi coincides with hemorrhage from the maternal vessels and results in the formation of hematomas (Buxton et al. 1990). These hematomas have been suggested as a means for chlamydia in the maternal circulation to make direct contact with the chorionic epithelium. The 30-day interval between the start of formation of hematomas and the first observations of pathological changes in the placenta has raised questions about whether endocrinological and/or immune-related changes may play a significant role (Buxton et al. 1990). The initial lesions in the placenta involve the limbus of the placentomas in the hilar region, where chlamydial inclusions are seen in the trophoblast (Buxton et al. 1990). Progression of the infection results in a considerable loss of chorionic epithelial cells in both the cotyledonary and intercotyledonary placenta. At this time, a mixed-cell inflammatory infiltrate is present. Fibrin deposits and a purulent arteritis are noted in the thickened placental mesenchyme underlying the lesions. Chlamydial inclusions are present in the endometrial epithelium, where affected chorion is in apposition to the maternal tissues. During the later stages, severe necrosis and sloughing of the endometrial epithelium occur.

Infection of the fetus is secondary to placentitis and is not a major factor in the disease. Chlamydial isolations are often made from fetuses; however, titers are low and infectious foci have not been reported. Necrotic foci, sometimes with inflammatory reactions, are frequently found in most fetal organs and tissues and may be embolic in origin (Buxton et al. 1990). Chlamydial antigen, if seen in the foci, is usually present only 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. Fetal lambs will respond with specific chlamydial antibodies that can be detected in the indirect fluorescent antibody or immunodiffusion tests.

Polyarthritis-Polyserositis in Ruminants

Polyarthritis in sheep and cattle is caused by strains of the *C. pecorum* species. The disease is readily reproduced by inoculating calves or lambs by oral, intramuscular, subcutaneous, intravenous, or intra-articular routes. Under field conditions, the organism is thought to be ingested and to subsequently multiply in the mucosa of the large and small intestines. It may produce a diarrhea during this phase. A chlamydemia follows the multiplication in the intestinal mucosa and distributes the chlamydiae to other parts of the body. Both periarticular and articular tissue changes are seen in the joints. Periarticular changes, including subcutaneous edema and fluid-filled synovial sacs, cause joint enlargement; these enlarged joints will contain excessive greyish-yellow turbid synovial fluid. Fibrin plaques will also be seen 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.

Histological changes are primarily an inflammatory reaction in the synovium, tendon sheaths, and subsynovial tissues (Shupe and Storz 1964; Cutlip and Ramsey 1973). In experimentally inoculated joints, granulation tissue replaces much of the fibropurulent exudate, with the formation of large fibrous villi by 21–24 days. By this time, the synovial surfaces are again covered by intact lining cells.

Conjunctivitis (Feline Pneumonitis)

C. felis infection in cats (feline pneumonitis) is characterized by a severe conjunctivitis with blepharospasm, conjunctival hyperemia, chemosis, and serous and mucopurulent ocular discharges (Wills et al. 1987). Mild respiratory signs with slight nasal discharge, coughing, and sneezing are often seen with the conjunctivitis. Following natural infections, the agent may colonize the gastrointestinal tract (primarily the superficial gastric) and reproductive tract.

The disease is transmitted by direct contact with infected secretions and by droplet infection. Clinical signs usually are seen on day 4 postinoculation (PI) and will last 30 days, after which they gradually subside. Long-term persistent infections are likely responsible for maintaining the agent in

the feline population. The agent has been recovered from the eyes, vagina, rectal swabs, and superficial gastric mucosa for over 150 days after infection (Wills et al. 1987).

Chlamydiosis in Turkeys

Transmission of chlamydiae in turkeys is thought to be primarily through inhalation of the organism, which is excreted in large quantities in both fecal material and nasal and ocular discharges. In a study using a virulent turkey serovar D isolate, only a few of the orally exposed turkeys initially developed a mild subclinical infection. The infection appeared then to spread to the remaining birds by the aerosol route (Page 1958). Chlamydial isolation patterns and serological data support the hypothesis of secondary transmission following infection of only a few of the birds by the oral route. The hypothesis that aerosol exposure is the primary method of exposure is supported by the findings that chlamydia is first isolated from the oral-pharyngeal and nasal secretions, and that titers in fecal material drop rapidly following drying.

Chlamydia is rapidly disseminated throughout the body following aerosol exposure and is recovered from the lung, air sacs, pericardial sac, and mesentery within 4 hours. Consistently detectable levels in the blood are not achieved until 72 hours, at which time chlamydia is found throughout the body and in cloacal materials (Page 1958). Clinical signs vary with the strain of chlamydia (Tappe et al. 1989). Turkeys given a virulent turkey strain (*C. psittaci,* TT3), a psittacine strain (*C. psittaci*, VS-1), or an ovine abortion strain (*C. abortus,* B577) showed significant differences in clinical signs. The TT3-infected turkeys showed clinical signs on postinoculation (PI) days 3 through 24 and experienced a 20 to 40% decrease in body weight compared with birds in other groups. The VS-1-infected birds experienced a mild dyspnea on days 4 through 11 and were slightly lethargic on day 7. Control birds and B577-infected birds remained normal throughout the study.

Gross lesions also varied with the chlamydial strain. Pericarditis was the most severe lesion seen in turkeys infected with TT3. Only mild pericardial lesions were seen in VS1-inoculated birds. However, air sac lesions were more severe in turkeys infected with VS-1 than in turkeys infected with TT3. Bronchopneumonia was characteristic of turkeys infected with the psittacine isolate but not of turkeys infected with the virulent turkey isolate.

Both the VS-1 strain and the TT3 strain produced an infection of the lateral nasal glands that was detectable by histological examination through PI day 50. This could be an important source for aerosolization of chlamydiae, since these glands are the main source of moisture for the nasal mucosa.

Immunity

During most chlamydial infections, the host develops an immune response that halts the infection and probably eliminates the organism from the body. This immunity provides solid protection from reinfection to the same chlamydial strain for 4 to 6 months, after which immunity subsides. Immunity likely involves both cell-mediated and antibody-mediated mechanisms. The role of each is not understood and may depend on the location of the parasite in the body (i.e., the eye, the respiratory tract, or the genital tract).

The response to the primary infection includes high levels of antibody to the chlamydial lipopolysaccharide (LPS) or genus-specific epitopes. The response is measured by complement-fixation (CF) and enzyme-linked immunosorbent assay (ELISA) tests; however, antibody levels do not correspond to immunity. In secondary infections, the anamnestic response may or may not give a CF response. The neutralizing or protective antibodies are thought to be primarily to the major outer-membrane protein (MOMP) and are likely serovar specific. With *C. trachomatis*, serovar-specific monoclonal antibodies (MAbs) to the MOMP have been shown to provide neutralization in vitro. Serovar-specific MAbs to the ovine abortion strain provide neutralization in *in vitro* tests and by passive immunization in mice (Andersen and van Deusen 1988; Buzoni-Gatel et al. 1990). However, the protein specificity of these MAbs has not been tested, for they fail to react by Western blot. The MAbs are thought to be to conformational epitopes on the MOMP, as the MOMP is believed to be the site of serovar specificity. High-molecular weight protein may also be a factor in immunity, for MAbs to an 89-kDa protein in the ovine abortion isolate have been shown to provide protection *in vitro* (Cevenini et al. 1991).

Currently there are commercial vaccines to the ovine abortion strain and to the feline pneumonitis strain. Two types of ovine abortion vaccine are produced. The formalin-inactivated bacterin is available in most parts of the world. In recent years, a live temperature-sensitive mutant vaccine

was introduced in some parts of Europe (Rodolakis and Souriau 1983; Chalmers et al. 1997). Neither vaccine gives complete protection. It is thought that the vaccine failure may be due to serotype variants in the field.

A subcellular vaccine for ovine abortion has provided good protection (Tan et al. 1990). The vaccine was prepared by procedures that provide a MOMP-enriched preparation while not denaturing the protein. The preparation has the advantage of reducing the response to the genus-specific antigen (LPS), which does not relate to protection. In early trials, the vaccine gave protection comparable to chlamydial bacterins prepared from purified elementary bodies.

Feline pneumonitis is the other chlamydial strain for which commercial vaccines are available. The vaccines include a live attenuated vaccine and a number of inactivated whole-cell preparations. The infection in cats is primarily an infection of the conjunctiva with secondary spread to the gastrointestinal and reproductive tracts. Reports on the specific inactivated products are not available; however, data are available on the use of the live attenuated vaccine and on experimental inactivated vaccines (Shewen et al. 1980; Wills et al. 1987). These vaccines reduce or eliminate clinical signs but do not prevent infection and shedding of the organism.

REFERENCES

Andersen, A. A. 1991. Serotyping of *Chlamydia psittaci* isolates using serovar-specific monoclonal antibodies with the microimmunofluorescence test. J. Clin. Microbiol. 29:707–11.

———. 1997. Two serovars of *Chlamydia psittaci* from North American birds. J. Vet. Diagn. Invest. 9:159–164.

Andersen, A. A., and A. Vanrompay. 2003. Avian chlamydiosis (psittacosis, ornithosis). In Diseases of poultry, 11th ed. Y. M. Saif (ed.). Ames: Iowa State Press, pp. 863–879.

Andersen, A. A., and R. A. van Deusen. 1988. Production and partial characterization of monoclonal antibodies to four *Chlamydia psittaci* isolates. Infect. Immun. 56:2075–2079.

Burnham, R. C. 1999. Human immunity to chlamydiae. In Chlamydia: intracellular biology, pathogenesis, and immunity. R. S. Stephens (ed.). Washington, DC: American Society for Microbiology, pp. 211–238.

Buxton, D., R. M. Barlow, J. Finlayson, I. E. Anderson, and A. Mackellar. 1990. Observations on the pathogenesis of *Chlamydia psittaci* infection of pregnant sheep. J. Comp. Pathol. 102:221–237.

Buzoni-Gatel, D., F. Bernard, F., A. Andersen, and A. Rodolakis. 1990. Protective effect of polyclonal and monoclonal antibodies against abortion in mice infected by *Chlamydia psittaci.* Vaccine 8:342–346.

Byrne, G. I., L. K. Lehmann, and G. J. Landry. 1986. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. Infect. Immun. 53:347–351.

Carlin, J. M., E. C. Borden, and G. I. Byrne. 1989. Interferon-induced indolamine 2,3-dioxygenase activity inhibits *Chlamydia psittaci* replication in human macrophages. J. Interferon Res. 9:329–337.

Cerrone, M. C., J. J. Ma, and R. S. Stephens. 1991. Cloning and sequence of the gene for heat shock protein 60 from *Chlamydia trachomatis,* and immunological reactivity of the protein. Infect. Immun. 59:79–90.

Cevenini, R., M. Donati, E. Brocchi, F. De Simone, and M. La Placa. 1991. Partial characterization of an 89-kDa highly immunoreactive protein from *Chlamydia psittaci* A/22 causing ovine abortion. Fed. Eur. Microbiol. Soc. 81:111–116.

Chalmers, W. S., J. Simpson, S. J. Lee, and W. Baxendale. 1997. Use of a live chlamydial vaccine to prevent ovine enzootic abortion. Vet. Rec. 141:63–67.

Cutlip, R. C., and F. K. Ramsey. 1973. Ovine chlamydial polyarthritis: sequential development of articular lesions in lambs after intraarticular exposure. Am. J. Vet. Res. 34:71–75.

Dean, D., and V. V. C. Powers. 2001. Persistent *Chlamydia trachomatis* infections resist apoptotic stimuli. Infect. Immun. 69:2442–2447.

Everett, K. D. E.. R. M. Bush, and A. A. Andersen. 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of the organisms. Int. J. Systematic Bacteriol. 49:415–440.

Fan, T., H. Lu, H. Hu, L. Shi, G. A. McClarty, D. M. Nance, A. H. Greenberg, and G. Zhong. 1998. Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. J. Exp. Med. 187:487–496.

Fields, K. A., and T. Hackstadt. 2000. Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. Mol. Microbiol. 38:1048–1060.

Fields, K. A., D. J. Mead, C. A. Dooley, and T. Hackstadt. 2003. *Chlamydia trachomatis* type III secretion: evidence for a functional apparatus during early-cycle development. Mol. Microbiol. 48:671–683.

Grayston, J. T. 1992. *Chlamydia pneumoniae,* strain TWAR pneumonia. Annu. Rev. Med. 43:317–23.

Hoelzle, L. E., G. Steinhausen, and M. M. Wittenbrink. 2000. PCR-based detection of chlamydial infection in swine and subsequent PCR-coupled genotyping of chlamydial *omp1*-gene amplicons by DNA-hybridization, RFLP analysis, and nucleotide sequence analysis. Epidemiol. Infect. 125:427–439.

Hsia, R. C., Y. Pannekoek, E. Ingerowski, and P. M. Bavoil. 1997. Type III secretion genes identify a putative virulence locus of Chlamydia. Mol. Microbiol. 25:351–359.

Huang, H.-S., D. Buxton, D. and I. E. Anderson. 1990. The ovine immune response to *Chlamydia psittaci;* histopathology of the lymph node. J. Comp. Pathol. 102:89–97.

