Eugene Rosenberg
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Editors

The Prokaryotes

Human Microbiology

Fourth Edition

Springer Reference

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With 178 Figures and 41 Tables

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Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These 11 volumes (planned) on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

This edition of *The Prokaryotes* recognizes the almost unbelievable impact that the work of Carl Woese has had in defining a phylogenetic basis for the microbial world. The concept that the ribosome is a highly conserved structure in all cells and that its nucleic acid components may serve as a convenient reference point for relating all living things is now generally accepted. At last, the phylogeny of prokaryotes has a scientific basis, and this is the first serious attempt to present a comprehensive treatise on prokaryotes along recently defined phylogenetic lines. Although evidence is incomplete for many microbial groups, these volumes make a statement that clearly illuminates the path to follow.

There are basically two ways of doing research with microbes. A classical approach is first to define the phenomenon to be studied and then to select the organism accordingly. Another way is to choose a specific organism and go where it leads. The pursuit of an unusual microbe brings out the latent hunter in all of us. The intellectual challenges of the chase frequently test our ingenuity to the limit. Sometimes the quarry repeatedly escapes, but the final capture is indeed a wonderful experience. For many of us, these simple rewards are sufficiently gratifying so that we have chosen to spend our scientific lives studying these unusual creatures. In these endeavors, many of the strategies and tools as well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

In this school, the principles of the selective, enrichment culture technique have been developed and diversified; they have been a major force in designing and applying new principles for the capture and isolation of microbes from nature. For me, the "organism approach" has provided rewarding adventures. The organism continually challenges and literally drags the investigator into new areas where unfamiliar tools may be needed. I believe that organism-oriented research is an important alternative to problem-oriented research, for new concepts of the future very likely lie in a study of the breadth of microbial life. The physiology, biochemistry, and ecology of the microbe remain the most powerful attractions. Studies based on classical methods as well as modern genetic techniques will result in new insights and concepts.

To some readers, this edition of *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly, those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the "nitrosome," a unique nitrogen-fixing organelle. The study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become "dog-eared" and worn as students seek basic information for the hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, that is, from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator, these volumes should generate excitement.

Happy hunting!

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Preface

During most of the twentieth century, microbiologists studied pure cultures under defined laboratory conditions in order to uncover the causative agents of disease and subsequently as ideal model systems to discover the fundamental principles of genetics and biochemistry. Microbiology as a discipline onto itself, e.g., microbial ecology, diversity, and evolution-based taxonomy, has only recently been the subject of general interest, partly because of the realization that microorganisms play a key role in the environment. The development and application of powerful culture-independent molecular techniques and bioinformatics tools has made this development possible. The fourth edition of *the Handbook of the Prokaryotes* has been updated and expanded in order to reflect this new era of microbiology.

The first five volumes of the fourth edition contain 34 updated and 43 entirely new chapters. Most of the new chapters are in the two new sections: Prokaryotic Communities and Bacteria in Human Health and Disease. A collection of microorganisms occupying the same physical habitat is called a "community," and several examples of bacterial communities are presented in the Prokaryotic Communities section, organized by Edward F. DeLong. Over the last decade, important advances in molecular biology and bioinformatics have led to the development of innovative culture-independent approaches for describing microbial communities. These new strategies, based on the analysis of DNA directly extracted from environmental samples, circumvent the steps of isolation and culturing of microorganisms, which are known for their selectivity leading to a nonrepresentative view of prokaryotic diversity. Describing bacterial communities is the first step in understanding the complex, interacting microbial systems in the natural world.

The section on Bacteria in Human Health and Disease, organized by Stephen Lory, contains chapters on most of the important bacterial diseases, each written by an expert in the field. In addition, there are separate general chapters on identification of pathogens by classical and non-culturing molecular techniques and virulence mechanisms, such as adhesion and bacterial toxins. In recognition of the recent important research on beneficial bacteria in human health, the section also includes chapters on gut microbiota, prebiotics, and probiotics. Together with the updated and expanded chapter on Bacterial Pharmaceutical Products, this section is a valuable resource to graduate students, teachers, and researchers interested in medical microbiology.

Volumes 6–11, organized by Erko Stackebrandt and Fabiano Thompson, contain chapters on each of the ca. 300 known prokaryotic families. Each chapter presents both the historical and current taxonomy of higher taxa, mostly above the genus level; molecular analyses (e.g., DDH, MLSA, riboprinting, and MALDI-TOF); genomic and phenetic properties of the taxa covered; genome analyses including nonchromosomal genetic elements; phenotypic analyses; methods for the enrichment, isolation, and maintenance of members of the family; ecological studies; clinical relevance; and applications.

As in the third edition, the volumes in the fourth edition are available both as hard copies and e-books, and as eReferences. The advantages of the online version include no restriction of color illustrations, the possibility of updating chapters continuously and, most importantly, libraries can place their subscribed copies on their servers, making it available to their community in offices and laboratories. The editors thank all the chapter authors and the editorial staff of Springer, especially Hanna Hensler-Fritton, Isabel Ullmann, Daniel Quiñones, Alejandra Kudo, and Audrey Wong, for making this contribution possible.

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Eugene Rosenberg holds a Ph.D. in biochemistry from Columbia University (1961) where he described the chemical structures of the capsules of *Hemophilus influenzae*, types B, E, and F. His postdoctoral research was performed in organic chemistry under the guidance of Lord Todd in Cambridge University. He was an assistant and associate professor of microbiology at the University of California at