Igietseme, J. U., G. A. Ananaba, J. Bolier, S. Bowers, T. Moore, T. Belay, F. O. Eko, D. Lyn and C. M. Black. 2000. Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development. J. Immunol. 164:4212–4219.

Jendro, M. C., F. Fingerle, T. Deutsch, A. Liese, L. Kohler, J. G. Kuipers, E. Raum, M. Martin, and H. Zeidler. 2003. *Chlamydia trachomatis*-infected macrophages induce apoptosis of activated T cells by secretion of tumor necrosis factor-alpha in vitro. Med. Microbiol. Immunol. (May 15, epub ahead of print)

Jendro, M. C., T. Deutsch, B. Korber, L. Kohler, J. G. Kuipers, B. Krausse-Opatz, J. Westermann, E. Raum, and H. Zeidler. 2000. Infection of human monocyte-derived macrophages with *Chlamydia trachomatis* induces apoptosis of T cells: a potential mechanism for persistent infection. Infect. Immun. 68:6704–6711.

Jones, G. E., and I. E. Anderson. 1988. *Chlamydia psittaci:* Is tonsillar tissue the portal of entry in ovine enzootic abortion? Res. Vet. Sci. 44:260–261.

Kalman, S., W. Mitchell, R. Marathe, C. Lammel, J. Fan, R. R. W. Hyman, L. Olinger, L., J. Grimwood, R. W. Davis, and R. S. Stephens. 1999. Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. Nat. Genet. 21:385–389.

Mayer, J.; M. L. Woods, Z. Vavrin, and J. B. Hibbs Jr. 1993. Gamma interferon-induced nitric oxide production reduces *Chlamydia trachomatis* infectivity in McCoy cells. Infect. Immun. 61:491–497.

Morrison, R. P., R. J. Belland, K. Lyng, and H. D. Caldwell. 1989. Chlamydial disease pathogenesis: the 57-kDa chlamydial hypersensitivity antigen is a stress response protein. J. Exp. Med. 170:1271–1283.

Moulder, J. W. 1984. Looking at chlamydiae without looking at their hosts. Am. Soc. Microbiol. News 50:353–62.

———. 1991. Interaction of chlamydiae and host cells in vitro. Microbiol. Rev. 55:143–90.

Moulder, J. W., T. P. Hatch, and C.–C. Kuo. 1984. Genus *Chlamydia.* In Bergey's manual of systematic bacteriology, vol. 1. N. R. Krieg and J. G. Holt (eds.). Baltimore: Williams and Wilkins.

Nietfeld, J. C., P. Leslie-Steen, D. H. Zeman, and D. Nelson. 1997. Prevalence of intestinal chlamydial infection in pigs in the midwest, as determined by immunoperoxidase staining. Am. J. Vet. Res. 58:260–264.

Ojcius, D. M., P. Souque, J. L. Perfettini, and A. Dautry-Varsat. 1998. Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen *Chlamydia psittaci.* J. Immunol. 161:4220–4226.

Page, L. A. 1958. Experimental ornithosis in turkeys. Avian Dis. 3:51–66.

Patton, D. L. 1985. Immunopathology and histopathology of experimental salpingitis. Rev. Infect. Dis. 7:746–753.

Perfettini, J. L., T. Darville, A. Dautry-Varsat, R. G. Rank, and D. M. Ojcius. 2002. Inhibition of apoptosis by gamma interferon in cells and mice infected with *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*). Infect. Immun. 70:2559–2665.

Rockey, D. D., M. A. Scidmore, J. P. Bannantine, and W. J. Brown. 2002. Proteins in the chlamydial inclusion membrane. Microbes Infect. 4:333–340.

Rodolakis, A., and A. Souriau. 1983. Response of ewes to temperature-sensitive mutants of *Chlamydia psittaci* (var ovis) obtained by NTG mutagenesis. Ann. Rech. Vet. 14:155–161.

Rogers, D. G., A. A. Andersen, and B. D. Hunsaker. 1996. Lung and nasal lesions caused by a swine chlamydial isolate in gnotobiotic pigs. J. Vet. Diagn. Invest. 8:45–55.

Rogers, D. G., and A. A. Andersen. 1996. Intestinal lesions caused by two swine chlamydial isolates in gnotobiotic pigs. J. Vet. Diagn. Invest. 8:433–440.

Rothermel, C. D., J. Schachter, P. Lavrich, E. C. Lipsitz, and T. Francus. 1989. *Chlamydia trachomatis*-induced production of interleukin-l by human monocytes. Infect. Immun. 57:2705–2711.

Schachter, J. 1999. Epidemiology. In Chlamydia. R. S. Stephens (ed.). Washington, DC: American Society of Microbiology, pp. 156–157.

Shewen, P. E., R. C. Povey, and M. R. Wilson. 1980. A comparison of the efficacy of a live and four inactivated vaccine preparations for the protection of cats against experimental challenge with *Chlamydia psittaci.* Can. J. Comp. Med. 44:244–51.

Shupe, J. L., and J. Storz. 1964. Pathologic study of psittacosis lymphogranuloma polyarthritis of lambs. Am. J. Vet. Res. 25:943–51.

Slepenkin, A., V. Motin, L. M. De La Maza, and E. M. Peterson. 2003. Temporal expression genes of *Chlamydia pneumoniae.* Infect. Immun. 71:2555–2562.

Stamp, J. T., A. D. McEwen, J. A. A. Watt, and D. J. Nisbet. 1950. Enzootic abortion in ewes. 1. Transmission of the disease. Vet. Rec. 62:251–54.

Stephens, R. S. 2003. The cellular paradigm of chlamydial pathogenesis. Trends in Microbiol. 11:44–51.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science 282:754–759.

Storz, J. 1988. Overview of animal diseases induced by chlamydial infections. In Microbiology of chlamydia. A. L. Barron (ed.). Boca Raton, FL: CRC Press.

Subtil, A., C. Parsot, and A. Dautry-Varsat. 2001. Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. Mol. Microbiol. 39:792–800.

Tan, T.-W., A. J. Herring, I. E. Anderson, and G. E. Jones. 1990. Protection of sheep against *Chlamydia psittaci* infection with a subcellular vaccine containing the major outer membrane protein. Infect. Immun. 58:3101–3108.

Tappe, J. P., A. A. Andersen, and N. F. Cheville. 1989. Respiratory and pericardial lesions in turkeys infected with avian or mammalian strains of *Chlamydia psittaci.* Vet. Pathol. 26:386–395.

Taylor, H. R., S. L. Johnson, J. Schachter, H. D. Caldwell, and R. A. Prendergast. 1987. Pathogenesis of trachoma: the stimulus for inflammation. J. Immunol. 38:3023–3027.

Tuffrey, M. 1992. Animal models of chlamydial infection. Proc. Eur. Soc. Chlamydia Res. 2:99–102.

Wagar, E. A., J. Schachter, P. Bavoil, and R. S. Stephens. 1990. Differential human serologic response to two 60,000 molecular weight *Chlamydia trachomatis* antigens. J. Infect. Dis. 162:922–927.

Wang, S., Y. Fan, R. C. Brunham, and X. Yang. 1999. IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. Eur. J. Immunol. 29:3782–3792.

Ward, M. E. 1988. The chlamydial developmental cycle. In Microbiology of chlamydia. A. L. Barron (ed.). Boca Raton: CRC Press.

———. 1999. Mechanisms of Chlamydia-induced disease. In Chlamydia: intracellular biology, pathogenesis, and immunity. R. S. Stephens (ed.). Washington, DC: American Society for Microbiology, pp. 171–210.

Watkins, N. D., W. J. Hadlow, A. B. Moos, and H. D. Caldwell. 1986. Ocular delayed hypersensitivity: a pathogenetic mechanism of chlamydial conjunctivitis in guinea pigs. Proc. Natl. Acad. Sci. U S A 83:74–80.

Wills, J. M., T. T. Gruffydd-Jones, S. J. Richmond, R. M. Gaskell, and F. J. Bourne. 1987. Effect of vaccination on feline *Chlamydia psittaci* infection. Infect. Immun. 55:2653–57.

Zhong, G., and R. C. Brunham. 1992. Antibody responses to the chlamydial heat shock proteins hsp60 and hsp70 are *H-2* linked. Infect. Immun. 60:3143–49.

Zhong, G., L. Liu, T. Fan, P. Fan, and H. Ji. 2000. Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in chlamydia-infected cells. J. Exp. Med. 191:1525–1534.

31
Rickettsiales

S. Harrus, T. Waner, S. Mahan, and H. Bark

Rickettsial organisms are responsible for several important and potentially fatal infectious diseases of domestic animals and humans. *Rickettsiales* taxonomy based on phenotypic characteristics, the type of cells and host they infect, and the clinical manifestations induced by the infectious agent has given way to reclassification based on sequence analysis of 16S rRNA gene, the citrate synthase gene, the *groESL* operon genes, and the outer membrane protein genes (Dasch and Weiss 1998; Dumler et al. 2001). This order now includes two families of organisms that are pathogenic to animals, *Anaplasmataceae*, the major family affecting animals, and *Rickettsiaceae* (table 31.1). These organisms are widely distributed around the world in endemic foci and are responsible for sporadic and often seasonal outbreaks of disease. They are implicated in some of the oldest and most recently recognized diseases in animals and humans (Raoult and Roux 1997; Dumler et al. 2001).

CHARACTERISTICS OF RICKETTSIAL ORGANISMS

Rickettsiae are small, obligately intracellular, pleomorphic, coccobacillary, gram-negative bacteria. They exist in nature as parasites of wild mammals, ticks, and trematodes and are transmitted to the host by the bite of the vector, a tick in most domestic rickettsial diseases, or by ingestion of infected flukes (McDade 1998). The adhesins involved in attachment have not been characterized for most rickettsial organisms. After invasion, rickettsiae replicate by binary fission in the host cell (erythrocytes, white blood cells, or endothelial cells in most cases).

Certain rickettsiae exert cytopathic effects that are thought to be due to peroxidation of membrane lipids by oxygen radicals. The rickettsiae do not produce exotoxins, and their limited lipopolysaccharide (LPS) content is considered inconsequential to the pathogenic process (McDade 1998). Direct rickettsial injury to infected endothelial cells and underlying smooth muscle cells is a common pathogenic mechanism (Dasch and Weiss 1998). Lysis of rickettsiae by interferon γ (IFN-γ)and cytotoxic T cells may contribute to tissue damage (Dasch and Weiss 1998). This process may result in vascular and perfusion disturbances, hemorrhages, edema, thrombosis, and necrosis, affecting most organ systems (Biberstein and Hirsh 1999). The clinical manifestation of rickettsial diseases may vary according to the rickettsial agent, the disease phase, the extent of cellular damage, and the host response (McDade 1998). Increasing evidence indicates that the predominant response to rickettsiae is a Th1-type cell-mediated immunity, with IFN-γ as the key modulator (Turco and Winkler 1997).

Thrombocytopenia is a common hematological finding in many rickettsial diseases. The pathogenesis of the thrombocytopenia involves sequestration in damaged tissues and thrombosed vessels, production disturbances, shortened life span, and immune destruction.

Many rickettsiae cause persistent infections. Frequent genetic recombinations leading to antigenic variants have been suggested to permit immune evasion and thereby allow persistent infections (Reddy and Streck 1999).

This chapter will review the current knowledge on the pathogenesis of major rickettsial organisms causing diseases in domestic animals.

Table 31.1. Rickettsial Organisms Pathogenic to Domestic Animals

	Animal species affected	Disease
Family: *Anaplasmataceae*		
Anaplasma genogroup		
A. phagocytophila	Ruminants, equine, canine, feline	Tickborne fever, Equine granulocytic ehrlichiosis, Canine granulocytic ehrlichiosis, Feline granulocytic ehrlichiosis
A. marginale	Bovine	Anaplasmosis
A. centrale	Bovine	Anaplasmosis
A. platys	Canine	Canine infectious cyclic thrombocytopenia
A. ovis	Ovine and caprine	Anaplasmosis
Ehrlichia genogroup		
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 or Cowdriosis
Neorickettsia genogroup		
N. helminthoeca	Canine	Salmon-poisoning disease
N. risticii	Horses	Potomac horse fever
	Canine	Atypical canine ehrlichiosis
Family: *Rickettsiaceae*		
Rickettsia genogroup		
R. rickettsii	Canine	Rocky Mountain spotted fever

ANIMAL RICKETTSIAL PATHOGENS—FAMILY *ANAPLASMATACEA*

Anaplasma Genogroup

Anaplasma marginale, A. centrale, and *A. ovis*

Background, Etiology, and Epidemiology. Anaplasmosis, or gall sickness, is an intra-erythrocytic rickettsial infection of cattle, sheep, and wild ruminants. The disease was first described by Sir Arnold Theiler in South Africa in 1910 (Potgieter and Stoltsz 1994). *Anaplasma marginale* and the less virulent *A. centrale* are erythrocytic parasites of cattle and wild ruminants, while *A. ovis* infects erythrocytes of sheep and goats. The *Anaplasma* species can be distinguished microscopically by their location in the red blood cell. *A. ovis* is microscopically indistinguishable from *A. marginale*. Anaplasmosis is endemic worldwide in most tropical and subtropical areas, and its distribution depends on its tick vectors.

Pathogenesis. Anaplasmosis is manifested by clinical signs of anemia, icterus without hemoglobinuria, fever, weakness, and depression. *A. marginale* is the most pathogenic of the *Anaplasma* species listed above, and therefore most of the discussion concerning the pathogenesis of anaplasmosis centers on this organism. Approximately 20 species of ticks have been incriminated as *A. marginale* vectors. Ixodid ticks are the main vectors in cattle, and *Dermacentor* species, including *D. variabilis*, *D. andersoni,* and *D. albipictus,* are the major vectors in the United States (de la Fuente et al. 2001). In South Africa, *Boophilus decoloratus* has been incriminated as the most important vector and *B. microplus, Rhipicephalus simus, R. everti everti,* and *Hyalomma marginatum rufipes* have been shown to transmit the infection experimentally. In Australia, *B. microplus* is the only tick recognized as a vector of bovine anaplasmosis. Among the ticks transmitting *A. ovis* are *R. bursa* and *D. andersoni* (Friedhoff 1997). Disease may also be transmitted mechanically by bloodsucking flies, iatrogenically, orally, and by the intrauterine route (Baumgartner et al. 1993).

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 are dependent on a number of factors: older animals are more prone to the severe form of anaplasmosis (Kocan 2001); animals on a reduced energy intake show less severe clinical disease (Wilson 1979); *Bos indicus* breeds appear to be more resistant than *B. taurus* breeds (Wilson et al. 1980).

The mode of entry of *Anaplasma* into the erythrocyte is unclear, but it appears that increased cell membrane permeability may be facilitated by a decrease in the acetylcholinesterase activity of the cell (Sharma and Tripathi 1985). The anemia is due to excessive erythrophagocytosis initiated by parasite-induced erythrocyte damage and the sensitizing effect of anti-erythrocytic 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 resultant anemia are: a factor stimulating nonspecific erythrophagocytosis; altered phosphofructokinase activity and lowered levels of adenosine triphosphate (ATP) in the erythrocytes resulting in functional disturbances (Silva et al. 1989), and changes in the protein and glycoprotein components of membranes of the infected (Nordelo and Ysern-Caldentey 1982).

The peracute form is rare, occurs most frequently in pure-bred animals, and is fatal within a few hours. In the acute form, the mucous membranes are pale, 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 recovering from *A. marginale* infection are persistently infected and are immune if reexposed to infection. These animals remain carriers of *A. marginale* and may serve as reservoirs for tick transmission. Persistent *A. marginale* infection is characterized by sequential, microscopically undetected cycles of rickettsemia (Eriks et al. 1993). During these cycles, *A. marginale* expresses antigenically variant major surface protein 2 (MSP2) encoded by the *msp2* gene, a member of a multigene family. The *msp2* gene consists of conserved regions that flank a single, central hypervariable region encoding B-cell epitopes (French et al. 1999). Generation of new MSP2 variants is a result of deletions, substitutions, and insertions in the central hypervariable region, and due to recombination between members of the *msp2* gene family (Brayton et al. 2002; Meeus and Barbet 2001). Thus, persistent infection is accomplished by the emergence of antigenic variants of *A. marginale,* which precede the variant-specific immune response that is responsible for clearing the rickettsemia.

The postmortem findings in fatal cases include anemia, icterus, splenomegaly, hepatomegaly, and possibly lymphadenomegaly. The splenic pulp is dark in color, and the cut surface of the liver varies in color from orange-brown to mottled yellow.

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.

Infection with *Anaplasma* results in both humoral and cellular immune responses. Splenectomized animals are considerably more susceptible to infection than intact animals. Antigenic variations have been reported between isolates of *A. marginale* (Wilson et al. 1980). *A. centrale* infected blood is used for vaccination against *A. marginale* in cattle. Complete serological cross-reactivity has been demonstrated for *A. marginale* and *A. ovis*, but infection with one does not provide protective immunity to the other (Splitter et al. 1955, 1956).

Anaplasma phagocytophila

Background, Etiology, and Epidemiology. Previously, *Ehrlichia phagocytophila, E. 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 *Anaplasma phagocytophila* (Dumler et al. 2001). Natural infection is transmitted by *Ixodes* ticks, mainly *I. ricinus* and *I. pacificus,* which feed on a wide range of vertebrate animals, giving the organism the opportunity to cause disease in a number of host species.

A. phagocytophila is the cause of tickborne fever (TBF) also known as "pasture disease" in cattle, goats, sheep, and wild ruminants. In horses, dogs, cats, and llamas, *A. phagocytophila* causes a granulocytic ehrlichiosis.

Pathogenesis. The natural incubation period of TBF is 3 to 13 days. The onset of disease is marked by a high fever of 1 to 2 weeks duration. In non-

pregnant and nonlactating animals, the signs tend to be mild and nonspecific. Clinical signs include reduced appetite, apathy, decreased milk production, and respiratory abnormalities. Death from uncomplicated disease is rare.

Following infection, a significant reduction in peripheral leukocytes is detected. This is 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 hematological changes in sheep were more severe than in goats (Gokce and Woldehiwet 1999a). Rickettsemia was demonstrated 3 days postinfection (PI) in sheep and goats with about 70% of leukocytes displaying intracytoplasmic morulae. The rickettsemia declined after 8 days PI and remained at low levels until about day 20 PI. In cattle, morulae appear 3 to 4 days PI. Peak rickettsemia appears on days 5 to 9 PI, and morulae can be detected until about day 23 PI (Braun-Hansen et al. 1998).

Pathological findings due to infection with *A. phagocytophila* are similar for humans, horses, and sheep. These findings include 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 histopathological lesions may not be directly related to *A. phagocytophila*, but rather to immunopathological mechanisms initiated by the organism. IFN-γ plays an important role in clearance of the organism in the early phases of infection, and may also play a role in the pathology associated with the infection. Interleukin–10 (IL-10) moderates the pathology, possibly through down-regulatory effects on IFN-γ or through other anti-inflammatory mechanisms (Martin et al. 2001).

An important consequence of *A. phagocytophila* infection is its ability to exacerbate concurrent infections probably due to its immunosuppressive effects. TBF increases susceptibility to tick-pyemia and pneumonic pasteurellosis and predisposes sheep to disease of increased severity with parainfluenza type 3, louping ill, and lambs to contagious ecthyma (Orf) (Gokce and Woldehiwet 1999b).

The mechanism of intracytoplasmic survival and multiplication of *A. phagocytophila* has been studied in polymorphonuclear cells of sheep. The findings indicated that *A. phagocytophila* is capable of inhibiting phagosome-lysosome fusion, attributable to an active bacterial process and not to lysosomal dysfunction (Gokce et al. 1999).

Sheep that recover from TBF remain persistently infected with *A. phagocytophila*, and may serve as a reservoir for the organism from one grazing season to the next (Stuen et al. 2001). The mechanism by which *A. phagocytophila* evades the host's immune system is probably similar to that described for *A. marginale* in cattle and HGE agent in humans. In both pathogens, the major immunodominant surface antigen, MSP2 and p44, respectively, is encoded by genes of a multigene family, which persistently generate antigenic variants (Caspersen et al. 2002).

Equine granulocytic ehrlichiosis 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, with a 2- to 3-week duration of illness (Gribble 1969). Clinical signs include fever, anorexia, listlessness, depression, limb edema, petechiae, and icterus. Stiffness of gait and incoordination may also occur. Young horses may cough and lose weight. Hematological changes include leukopenia, thrombocytopenia, and anemia. Morulae are present in the neutrophils of acutely ill horses.

Infection of dogs with *A. phagocytophila* (previously *E. equi*) causes canine granulocytic ehrlichiosis. 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. phagocytophila*, fever developed 4 to 7 days postinfection (Egenvall et al. 1998). Leukopenia, lymphopenia, neutropenia, and moderate to severe thrombocytopenia were detected. Persistent infection with *A. phagocytophila* 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.

Feline granulocytic ehrlichiosis has been described in several cats as a nonspecific clinical entity. Reported signs included lethargy, anorexia, fever, tachypnea, and dehydration. In Sweden, the etiological agent was visualized in neutrophils of a cat infested with *I. ricinus* ticks. DNA from the feline organism showed 100% identity with the DNA sequences of Swedish canine, equine, and human granulocytic ehrlichioses agents (Bjoersdorff et al. 1999).

An ehrlichial organism was identified in the blood of a sick llama (*Lama glama*). Symptoms

were nonspecific and included partial anorexia, slight ataxia, and lethargy. Rare cytoplasmic inclusions were present. Buffy-coat cells were strongly positive by nested PCR for *A. phagocytophila*. 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). The disease was first reported in the United States in 1978 (Harvey et al. 1978). *A. platys* is the only known rickettsia with a tendency to infect canine platelets.

ICCT has been reported in the United States, Japan, Thailand, Venezuela, China, Spain, Australia, South Africa, Greece, and Israel. *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). Additional evidence for the role of *R. sanguineus* was the detection of *A. platys* in ticks from free-roaming dogs in Japan (Inokuma et al. 2000).

Pathogenesis. *A. platys* infection may manifest as a subclinical or as an acute disease. The latter form of the disease with distinct clinical signs has been described only 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 in cases of natural infection included pale mucous membranes, fever, and lymphadenomegaly. The main hematological and biochemical findings were thrombocytopenia and the presence of giant platelets, anemia, monocytosis, and lowered albumin concentrations. Uveitis has been associated with *A. platys* in natural infection (Glaze and Gaunt 1986).

The incubation period after experimental intravenous infection is 8 to 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 does not seem to be dependent on the rate of parasitemia, and even in cases with a low parasitemia, the thrombocytopenia may be severe. It was 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* developed a mild normocytic normochromic anemia (Baker et al. 1988). Changes in erythrocytes and serum iron concentrations were similar to those described for dogs with anemia of inflammation. Dogs experimentally infected with *A. platys* showed significant increases in serum alanine aminotransferase and alkaline phosphatase activities 7 days PI. These enzyme levels were unchanged for up to 35 days postinfection.

Gross pathological findings were limited to generalized lymphadenomegaly, in which lymph nodes showed follicular hyperplasia with mild necrosis of follicular lymphocytes, later in the infection. Plasmacytosis was evident 2 weeks PI. Follicular hyperplasia was also evident in the spleens of artificially infected dogs. Crescent-shaped splenic hemorrhages were seen in dogs 14 days PI. Livers contained multifocal lymphocytic accumulations and the kidneys showed mild multifocal lymphocytic and plasmacytic infiltrations. Bone marrow cellularity was increased (Baker et al. 1987).

The first antibody titers are detectable coincidentally with the initial parasitemia. There is no serological cross-reaction between *Ehrlichia canis* and *A. platys* (Waner et al. 2001).

EHRLICHIA GENOGROUP

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 was first identified in Algeria in 1935 (Donatien and Lestoquard 1935) and has since been recognized worldwide as an important infectious agent of dogs and other canids.

E. canis is transmitted by *R. sanguineus* (Groves et al. 1975). 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

salivary gland secretions into the feeding site. Adult ticks transmit infection as long as 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 incubation period of CME is 8 to 20 days. During this period, the organisms multiply in macrophages of the monocytic phagocytic system, by binary fission, spreading throughout the body (Ristic and Holland 1993). The incubation period is followed by three consecutive stages: an acute, a subclinical, and a chronic phase. The acute phase may last 1 to 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 subsequent subclinical phase. Dogs in this stage show no clinical signs but their platelet counts may be subnormal (Waner et al. 1997; Harrus et al. 1998a). Previous studies have shown that dogs in this phase may remain persistent carriers of *E. canis* for months and even for years (Harrus et al. 1998a). Persistently infected dogs may recover from the disease spontaneously, however, others may subsequently develop the chronic severe form of the disease. Not all dogs develop the chronic phase of CME, and the conditions leading to the development of this phase remain unclear. Dogs suffering the chronic phase may develop clinical signs similar to those of the acute disease with greater severity. Severe pancytopenia is a typical finding of the chronic disease, which occurs as a result of hypocellular bone marrow. Death may occur as a consequence of hemorrhages and/or secondary infections.

Gross pathologic 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. 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 color 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).

Most dogs infected with *E. canis* develop hyperproteinemia, which occurs due to hypergammaglobulinemia. The hypergammaglobulinemia is usually polyclonal but some dogs may develop monoclonal gammopathy (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. 1998b).

Immunological mechanisms seem to be involved in the pathogenesis of the disease. *E. canis* may induce the development of antierythrocyte and/or antiplatelet antibodies and immune complexes (Harrus et al. 1996a, 2001). Platelet-bindable and platelet-bound antiplatelet antibodies were demonstrated postinfection and were suggested to play a role in the pathogenesis of thrombocytopenia in CME (Harrus et al. 1996a; 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). Platelet aggregation studies have shown 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. 1996b).

Following experimental infection, immunoglobulin (Ig) M and IgA antibodies appear 4–7 days after infection, while IgG antibodies generally appear 15 days postinfection (Weisiger et al. 1975; Waner et al. 2001). The role of the humoral immune response in *E. canis* infections is unclear. High *E. canis*-antibody titers do not provide protection for animals when challenged and may have a detrimental effect on the pathogenesis of the disease (Ristic and

Holland 1993; Breitschwerdt et al. 1998a). German shepherd dogs were shown to be more susceptible than other breeds to CME. Moreover, the disease in this breed tends to be more severe with a high mortality rate. The cellular immune response of German shepherd dogs was shown to be depressed compared to 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.

An immunoblot analysis of the IgG response to *E. canis* has indicated antigenic diversity among *E. canis* organisms from different parts of the world (Hegarty et al. 1997). The severity of the disease may be affected by the strain of the organism. A 30-kDa major outer protein is the major antigen recognized by both naturally and experimentally infected dog sera (Ohashi et al. 1998). The inoculum size influences the course of infection (Gaunt et al. 1996). Concurrent disease with other tickborne parasites or other pathogens may also affect the severity and manifestation of the disease.

Ehrlichia chaffeensis

Background, Etiology, and Epidemiology. *Ehrlichia chaffeensis* is the etiologic agent of human monocytic ehrlichiosis (HME) (Dumler et al. 2001). The first diagnosed HME case occurred in Arkansas in 1986, 2 weeks after a tick bite (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* (Maeda et al. 1987). 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).

E. chaffeensis has been convincingly identified from humans, deer, dogs, and ticks only in the United States, mainly in the south-central, southeastern, and mid-Atlantic states, and California. Detection of *E. chaffeensis* DNA by PCR amplification 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 (*Odocoileus virginianus*) and possibly canines, serve as reservoirs (Dumler and Walker 2001).

Pathogenesis. The clinical significance of natural canine infection with *E. chaffeensis* has yet to be determined. Experimentally infected pups have shown fever and no other signs (Dawson and Ewing 1992). One report of three dogs naturally infected with *E. chaffeensis* documented more serious signs including vomiting, epistaxis, lymphadenomegaly, and uveitis, signs indistinguishable from ehrlichiosis caused by *E. canis* (Breitschwerdt et al. 1998b).

In HME, *E. chaffeensis* resides primarily in monocytes and macrophages, and granulomas have been observed in tissues of patients who subsequently recovered from the illness (Feng and Walker 1999). *E. chaffeensis* causes cytopathic effects and necrosis of heavily infected cells *in vitro* and necrosis in immunocompromised patients (Brouqui et al. 1994). Pathologic 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 were 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. *E. chaffeensis* was shown to modulate the host-cell expression of transferrin receptor by up-regulation of host-cell transferrin receptor mRNA, and accumulation of iron in the morula membrane. INF-γ-activated human monocytes were shown to inhibit *E. chaffeensis* infection by down-regulation of surface transferrin receptors, which led to the limitation of available cytoplasmic iron (Barnewall and Rikihisa 1994). This effect of IFN-γ indicated the predominant role of cell-mediated immunity in *E. chaffeensis* infections. However, outer-membrane protein-specific monoclonal antibodies protect severe combined immunodeficiency (SCID) mice from fatal infection of *E. chaffeensis*. This indicated 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 the active host immune response (Telford and Dawson 1996). The multigene locus encoding 28 kDa surface antigen proteins of two *E. chaffeensis* isolates was characterized. Variant forms of the genes for the 28-kDa surface protein 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).

Ehrlichia ewingii

Background, Etiology, and Epidemiology. Although classified in the *E. canis* genogroup, *E. ewingii* infects granulocytes, causing a granulocytic ehrlichiosis in canines. In 1999 it was apparent that it also infects humans (Buller et al. 1999). Based on 16S rRNA sequence, *E. ewingii* is most closely related to *E. chaffeensis* (98.1%) and *E. canis* (98.0%) (Anderson et al. 1992). *E. ewingii* has not yet been cultured *in vitro*.

E. ewingii was identified in a large variety of ticks including *R. sanguineus, A. americanum, D. variabilis, Ixodes scapularis,* and *I. pacificus*. Of these tick species, *A. americanum* is the only proven vector for *E. ewingii* (Anziani et al. 1990; Murphy et al. 1998). The role of the other tick species in transmission of the rickettsia warrants further investigation. Recently, it has been suggested that white-tailed deer (*Odocoileus virginianus*) may be an important reservoir for *E. ewingii* (Yabsley et al. 2002). Ehrlichiosis caused by *E. ewingii* occurs mainly in the spring and early summer.

Pathogenesis. The disease caused by *E. ewingii* in dogs is usually a mild disease that may lead to polyarthritis in chronically infected animals. Anorexia, fever, and lameness are common clinical signs. Thrombocytopenia and mild nonregenerative anemia are typical laboratory findings (Neer 1998).

Ehrlichia ruminantium

Background, Etiology, and Epidemiology. *Ehrlichia ruminantium,* previously known as *Cowdria ruminantium* (Dumler et al. 2001), is a causative agent of heartwater, or cowdriosis, an acute lethal infection of domestic and wild ruminants. It is transmitted to ruminants by *Amblyomma* ticks. At least 12 *Amblyomma* tick species are able to transmit *E. ruminantium,* although at different vector efficiencies (Camus et al. 1996; Walker and Olwage 1987). The most important and widespread are *A. hebraeum* and *A. 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). Heartwater causes severe economic losses through high mortality rate of up to 90% in susceptible hosts (Mahan et al. 2001), and it hinders improvement of livestock to enhance productivity due to their increased susceptibility to the disease. Heartwater occurs all year where seasons are not distinct, as in eastern Africa and the Caribbean, or occurs 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 (Deem et al. 1996a, 1996b). *Amblyomma* ticks are important reservoirs of infection because they can harbor *E. ruminantium* organisms for 15 months (Camus et al. 1996). The abundance of infection in an animal population (clinical or carrier) and the level of tick control influence the infection intensity in the ticks. In Africa, wildlife frequently share pastures with cattle, sheep, and goats and contribute to the epidemiology of the disease (Peter et al. 2002).

Although *E. ruminantium* was only discovered in 1925 by Cowdry, heartwater was recognized as a major constraint to farmers in South Africa in the early 1800s and was associated with transmission by *A. hebraeum* ticks (Camus et al. 1996). *E. ruminantium* is a gram-negative coccus (0.2 to 2.5 μm in size) and has a predilection for monocytes, neutrophils, and endothelial cells. *E. ruminantium* multiplies by binary fission and forms colonies inside a cytoplasmic vacuole in a host cell. *In vitro*, three stages 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 pathogenicity of *E. ruminantium* infection in domestic ruminants varies based on age and breed of the animal. Calves, lambs, and kids possess an innate immunity during the first few weeks of life (1 to 4 weeks). Following this period, these species become totally susceptible to *E. ruminantium* (Du Plessis and Malan 1987). Sheep and goats are more susceptible than cattle, and European breeds are more susceptible to infection than indigenous breeds (Camus et al. 1996; Mahan unpublished observations). Several wildlife species are also susceptible to *E. ruminantium* infection, but the majority remain refractory and serve as reservoirs of infection (Peter et al. 2002).

Pathogenesis. It is believed that the first host cell to be infected by *E. ruminantium* is the monocyte, in

which multiplication occurs in a cytoplasmic vacuole. The organism is carried to the local draining lymph node and comes in contact with endothelial cells, which are primarily associated with *E. ruminantium* infection. *E. ruminantium* enters the blood circulation via the lymph and is disseminated systemically. It undergoes cycles of invasion of host cells, multiplication, expansion into colonies, and release from cells by cellular disruption. This permits invasion of organs and tissue damage. An incubation period of 10–21 days (average of 18 days) is typically observed. The clinical signs of the disease are nonspecific and are initially characterized by onset of fever (>41°C). Depending on the species, breed, age of host, and organism virulence, the clinical disease that ensues can be classified as being either peracute, acute, subacute, or mild. Death is a common sequel to the acute forms of disease, but recovery is common after a 2- to 3-day febrile period in the mild form (Mahan, unpublished observations).

In the peracute form (which lasts less than 2 days), death is sudden without any clinical signs. The acute form of the disease is most commonly observed and lasts for 2 to 6 days. The febrile period commences with a fever of 41–42°C, which develops within 12 to 24 hours, and is accompanied by nonspecific signs such as dyspnea, rapid heart rate, anorexia, coughing, oculonasal discharge and dullness. The latter signs may be followed by neurological signs, which include leg paddling, muscle tremors, circling, hyperesthesia, twitching of eyes, head butting, and recumbency, followed a few hours later by death. Diarrhea may be observed in terminal cases, especially in cattle. The subacute form lasts 7 to 10 days, and is similar to the acute form, except the disease process is prolonged.

The clinical pathology of *E. ruminantium* infection is associated with the acute phase of the disease, which coincides with the febrile reaction, when *E. ruminantium* rickettsemia is the highest (Byrom et al. 1991). *E. ruminantium* infection causes progressive anemia (normocytic, normochromic) thought to be associated with bone marrow depression. There is usually a fluctuation in the total and differential white cell count, which is characterized by a neutropenia, eosinopenia, and marked lymphocytosis, usually at the end of the disease (Van Amstel et al. 1988; 1994). Lymphocytes, which increase in numbers, include CD4+ T cells, CD8+ T cells, and IgG and IgM surface positive B lymphocytes (Mahan, unpublished observations). Thrombocytopenia, increased prothrombin time, increased activated partial thromboplastin time, and fibrinogen degradation products are also detected. A decline in total calcium, total serum protein, and albumin and globulin levels may also occur during the acute phase of the disease.

E. ruminantium causes widespread systemic infection, however, it may not be easily detected in all organs. At postmortem, the typical signs of *E. ruminantium* infection are hydropericardium (hence termed "heartwater"), hemorrhages on epicardium, endocardium, and visceral and mucosal surfaces, hydrothorax, froth in the trachea, ascites, edema of the mesenteric and mediastinal lymph nodes, and splenomegaly. The fluid in the pericardial sac, and/or thoracic cavity is usually clear with a yellow color, sometimes blood-tinged, especially in the former. The amount of fluid varies between 50 to 1,000 ml. The lungs and kidneys may be congested and enlarged. Brain congestion and edema are also observed in some animals. This finding is thought to be associated with the nervous signs seen in the course of the disease. Effusion of fluids into the body cavities is due to seepage of plasma proteins, which is thought to occur due to 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 involvement of an endotoxin, release of vasoactive amines, or pathology mediated by cytokines that are released in response to infection. *E. ruminantium* has a profound effect on endothelial cells and induces cytokine production. Infection causes a down-regulation of MHC class I and II antigens, which is thought to assist *E. ruminantium*'s persistence and dampens the host's immune response to infection. In bovine endothelial cells, *E. ruminantium* infection elicits the *de novo* synthesis of interleukin 1–β (IL-1β), IL-6, and IL-8 mRNA. Although IL-1 and IL-6 can act as costimulators of T and B cells, uncontrolled production of these cytokines and other mediators can contribute to the pathogenesis of *E. ruminantium* infection (Totté et al. 1999).

Microscopically, the most definitive finding is the identification of *E. ruminantium* in brain capillary endothelium (Purchase 1945). These organisms occur in colonies in the cytoplasm or as free organisms in the capillary lumen in material taken from any part of the brain, although the hippocampus is

the preferred choice for a diagnostic sample. Perivascular infiltrates of leukocytes are also seen in the brain tissue from infected animals. Renal nephrosis is common, and *E. ruminantium* can also be found in the glomerular capillaries (Camus et al. 1996).

Immunity to *E. ruminantium* is acquired following recovery from infection. In addition, immunity can be acquired passively via colostrum by calves born to immune dams from heartwater endemic areas (Deem et al. 1996a). A carrier subclinical state develops and does not have any effect on the well-being or productivity of the animal. An antibody response develops following the febrile reaction in animals that survive infection and recognizes the immunodominant 28-kDa major antigenic protein (MAP)-1 (Barbet et al. 1994; Jongejan et al. 1989), the 21-kDa MAP-2 antigen (Mahan et al. 1994), the 27-kDa 18HW antigen(analogous to the outer-membrane protein of *Coxiella burnetti*) (Barbet et al. 2001), the 58-kDa heat shock protein (Lally et al. 1995), and other proteins that have yet to be fully defined (Barbet et al. 2001). These immunogenic proteins are highly conserved among *E. ruminantium* strains. Humoral immunity is unlikely to provide protection against *E. ruminantium*, because it is an obligate intracellular organism and is therefore inaccessible to antibodies. This has been demonstrated by the failure to transfer immunity by hyperimmune serum (Du Plessis 1970).

Activation of T lymphocytes of the Th1 immune response pathway (CD4+ and or CD8+ T cells) is key to the induction and maintenance of protective immunity to *E. ruminantium* infection (Byrom et al. 2000a, 2000b; Mwangi et al. 1998, 2002). 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. INF-γ is produced when a classical Th1-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 antigenic stimulation with MAP-1 and MAP-2. CD4+ and γδ-T lymphocyte cell lines were eventually isolated from these cultures (Mwangi et al. 1998, 2002). These enriched activated T cells secreted IFN-γ, and contained mRNA of IFN-γ, TNF-α, TNF-β, IFN-α, and IL2-receptor-α 36–48 hours after *in vitro* stimulation (Mwangi et al. 1998, 2002). IFN-γ is an important protective cytokine, which is active against *E. ruminantium* (Totté et al. 1993; Mahan et al. 1996) and other intracellular organisms. It mediates its effect by direct killing of infected cells, by rendering host cells less "infection-friendly," and by activating other pathways that kill intracellular organisms, in particular the activation of the phagocytes that release TNF-α and activate the nitric oxide pathway.

Development of vaccines against heartwater will require the activation of T cells that belong to the Th1 pathway. Two vaccines have been developed so far. A vaccine containing inactivated *E. ruminantium* in combination with an adjuvant that stimulates cell-mediated immunity has been shown to protect sheep, goats, and cattle against lethal challenge (Martinez et al. 1996; Totté et al. 1997; Mahan et al. 1998, 2001). This vaccine induces T cells of the Th1 immune pathway. DNA vaccines have also been tested against *E. ruminantium* infection. The MAP-1 DNA vaccine activates the Th1 pathway (secretion of high levels of IFN-γ) and protects inbred DBA/2 mice against *E. ruminantium* challenge. Vaccination with the recombinant MAP-1 protein alone induces nonprotective Th2 responses, further endorsing the fact that protective immunity against *E. ruminantium* is cell mediated (Nyika et al. 2002).

Ehrlichia muris

Background, Etiology, and Epidemiology. *Ehrlichia muris* was first isolated from the spleen of a wild mouse (*Eothenomys kageus*) in 1983 in Japan (Kawahara et al. 1993). Molecular evidence suggests that it is most closely related to *E. chaffeensis* (Wen et al. 1995). Inclusion bodies were observed in the cytoplasm of splenic and peritoneal macrophages (Wen et al. 1995). A serological survey in central Japan revealed antibodies reactive with *E. muris* among mice, dogs, monkeys, bears, deer, and wild boars (Kawahara et al. 1999). The vector of *E. muris* is unknown, however, the tick *Haemaphysalis flava* has been suggested as a potential vector (Kawahara et al. 1999).

Pathogenesis. When injected intraperitoneally, the organism can establish infection in mice and cause severe clinical signs including splenomegaly, hepatomegaly, lymphadenomegaly, and mortality in rare cases (Kawahara et al. 1993). The level of *E. muris* infection in the spleen and peritoneal cavity was shown to be greatest at day 10 post-artificial infection (Kawahara et al. 1996). Infected mice developed marked IgM and IgG hypergammaglobulinemia that peaked at day 20 postinfection. The

hypergammaglobulinemia did not correlate with antibody titers against *E. muris,* which remained low through a 30-day study. Serologically, *E. muris* cross-reacts with *E. chaffeensis* and *E. canis*, and weakly with *Neorickettsia sennetsu* (Wen et al. 1995).

Th1-type response has been demonstrated to be the predominant immune response to *E. muris* infection as indicated by the increase in blood IFN-γ levels, which peaked at day 10 postinfection. *E. muris* was shown to rapidly induce a protective immune response, which reduced the organism numbers below the threshold level of a clinical disease. Clearance of the organism was however incomplete, and mice remained persistent carriers for up to 400 days postinfection (Kawahara et al. 1996).

Ehrlichia ovina

Background, Etiology, and Epidemiology. *Ehrlichia ovina* is the etiologic agent of ovine ehrlichiosis. The organism has not been formally proposed and accepted according to the rules of the International Bacteriological Code. The organism was first encountered in 1930 in Algerian sheep. Since then, it has been reported in other countries in Africa and Asia (Neitz 1968). Information regarding this organism is limited.

E. ovina parasitizes mononuclear cells. It has been artificially transmitted by intravenous and subcutaneous injections of emulsions of engorged adult female *Rhipicephalus bursa* ticks that had fed in the preceding stages on *E. ovina*-infected sheep, and by adult *R. evertsi* ticks (Neitz 1968).

Pathogenesis. *E. ovina* is not recognized as a highly pathogenic organism, and it seems that sheep suffering other diseases, malnutrition, or coinfection with other organisms may show signs of disease. Affected sheep may show pyrexia, anorexia, listlessness, and anemia, however, the disease may be fatal in splenectomized animals. In untreated animals, recovery is followed by a durable premunity. Protective immunity may persist for a period of 18 months, and splenectomy in these animals may be followed by a relapse within 3 weeks (Neitz 1968).

NEORICKETTSIA GENOGROUP

Neorickettsia helminthoeca

Background, Etiology, and Epidemiology. Salmon-poisoning disease (SPD) is a highly fatal disease of dogs and wild canids caused by *Neorickettsia helminthoeca*. Antigenically, *N. helminthoeca* is closely related to *Ehrlichia canis* (Rikihisa 1991). The Elokomin fluke fever agent, which causes a disease with a high morbidity but lower mortality than SPD, is considered to be a less-pathogenic strain of *N. helminthoeca*. Both these agents are transmitted by the ingestion of fish containing the metacercariae of the trematode *Nanophyetus salmincola.* The disease is restricted geographically by the distribution of the fluke's first intermediate host, the snail *Oxytrema silicula,* to the west coast of Northern California, Washington, Oregon, and British Columbia.

Pathogenesis. Dogs are infected by eating raw fish containing infected metacercariae of *N. salmincola.* The species of fish involved are salamoid fish, some species of nonsalamoid fishes, and the Pacific giant salamander. The metacercariae develop into adults about 6 days postinfection, at which time the rickettsiae are inoculated into the intestinal mucosa. Initial replication of the rickettsia takes place in the epithelial cells of the villi or in the intestinal lymphoid tissue and Peyer's patches, and may cause enteritis. Rickettsiae subsequently enter the bloodstream, spreading to the lymph nodes, tonsils, thymus, spleen, liver, lungs, and brain, infecting macrophages of the monocytic phagocytic system (Frank et al. 1974). The incubation period of SPD usually varies between 5 to 7 days, but can be as long as a month. SPD is characterized by a fever greater than 40°C accompanied by depression and anorexia. Vomiting and diarrhea are common signs, and a seronasal and mucopurulent ocular discharge may be present in some dogs. A generalized lymphadenopathy may be evident.

The major gross pathological findings occur in the lymphoid tissues, which are markedly enlarged. Other changes include splenomegaly and petechiae in the wall of the gallbladder, in the urinary bladder, and along the intestinal tract. Microscopically, the lymph nodes show a marked and consistent depletion of the number of mature lymphocytes (Frank et al. 1974). The central nervous system often presents with nonsuppurative meningitis and meningoencephalitis.

Neorickettsia risticii

Background, Etiology, and Epidemiology. *Neorickettsia risticii,* previously known as *Ehrlichia risticii,* is the etiological agent of Potomac horse fever (PHV), also known as equine monocytic ehrlichiosis and equine ehrlichial colitis. The organism initially infects monocytes and macrophages, and later

in the disease can be identified in colonic and small intestinal epithelial cells. The mode of transmission of *N. risticii* and the reservoir of infection are uncertain, however, the disease has been linked to freshwater snails and aquatic insects, and the proximity of horses to freshwater streams or ponds increases the risk of infection. *N. risticii*-DNA was found in trematodes (virgulate cercariae from freshwater snails in California and in virgulate xiphidiocercariae from *Elimia* snails in Ohio) and in various aquatic insects (Barlough et al. 1998; Reubel et al. 1998; Kanter et al. 2000; Chae et al. 2000). These findings suggest that they play an important role in the transmission of the rickettsia. Although first diagnosed in the Potomac River area of Maryland, the disease is prevalent throughout the United States of America. Recently, the disease has been identified in Uruguay and Brazil where it has a peak prevalence in summer and appears to be associated with the presence of the snail *Pomacea* species (Dutra et al. 2001).

Pathogenesis. The clinical disease is associated with pyrexia, anorexia, mild to severe gastrointestinal signs (ranging from mild colic and soft stools to severe diarrhea), and laminitis. Leukopenia is usually present. The possibility of subclinical cases has been proposed based on the findings of seropositive horses without any history of disease.

A single report of dogs infected by *N. risticii* has been made. Serologic evidence was presented and fatalities were documented. The condition has been referred to as atypical canine ehrlichiosis (Kakoma et al. 1991). Several reports have documented cats seropositive to *N. risticii* by both immunofluorescence antibody and Western blot studies (Peavy et al. 1997). In an experimental infection study, cats seroconverted and some developed fever and mild gastrointestinal signs. *N. risticii* was isolated from the blood of clinically ill cats and was able to infect a pony (Dawson et al. 1988).

Studies of the binding, internalization, and proliferation of *N. risticii* have indicated that *N. risticii* is internalized by receptor-mediated endocytosis and not by phagocytosis (Messick and Rikihisa 1993, 1994). Internalization occurred within 3 hours after infection, and proliferation by 48 hours of incubation. The pathogenesis of the disease caused by *N. risticii* has been studied by investigating the inflammation-associated products in infected cells. Significant amounts of interleukin-1α (IL-1α), IL-6, and prostaglandin-E2 were detected in infected mouse peritoneal macrophages, and were proposed to be primarily involved in the pathogenesis of the disease. Increased intracellular concentrations of cyclic AMP were documented in the infected macrophages and have been proposed to be responsible for the high levels of IL-1α and the inhibition of tumor necrosis factor α (TNF-α) (Van Heeckeren et al. 1993). Intracellular survival of *N. risticii* is considered to take place by the inhibition of phagosome-lysosome fusion. Treatment of infected cells with doxycycline restores phagosome-lysosome fusion, probably by inhibiting a protein secreted by the rickettsia that hinders fusion (Wells and Rikihisa 1988).

ANIMAL RICKETTSIAL PATHOGENS—FAMILY *RICKETTSIACEAE*

RICKETTSIA GENOGROUP

Rickettsia rickettsii

Background, Etiology, and Epidemiology. *Rickettsia rickettsii* is the etiological agent of Rocky Mountain Spotted Fever (RMSF) in animals and humans. The organism replicates in endothelial cells of small blood vessels and capillaries. RMSF in humans was first described in 1899 by Maxey in Idaho (Ricketts 1909). It was reported for the first time in dogs only in 1979 and occurs primarily in the southeastern United States, with cases reported throughout the United States, western Canada, Mexico, and South America. This distribution is related to the distribution of the vector ticks *Dermacentor variabilis* (American dog tick) found primarily in the eastern United States and *D. andersoni* (wood tick) found primarily in the western United States. *Rhipicephalus sanguineus* (brown dog tick) and *Amblyomma cajennense* have been incriminated as vectors in Central and South America. Other ticks that have been found to harbor *R. rickettsii* include *A. americanum, D. occidentalis, D. parumapertus, Ixodes pacificus, I. dammini, I. dentatus, I. texanus, I. brunneus, I. scapularis, I. cookei, Octobius lagophilus,* and *Haemaphysalis leporispalustris* (Anderson et al. 1986; McDade and Newhouse 1986). Ticks are in fact the natural hosts, reservoirs, and vectors of *R. rickettsii*.

Transmission in the tick may be transovarial or transstadial. Larvae and nymphs feed on small mammals, and adults feed on larger mammals including dogs and humans. All stages may become

infected, and infections may be maintained for several generations. Larvae and nymphs may survive the winter, however, the eggs do not (Comer 1991). Rickettsemia in infected dogs is low and short lasting (5–14 days), therefore it is unlikely that dogs serve as reservoirs. However, dogs are important in the epidemiology of human RMSF as they may transport ticks into the home environment. The phenomenon known as reactivation to virulence in which the dormant bacteria become reactivated and pass to the salivary glands requires that the tick be attached to the host for 5 to 20 hours before rickettsial transmission can take place. Thus, early removal of the tick can prevent infection (Greene 1987). The disease occurs more frequently in the spring and summer months when the vector ticks are active.

Pathogenesis. After tick facilitated introduction into the host, the rickettsia enter the lymph and blood circulation and spread to virtually all organs where they invade the endothelial cells and replicate (Rao et al. 1988; Silverman and Santucci 1988). Later they move deeper into the vessel wall and infect the smooth muscle layer and perivascular adventitia causing a necrotizing vasculitis with neutrophilic and lymphocytic infiltrations (Keenan et al. 1977). Phospholipase and proteases have been incriminated as mechanisms for rickettsial damage to cell membranes (Greene and Breitschwerdt 1998). The vascular injury caused by the rickettsia results in increased permeability with extravasation of fluid and blood cells into the extravascular space causing edema, hemorrhage, hypotension, and shock. Central nervous system hypoxia and brain edema may result in nervous signs followed by rapid deterioration and death. Myocardial involvement frequently results in conduction disturbances or life-threatening arrhythmias. Pulmonary edema associated with tachypnea and dyspnea may occur.

Ophthalmologic involvement such as subconjunctival hemorrhage, retinal petechiae, and edema is seen in some cases (Breitschwerdt et al. 1990). Gangrene of the extremities, nose, scrotum, ears, and lips may occur. Decreased renal perfusion as a result of hypotension can result in acute renal failure. The major clinical signs of RMSF include fever, often greater than 40°C, which develops 2–3 days after infection, scleral and conjunctival congestion, anorexia, depression, mucopurulent ocular discharge, tachypnea, vomiting, diarrhea, muscle pain, and various neurological signs. Lymphadenopathy is a common finding in infected dogs (Greene 1987). Lymph node aspirations examined microscopically reveal reactive lymphocytes. In some cases, epistaxis, melena, petechiae, and ecchymoses may occur. The mortality rate in humans is reported to be 4% (Center for Disease Control 1988). In one study in dogs, it was reported at 3% (Greene 1987). Hematological and biochemical changes are diverse and nonspecific. Thrombocytopenia is the most consistent hematological finding, and hypercholesterolemia and hypoalbuminemia are the most consistent biochemical changes. Decreased plasma levels of antithrombin III and fibrinogen, and increased fibrinogen degradation products are also detected, due to activation of the coagulation system (Greene and Breitschwerdt 1998). RMSF may occur concurrently with other tickborne diseases further complicating the diagnosis.

Dogs experimentally infected with RMSF and rechallenged with *R. rickettsii* organisms failed to produce a clinical or serological response suggesting that infection implies lifelong immunity. It is also possible that subclinically infected dogs, dogs with a mild undiagnosed disease, and dogs exposed to nonpathogenic spotted fever group rickettsia develop immunity, which prevents disease in endemic areas (Breitschwerdt et al. 1991).

Rickettsial organisms have developed the ability to replicate in the host cell and thereby evade the humoral immune response. This is supported by the fact that rickettsial infections can continue in the face of vigorous antibody production (Kenyon and Pedersen 1980; Kokorin et al. 1982). This does not mean that T-cell-dependent antibodies play no role in the immune response. It has been shown that pretreatment of rickettsiae with immune serum or monoclonal antibodies prevents or limits infectivity. It seems that antibodies form part of the secondary immune response by binding to the organism and preventing penetration into the host cell (Messick and Rikihisa 1994). *In vivo* it has been shown that penetration of the host endothelial cell by *R. rickettsii* causes the production of cytokines, mainly IL-6, IL-8, and IL-1, which are responsible for the local inflammatory response and possibly the ultimate elimination of the organism. It has now been shown that human cells are capable of controlling rickettsial infections intracellularly by one or a combination of three mechanisms: nitric oxide synthesis, hydrogen peroxide production, and tryptophan degradation (Feng and Walker 2000). Thus, it appears that immunity to primary *R. rickettsii* infection is a Th1-type cellular immunity, and IFN-γ is

the most important but not exclusive cytokine involved in the clearance of the organism (Sporn and Mader 1996). Recently it was observed that serologically unrelated spotted fever group organisms have the ability to cross-stimulate T cells. This cross-reaction provided protective immunity to guinea pigs challenged with virulent *R. rickettsii* (Cage and Jerrells 1992).

CONCLUSION

Rickettsial organisms have been documented to cause a wide variety of clinical syndromes in domestic animals and humans, causing significant health problems and major economic losses. Their harmful effect results from their ability to induce immunosuppression and cause persistent infections despite the presence of a vigorous hostile immune response by the host, which is directed at their immunodominant outer-membrane proteins. Persistent infection by many rickettsiae results from their ability to evade the immune system, using the mechanism of antigenic variation. These rickettsiae can exhaustively generate antigenically distinct variants as a result of sequence modifications and recombinations within the hypervariable regions of genes that encode their major outer-membrane proteins. The constant generation of new antigenic variants complicates the development of improved vaccines to most pathogenic rickettsiae. An in-depth understanding of the immune responses to rickettsial organisms, the antigens that provoke them, and the molecular processes that occur in the host-pathogen interactions is likely to facilitate the development of new antirickettsial vaccines. It may also facilitate more efficacious treatment regimens for use in infected animals.

REFERENCES

Abeygunawardena, I. S., I. Kakoma, and R. D. Smith. 1990. Pathophysiology of canine ehrlichiosis. In Ehrlichiosis: a vector-borne disease of animals and humans. J. C. Williams and I. Kakoma (eds.). Dordrecht: Kluwer, pp.78–92.

Anderson, B. E., C. E. Greene, D. C. Jones, and J. E. Dawson. 1992. *Ehrlichia ewingii* sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. Int. J. Syst. Bacteriol. 42:299–302.

Anderson, J. F., L. A. Magnarelli, R. N. Pillip, and W. Burgdorfer. 1986. *Rickettsia rickettsii* and *Rickettsia montana* from ixodid ticks in Connecticut. Am. J. Trop. Med. Hyg. 35:187–191

Anziani, A. S., S. A. Ewing, and R. W. Barker. 1990. Experimental transmission of a granulocytic form of the tribe Ehrlichieae by *Dermacentor variabilis* and *Amblyomma americanum* to dogs. Am. J. Vet. Res. 51:929–931.

Baker, D. C., M. Simpson, S. D. Gaunt, and R. E. Corstvet. 1987. Acute *Ehrlichia platys* infection in the dog. Vet. Pathol. 24:449–453.

Baker, D. C., S. D. Gaunt, and S. S. Babin. 1988. Anemia of inflammation in dogs infected with *Ehrlichia platys*. Am. J. Vet Res. 49:1014–1016.

Barbet, A. F, S. M. Semu, N. Chigagure, P. J. Kelly, F. Jongejan, and S. M. Mahan. 1994. Size variation of the major immunodominant protein of *Cowdria ruminantium*. Clin. Diagn. Lab. Immunol. 1:744–746.

Barbet, A. F., W. M. Whitmire, S. M. Kamper, B. H. Simbi, R. R. Ganta, A. L. Moreland, D. M. Mwangi, T. C. McGuire, and S. M. Mahan. 2001. A subset of *Cowdria ruminantium* genes important for immune recognition and protection. Gene 275:287–298.

Barlough, G. E., G. H. Reubel, J. E. Madigan, L. K. Vredevoe, P. E. Miller, and Y. Rikihisa. 1998. Detection of *Ehrlichia risticii*, the agent of Potomac horse fever, in freshwater stream snails (*Pleuroceridae*: *Juga spp*.) from northern California. Appl. Environ. Microbiol. 64:2888–2893.

Barlough, J. E., J. E. Madigan, D. R. Turoff, J. R. Clover, S. M. Shelly, and J. S. Dumler. 1997. An Ehrlichia strain from a llama (*Lama glama*) and llama-associated ticks (*Ixodes pacificus*). J. Clin. Microbiol. 35:1005–1007.

Barnewall, R. E., and Y. Rikihisa. 1994. Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron transferrin. Infect. Immun. 62:4804–4810.

Baumgartner, W., J. Stoger, and W. Marktl. 1993. Demonstration of the oral path of infection with *Anaplasma marginale* in calves. Vet. Rec.133:64–66.

Biberstein, E. L., and D. C. Hirsh. 1999. Rickettsial agents of animal disease; the *Rickettsiae*. In Veterinary microbiology. D. C. Hirsh and Y. C. Zee (eds.). Oxford: Blackwell Science, pp. 291–293.

Bjoersdorff, A., L. Svendenius, J. H. Owens and R. F. Massung. 1999. Feline granulocytic ehrlichiosis—a report of a new clinical entity and characterization of the infectious agent. J. Small Anim. Pract. 40:20–24.

Braun-Hansen, H., H. Gronstol, and F. Hardeng. 1998. Experimental infection with *Ehrlichia phagocytophila* in cattle. J. Vet. Med. B. 45:193–203.

Brayton, K. A., G. H. Palmer, A. Lundgren, J. Yi, and A. F. Barbet. 2002. Antigenic variation of

Anaplasma marginale msp2 occurs by combinatorial gene conversion. Mol. Microbiol. 43:1151–1159.

Breitschwerdt, E. B., B. Hegarty, and S. Hancock. 1998a. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. J. Clin. Microbiol. 36:2645–2651.

Breitschwerdt, E. B., C. H. Hegarty, and S. I. Hancock. 1998b. Doxycycline Hyclate treatment of experimental canine ehrlichiosis followed by challenge inoculation with two *Ehrlichia canis* strains. Antimicrob. Agents Chemother. 42:362–368.

Breitschwerdt E. B., M. G. Davidson, D. P. Aucoin, M. G. Levy, N. S. Szabados, B. C. Hegarty, A. L. Kuehne, and R. L. James. 1991. Efficacy of chloramphenicol, enrofloxacin and tetracycline for treatment of experimental Rocky Mountain Spotted Fever in dogs. Antimicrob. Agents Chemother. 35:2375– 2389.

Breitschwerdt, E. B, M. Levy, M. G. Davidson, D. H. Walker, W. Burgdorfer, B. C. Curtis and C. A. Babineau. 1990. Kinetics of IgM and IgG responses to experimental and naturally occurring *Rickettsia rickettsii* infection in dogs. Am. J. Vet. Res. 51:1312–1319.

Brouqui, P., and J. S. Dumler. 1997. The immune response to *E. chaffeensis*. In Rickettsial infection and immunity. B. Anderson, H. Friedman, and M. Bendinelli (eds.). New York: Plenum Press, pp. 163–172.

Brouqui, P., M. L. Birg, and D. Raoult. 1994. Cytopathic effect, plaque formation, and lysis of *Ehrlichia chaffeensis* grown on continuous cell lines. Infect. Immun. 62:405–411.

Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikihisa, A. Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddel, N. Shmulewitz, and G. A. Storch. 1999. *Ehrlichia ewingii,* a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. 341:148–155.

Byrom, B., A. F. Barbet, M. Obwolo and S. M. Mahan. 2000a. $CD8^+$ T cell knockout mice are less susceptible to *Cowdria ruminantium* infection than athymic, $CD4^+$ T cell knockout mice and normal C57BL/6 mice. Vet. Parasitol. 93:159–172.

Byrom, B., C. E. Yunker, P. L. Donovan, and G. E. Smith. 1991. *In vitro* isolation of *Cowdria ruminantium* from plasma of infected ruminants. Vet. Microbiol. 26:263–268.

Byrom, B, M. Obwolo, A. F. Barbet, and S. M. Mahan. 2000b. A polarized Th1 type immune response to *Cowdria ruminantium* is detected in immune DBA/2 mice. J. Parasitol. 86:983–992.

Cage, K. L., and T. R. Jerrells. 1992. Demonstration and partial characterization of antigens of *Rickettsia rhipicephali* that induce cross-reactive cellular and humoral immune responses to *Rickettsia rickettsii*. Infect. Immun. 60:5099–5106.

Camus, E., N. Barre, D. Martinez, and G. Uilenberg. 1996. Heartwater (Cowdriosis). A review. 2nd ed., Office International des Epizooties, Paris.

Caspersen, K., J. H. Park, S. Patil, and J. S. Dumler. 2002. Genetic variability and stability of *Anaplasma phagocytophila* msp2 (p44). Infect. Immun. 70:1230–1234.

Centers for Disease Control. 1988. Rocky Mountain spotted fever: United States MMWR 37:388–389.

Chae, J. S., N. Pusterla, E. Johnson, E. Derock, S. P. Lawler, and J. E. Madigan. 2000. Infection of aquatic insects with trematode metacercariae carrying *Ehrlichia risticii,* the cause of Potomac horse fever. J. Med. Entomol. 37:619–625.

Comer, K. M. 1991. Rocky Mountain spotted fever. Vet. Clin. North Am. Small Anim. Pract. 21:27–44

Dasch, G. A., and E. Weiss. 1998. The rickettsiae. In Topley and Wilson's microbiology and microbial infections: systematic bacteriology. L. Collier, A. Balows, and M. Sussman (eds.). New York: Oxford University Press, pp. 853–876.

Dawson, J. E., and S. Ewing. 1992. Susceptibility of dogs to infection with *Ehrlichia chaffeensis*, causative agent of human ehrlichiosis. Am. J. Vet. Res. 53:1322–1327.

Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley. 1991. Isolation and characterization of an *Ehrlichia sp*. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. 29:2741–2745.

Dawson, J. E., I. Abeygunawardena, C. J. Holland, M. M. Buese, and M. Ristic. 1988. Susceptibility of cats to infection with *Ehrlichia risticii*, causative agent of equine monocytic ehrlichiosis. Am. J. Vet. Res. 49:2096–2100.

Deem, S. L., P. L. Donachie, R. A. I. Norval, and S. M. Mahan. 1996a. Colostrum from dams living in heartwater-endemic area influences calfhood immunity to *Cowdria ruminantium*. Vet. Parasitol. 61:133–144.

Deem, S. L., R. A. I. Norval, P. L. Donachie, and S. M. Mahan. 1996b. Demonstration of vertical transmission of *Cowdria ruminantium*, the causative agent of heartwater, from cows to their calves. Vet. Parasitol. 61:119–132.

de la Fuente, J., R. A. Van Den Bussche, and K. M. Kocan. 2001. Molecular phylogeny and biology of North American isolates of *Anaplasma marginale* (Rickettsiacea: Ehrlichieae). Vet. Parasitol. 97:65–76.

Donatien, A., and A. Lestoquard. 1935. Existance en Algerie d'une rickettsia du chien. Bul. de la Soc. de Path. Exot. 28:418–419.

Dumler, J. S., A. F. Barbet, C. P. J. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa, and F. Rurangirwa. 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. 51:2145–2165.

Dumler, J. S., and D. H. Walker. 2001. Tick-borne ehrlichioses. Lancet Infect. Dis. preview issue, 21–28.

Du Plessis, J. L. 1970. Immunity in heartwater: I. A preliminary note on the role of serum antibodies. Onderstepoort. J. Vet. Res. 37:147–150.

Du Plessis, J. L., and L. Malan. 1987. The non-specific resistance of cattle to heartwater. Onderstepoort. J. Vet. Res. 54:333–336.

Dutra, F., L. F. Schuch, E. Delucchi, B. R. Curcio, H. Coimbra, M. B. Raffi, O. Dellagostin, and F. Riet-Correa. 2001. Equine monocytic ehrlichiosis (Potomac horse fever) in horses in Uruguay and southern Brazil. J. Vet. Diagn. Invest. 13:433–437.

Egenvall, A., A. Bjoersdorff, I. Lilliehook, E. Olsson-Engvall, E. Karlstam, K. Artursson, A. Hedhammar, and A. Gunnarsson. 1998. Early manifestations of granulocytic ehrlichiosis in dogs inoculated experimentally with a Swedish *Ehrlichia* species isolate. Vet. Rec. 143:412–417.

Egenvall, A., I. Lilliehook, A. Bjoersdorff, E. Olsson-Engvall, E. Karlstam, K. Artursson, M. Heldtander, and A. Gunnarsson. 2000. Detection of granulocytic *Ehrlichia* species DNA by PCR in persistently infected dogs. Vet. Rec. 146:186–190.

Eriks, I. S., D. Stiller, and G. H. Palmer. 1993. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J. Clin. Microbiol. 31:2091–2096.

Feng, H., and D. H. Walker. 2000. Mechanisms of intracellular killing of *Rickettsia conorii* in infected human endothelial cells, hepatocytes and macrophages. Infect. Immun. 68:6729–6736.

Feng, H. M., and D. H. Walker. 1999. Mechanisms of Immunity to *Ehrlichia chaffeensis* in mice. In Rickettsiae and rickettsial diseases at the turn of the third millennium. D. Raults and P. Brouqui (eds.) Paris: Elsevier, pp. 145–150.

Frank, D. W., T. C. McGuire, J. R. Gorham, and R. K. Farrell. 1974. Lymphoreticular lesions of canine neorickettsiosis. J. Infect. Dis. 129:163–171.

French, D. M., W. C. Brown, and G. H. Palmer. 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. Infect. Immun. 67:5834–5840.

Friedhoff, K. T. 1997. Tick-borne disease of sheep and goats, *Babesia, Theileria* or *Anaplama* spp. Parasitiologia. 93:99–109.

Gaunt, S. D., R. E. Corstvet, C. M. Berry, and B. Brennan. 1996. Isolation of *Ehrlichia canis* from dogs following subcutaneous inoculation. J. Clin. Microbiol. 34:1429–1432.

Glaze, M. B., and S. D. Gaunt. 1986. Uveitis associated with *Ehrlichia platys* infection in a dog. J. Am. Vet. Med. Assoc. 189:916–917.

Gokce, H. I., and Z. Woldehiwet. 1999a. Differential haemtological effects of tick-borne-fever in sheep and goats. J. Vet. Med. B. 46:105–115.

———. 1999b. *Ehrlichia* (*Cytoecetes*) *phagocytophila* predisposes to severe contagious ecthyma (Orf) in lambs. Comp. Path. 121:227–240.

Gokce, H. I., G. Ross, and Z. Woldehiwet. 1999. Inhibition of phagosome-lysosome fusion in ovine polymorphonuclear leucocytes by *Ehrlichia (Cytoecetes) phagocytophila*. J. Comp. Pathol. 120:369–381.

Greene, C. E. 1987. Rocky Mountain spotted fever. J. Am. Vet. Med. Assoc. 15:666–671.

Greene, C. E., and E. B. Breitschwerdt. 1998. Rocky Mountain spotted fever, Q fever, and typhus. In Infectious diseases of the dog and cat, 2nd ed. C. E. Greene (ed.). Philadelphia: W. B. Saunders Co., pp. 155–165.

Gribble, D. H. 1969. Equine ehrlichiosis. J. Am. Vet. Med. Assoc. 155:462–469.

Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll. 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanuineus*). Am. J. Vet. Res. 36:937–940.

Harrus, S., I. Aroch, E. Lavy, and H. Bark. 1997. Clinical manifestations of infectious canine cyclic thrombocytopenia. Vet. Rec. 141:247–250.

Harrus S., M. J. Day, T. Waner, and H. Bark. 2001. Presence of immune complexes, and absence of antinuclear antibodies, in sera of dogs naturally and experimentally infected with *Ehrlichia canis*. Vet. Microbiol. 83:343–349.

Harrus, S., R. Ofri, I. Aizenberg, and T. Waner. 1998b. Acute blindness associated with monoclonal gammopathy induced by *Ehrlichia canis* infection. Vet. Parasitol. 78:155–160.

Harrus, S., T. Waner, A. Eldor, E. Zwang, and H. Bark. 1996b. Platelet dysfunction associated with experimental acute canine ehrlichiosis. Vet. Rec. 139:290–293.

Harrus, S., T. Waner, D. J. Weiss, A. Keysary, and H. Bark. 1996a. Kinetics of serum antiplatelet antibodies in experimental acute canine ehrlichiosis. Vet. Immunol. Immunopathol. 51:13–20.

Harrus S., T. Waner, H. Bark, F. Jongejan, and A. W. C. A. Cornelissen. 1999. Recent advances in determining the pathogenesis of canine monocytic ehrlichiosis. J. Clin. Microbiol. 37:2745–2749.

Harrus, S., T. Waner, I. Aizenberg, J. E. Foley, A. M. Poland, and H. Bark. 1998a. Ehrlichial DNA amplification from dogs, thirty four months post infection with *E. canis*. J. Clin. Microbiol. 36:73–76.

Harvey, J. W., C. F. Simpson, and J. M. Gaskin. 1978. Cyclic thrombocytopenia induced by a *Rickettsia*-like agent in dogs. J. Infect. Dis. 137:182–188.

Hegarty, B. C., M. G. Levy, R. F. Gager, and E. B. Breitschwerdt. 1997. Immunoblot analysis of the immunoglobulin G response to *Ehrlichia canis* in dogs: an international survey. J. Vet. Diagn. Invest. 9:32–38.

Hildebrandt, P. K., D. L. Huxsoll, J. S. Walker, R. M. Nims, R. Taylor, and M. Andrews. 1973. Pathology of canine ehrlichiosis (Tropical canine pancytopenia). Am. J. Vet. Res. 34:1309–1320.

Hoskins, J. D., O. Barta, and J. Rothschmitt. 1983. Serum hyperviscosity syndrome associated with *Ehrlichia canis* infection in a dog. J. Am. Vet. Med. Assoc. 183:1011–1012.

Inokuma, H., D. Raoult, and P. Brouqui. 2000. Detection of *Ehrlichia platys* DNA in brown dog ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. J. Clin. Microbiol. 38:4219–4221.

Jongejan, F., and M. J. C. Theilemans. 1989. Identification of an immunodominant, antigenically-conserved 32-kilodalton protein from *Cowdria ruminantium*. Infect. Immun. 57:3243–3246.

Kakoma, I., R. Hansen, L. Liu, S. Fedrigon, and K. Bellamy. 1991. Serologically atypical canine ehrlichiosis associated with *Ehrlichia risticii* infection. J. Am. Vet. Med. Assoc. 199:1120.

Kanter, M., J. Mott, N. Ohashi, B. Fried, S. Reed, Y. C. Lin, and Y. Rikihisa. 2000. Analysis of 16S rRNA and 51-kilodalton antigen gene and transmission in mice of *Ehrlichia risticii* in virgulate trematodes from *Elimia livescens* snails in Ohio. J. Clin. Microbiol. 38:3349–3358.

Kawahara, M., C. Suto, S. Shibata, M. Futohashi, and Y. Rikihisa. 1996. Impaired antigen specific responses and enhanced polyclonal stimulation in mice infected with *Ehrlichia muris*. Microbiol. Immunol. 40:575–581.

Kawahara, M., C. Suto, Y. Rikihisa, S. Yamamoto, and Y. Tsuboi. 1993. Characterization of ehrlichial organisms isolated from a wild mouse. J. Clin. Microbiol. 31:89–96.

Kawahara, M., T. Ito, C. Suto, S. Shibata, Y. Rikihisa, K. Hata, and K. Hirai. 1999. Comparison of *Ehrlichia muris* strains isolated from wild mice and ticks and serologic survey of humans and animals with *E. muris* as antigen. J. Clin. Microbiol. 37:1123–1129

Keenan, K. P., W. C. Buhles, D. L. Huxsol, R. G. Williams, P. K. Hildebrandt, J. M. Campbell, and E. H. Stephenson. 1977. Pathogenesis of infection with *Rickettsia rickettsii* in the dog: a disease model for Rocky Mountain Spotted Fever. J. Infect. Dis. 135:911–917.

Kenyon, R. H., and C. E. Pedersen, Jr. 1980, Immune responses to *Rickettsia akari* infection in congenitally athymic nude mice. Infect. Immun. 28:310–313.

Kocan, K. M. 2001. Anaplasmosis. In The encyclopedia of arthropod transmitted infections. M. W. Service (ed.). Oxon: CABI Publishing, pp. 28–33.

Kokorin, I., N. E. A. Kabanova, E. M. Shirokova, G. E. Abrosimova, N. N. Rybkina, and V. I. Pushkareva. 1982. Role of T lymphocytes in *Rickettsia conorii* infection. Acta Virol. 26:91–97.

Kontos, V. I., O. Papadopoulos, and T. W. French. 1991. Natural and experimental infections with a Greek strain of *Ehrlichia platys.* Vet. Clin. Pathol. 20:101–105.

Lally, N. C., S. Nicoll, E. A. Paxton, C. M., Cary, and K. J. Sumption. 1995. The *Cowdria ruminantium groE* operon. Microbiology 141:2091–2100.

Lepidi, H., J. E. Bunnell, M. E. Martin, J. E. Madigan, S. Stuen, and J. S. Dumler. 2000. Comparative pathology, and immunohistology associated with clinical illness after *Ehrlichia phagocytophila*-group infections. Am. J. Trop. Med. Hyg. 62:29–37.

Lewis, G. E., M. Ristic, R. D. Smith, T. Lincoln, and E. H. Stephenson. 1977. The brown dog tick *Rhipicephalus sanuineus* and the dog as experimental host of *Ehrlichia canis.* Am. J. Vet. Res. 38:1953–1955.

Li, J. S., E. Yager, M. Reilly, C. Freeman, G. R. Reddy, A. A. Reilly, F. K. Chu, and G. Winslow. 2001. Outer membrane protein-specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis*. J. Immunol. 166:1855–1862.

Maeda, K., N. Markovitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade. 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N. Engl. J. Med. 316:853–856.

Mahan, S. M., D. Kumbula, M. J. Burridge, and A. F. Barbet. 1998. The inactivated *Cowdria ruminantium* vaccine for heartwater protects against

heterologous strains and against laboratory and field tick challenge. Vaccine 16:203–1211.

Mahan, S. M., G. E. Smith, D. Kumbula, M. J. Burridge, and A. F. Barbet. 2001. Reduction in mortality from heartwater in cattle, sheep and goats exposed to field challenge using an inactivated vaccine. Vet. Parasitol. 97:295–308.

Mahan, S. M., M. Sileghem, G. E. Smith, and B. Byrom. 1996. Neutralization of bovine concanavalin-A T cell supernatant-mediated anti-*Cowdria ruminantium* activity with antibodies specific to interferon gamma but not to tumor necrosis factor. Parasite Immunol. 18:317–324.

Mahan, S. M., T. McGuire, F. Jongejan,, and A. F. Barbet. 1994. Molecular cloning of a gene for an immunogenic 21kD protein of *Cowdria ruminantium*. Microbiology 140:2135–2142.

Martin, M. E., K. Caspersen, and J. S. Dumler. 2001. Immunopathology and ehrlichial propagation are regulated by interferon-gamma and interleukin-10 in a murine model of human granulocytic ehrlichiosis. Am. J. Pathol. 158:1881–1888.

Martinez, D., J. M. Perez, C. Sheikboudou, A. Debus, and A. Bensaid. 1996. Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. Vet. Parasitol. 67:175–184.

McDade, J. E. 1998. Rickettsial diseases. In Topley and Wilson's microbiology and microbial infections, bacterial infections. L. Collier, A. Balows, and M. Sussman (eds.). New York: Oxford University Press, pp. 995–1011.

McDade J. E., and V. F. Newhouse. 1986. Natural history of *Rickettsia rickettsii*. Ann. Rev. Microbiol. 40:287–309.

Meeus, P. F., and A. F. Barbet. 2001. Ingenious gene generation. Trends Microbiol. 9:353–355.

Messick, J. B., and Y. Rikihisa. 1993. Characterization of *Ehrlichia risticii* binding, internalization, and proliferation in host cells by flow cytometry. Infect. Immun. 61:3803–3810.

———. 1994. Inhibition of binding, entry or intracellular proliferation of *Ehrlichia risticii* in P388D1 cells by anti–*E. risticii* serum, immunoglobulin G, or Fab fragment. Infect. Immun. 62:3156–3161.

Mosmann, T. R., and R. F. Coffman. 1989. Heterogenicity of cytokine secretion patterns and functions of helper T-cells. Adv. Immunol. 46:111–143.

Murphy, G. L., S. A. Ewing, L. C. Whitworth, J. C. Fox, and A. A. Kocan. 1998. A molecular and serologic survey of *Ehrlichia canis* and *E. ewingii* in dogs and ticks from Oklahoma. Vet. Parasitol. 79:325–329.

Mwangi, D. M., D. J. McKeever, J. K. Nyanjui, A. F. Barbet, and S. M. Mahan. 2002. Immunisation of cattle against heartwater by infection with *Cowdria ruminantium* elicits T lymphocytes that recognise major antigenic proteins 1 and 2 of the agent. Vet. Immunol. Immunopathol. 85:23–32.

Mwangi, D. M., S. M. Mahan, J. K. Nyanjui, E. L. N. Taracha, and D. J. McKeever. 1998. Immunization of cattle by infection with *Cowdria ruminantium* elicits T lymphocytes that recognize autologous, infected endothelial cells and monocytes. Infect. Immun. 66:1855–1860.

Neer, T. M. 1998. Canine monocytic and granulocytic ehrlichiosis. In Infectious diseases of the dog and cat, 2nd ed. C. E. Greene (ed.). Philadelphia: W. B. Saunders Co., pp. 139–147.

Neitz, W. O. 1968. *Ehrlichia ovina* infection. Bull. Off. Int. Epizoot. 70:337–340.

Nordelo, M., and M. Ysern-Caldentey. 1982. Abnormal bovine erythrocyte membrane proteins and glycoproteins during and after injection with *Anaplasma marginale*. Biochem. Biophys. Res. Comm. 104:664–672.

Nyika, A., A. F. Barbet, M. J. Burridge, and S. M. Mahan. 2002. DNA vaccination with *map1* gene followed by protein boost augments protection against challenge with *Cowdria ruminantium,* the agent of heartwater. Vaccine 20:1215–1225.

Nyindo, M., D. L. Huxsoll, M. Ristic, I. Kakoma, J. L. Brown, C. A. Carson, and E. H. Stephenson. 1980. Cell mediated and humoral immune responses of German shepherd dogs and beagles to experimental infection with *Ehrlichia canis*. Am. J. Vet. Res. 41:250–254.

Ohashi, N., A. Unver, N. Zhi, and Y. Rikihisa. 1998. Cloning and characterization of multigenes encoding the immunodominant 30-kilodalton major outer membrane proteins of *Ehrlichia canis* and application of the recombinant protein for serodiagnosis. J. Clin. Microbiol. 36:2671–2680.

Panciera, R. J., S. A. Ewing, and A. W. Confer. 2001. Ocular histopathology of ehrlichial infections in the dog. Vet. Pathol. 38:43–46.

Peavy, G. M., C. J. Holland, S. K. Dutta, G. Smith, A. Moore, L. J. Rich, M. R. Lappin, and K. Richter. 1997. Suspected ehrlichial infection in five cats from a household. J. Am. Vet. Med. Assoc. 210:231–234.

Peter, T. F., M. J. Burridge, and S. M. Mahan. 2002. *Ehrlichia ruminantium* infection (heartwater) in wild animals. Trends Parasitol. 18:214–218.

Potgieter, F. T, and W. H. Stoltsz, 1994. Bovine anaplasmosis. In Infectious diseases of livestock with special reference to southern africa. J. A. W. Coetzer, G. R. Thomson, and R. C. Tustin (eds.). Oxon: Oxford University Press, pp.408–430.

Purchase, H. S. 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry 1926) in heartwater brains. Vet. Rec. 57:413–414.

Rao, A. K., M. Schapira, M. L. Clements, S. Niewiarowski, A. Z. Budzynski, A. H. Schmaier, P. C. Harpel, W. C. Blackwelder, J. R. Scherrer, E. Sobel, and R. W. Colman. 1988. A prospective study of platelets and plasma proteolytic systems during the early stages of Rocky Mountain spotted fever. N. Engl. J. Med. 318:1021–1028

Raoult, D., and V. Roux. 1997. Rickettsioses as paradigms of new or emerging infectious diseases. Clin. Mic. Rev. 10:694–719.

Reddy, G. R., and C. P. Streck. 1999. Variability in the 28-kDa surface antigen multigene locus of isolates of the emerging disease agent *Ehrlichia chaffeensis* suggests that it plays a role in immune evasion. Mol. Cell. Biol. Res. Commun. 1:167–175.

Reubel, G. H., G. E. Barlough, and J. E. Madigan. 1998. Production and characterization of *Ehrlichia risticii,* the agent of Potomac horse fever, from snails (*Pleuroceridae*: *Juga* spp.) in aquarium culture and genetic comparison to equine strains. J. Clin. Microbiol. 36:1501–1511.

Ricketts, H. T. 1909. Some aspects of Rocky Mountain spotted fever as shown by recent investigations. Med. Rec. 16:843–855.

Rikihisa, Y. 1991. Cross-reacting antigens between *Neorickettsia helminthoeca* and *Ehrlichia* species, shown by immunofluorescence and Western immunoblotting. J. Clin. Microbiol. 29:2024–2029.

Ristic, M., and C. J. Holland. 1993. Canine ehrlichiosis. In Rickettsial and chlamydial diseases of domestic animals. Z. Woldehiwet and M. Ristic (ed.). Oxford: Pergamon Press, pp. 169–186.

Sharma, S. P., and H. C. Tripathi. 1985. Observations on erythrocytic acetylcholinesterase activity in experimentally induced anaplasmosis in calves. Vet. Parasitol. 17:251–254.

Silva, I. M., C. Hubsch, and M. Ysern-Caldentey. 1989. Erythrocyte osmotic fragility and cation concentrations during experimentally induced bovine anaplasmosis. Comp. Biochem. Physiol. A. 94:455–459.

Silverman, D. J., and L. A. Santucci. 1988. Potential for free radical-induced lipid peroxidation as a cause of endothelial cell injury in Rocky Mountain spotted fever. Infect. Immunol. 56:3110–3115.

Simpson, R. M., S. D. Gaunt, J. A. Hair, K. M. Kocan, W. G. Henk, and H. W. Casey. 1991. Evaluation of *Rhipicephalus sanguineus* as a potential biologic vector of *Ehrlichia platys*. Am. J. Vet. Res. 52:1537–1541.

Smith, R. D., D. M. Sells, E. H. Stephenson, M. R. Ristic, and D. L. Huxsoll. 1976. Development of *Ehrlichia canis*, causative agent of canine ehrlichiosis, in the tick *Rhipicephalus sanguineus* and its differentiation from a symbiotic rickettsia. Am. J. Vet. Res. 37:119–126.

Splitter, E. J., H. D. Anthony, and M. J. Twiehaus. 1956. *Anaplasma ovis* in the USA. Experimental studies with sheep and goats. Am. J. Vet. Res. 17:478–491.

Splitter, E. J., M. J. Twiehaus, and E. R. Castro. 1955. Anaplasmosis in sheep in the USA. J. Am. Vet. Med. Assoc. 127:244–245.

Sporn, L. A., and V. J. Mader. 1996. Interleukin-1α production during *Rickettsia rickettsii* infection of cultured endothelial cells: potential role of autocrine cell stimulation. Infect. Immun. 64:1609–1613.

Stoltsz, W. H. 1994. Ovine and caprine Anaplasmosis. In Infectious diseases of livestock with special reference to southern africa. J. A. W. Coetzer, G. R. Thomson, and R. C. Tustin (eds.). Oxon: Oxford University Press, pp. 431–438.

Stuen, S, R. Djuve, and K. Bergstrom. 2001. Persistence of granulocytic *Ehrlichia* infection during wintertime in two sheep flocks in Norway. Acta. Vet. Scand. 42:347–353.

Telford, S. R., and J. E. Dawson. 1996. Persistent infection of C3H/HeJ mice by *Ehrlichia chaffeensis*. Vet. Microbiol. 52:103–112.

Totté, P., A. Bensaid, S. M. Mahan, D. Martinez, and D. J. McKeever. 1999. Immune responses to *Cowdria ruminantium* infections. Parasitology Today. 15:286–290.

Totté, P., D. Blankaert, P. Zilimwabagabo, and J. Werenne. 1993. Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells. Rev. Elev. Méd. Vét. Pays trop. 46:189–194.

Totté, P., D. McKeever, D. Martinez, and A. Bensaid. 1997. Analysis of T-cell responses in cattle immunised against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. Infect. Immun. 65:236–241.

Turco, J., and H. H. Winkler. 1997. Cytokines influencing infections by Rickettsia species. In Rickettsial infection and immunity. B. Anderson, H. Friedman and M. Bendinelli (eds.). New York: Plenum Press, pp. 29–52.

Van Amstel, S. R., F. Reyers, A. J. Guthrie, P. T. Oberem, and H. Bertschinger. 1988. The clinical pathology of heartwater. I. Haematology and blood chemistry. Onderstepoort. J. Vet. Res. 55:37–45.

Van Amstel, S. R., F. Reyers, E. Myburgh, G. Pretorius, and P. Sacks. 1994. The clinical pathology of heartwater. III. Changes in blood clotting, blood calcium, blood protein, haematocrit, and

white cell counts in sheep with experimentally induced heartwater. Onderstepoort. J. Vet Res. 61:21–27.

Van Heeckeren, A. M., Y. Rikihisa, J. Park, and R. Fertel. 1993. Tumor necrosis factor alpha, interleukin-1 alpha, interleukin-6, and prostaglandin E2 production in murine peritoneal macrophages infected with *Ehrlichia risticii*. Infect. Immun. 61:4333–4337.

Walker, J. B., and A. Olwage. 1987. The tick vectors of *Cowdria ruminantium* (*Ixodoidea*, *Ixodidae*, genus *Amblyomma*) and their distribution. Onderstepoort J. Vet. Res. 54:353–379.

Waner T., I. Leykin, M. Shinitsky, E. Sharabani, H. Buch, A. Keysary, H. Bark, and Harrus S. 2000. Platelet-bound antibodies in dogs after experimental infection with *Ehrlichia canis*. Vet. Immunol. Immunop. 77:145–150.

Waner, T., S. Harrus, F. Jongejan, H. Bark, A. Keysary, and A. W. Cornelissen. 2001. Significance of serological testing for ehrlichial diseases in dogs with special emphasis on the diagnosis of canine monocytic ehrlichiosis caused by *Ehrlichia canis*. Vet. Parasitol. 95:1–15.

Waner, T., S. Harrus, H. Bark, E. Bogin, Y. Avidar, and A. Keysary. 1997. Characterization of the subclinical phase of canine ehrlichiosis in experimentally infected beagle dogs. Vet. Parasitol. 69:307–317.

Weisiger, R. M., M. Ristic, and D. L. Huxsoll. 1975. Kinetics of antibody response to *Ehrlichia canis* assayed by the indirect fluorescent antibody method. Am. J. Vet. Res. 36:689–694.

Wells, M. Y., and Y. Rikihisa. 1988. Lack of lysosomal fusion with phagosomes containing *Ehrlichia risticii* in P388D1 cells: abrogation of inhibition with oxytetracycline. Infect. Immun. 56:3209–3215.

Wen, B., Y. Rikihisa, J. Mott, P. A. Fuerst, M. Kawahara, and C. Suto. 1995. *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biological characteristics. J. Clin. Microbiol. 45:250–254.

Wilson, A. J. 1979. Observations on the pathogenesis of anaplasmosis in cattle with particular reference to nutrition, breed and age. J. South Afric. Vet. Assoc. 50:293–295.

Wilson, A. J., R. Parker, and K. F. Trueman. 1980. Susceptibility of *Bos indicus* crossbred and *Bos taurus* cattle to *Anaplasma marginale* infection. Trop. Anim. Health Prod. 12:90–94.

Yabsley, M. J., A. S. Varela, C. M. Tate, V. G. Dugan, D. E. Stallknecht, S. E. Little, and W. R. Davidson. 2002. *Ehrlichia ewingii* infection in white-tailed deer (*Odocoileus virginianus*). Emerg. Infect. Dis. 8:668–671.

Index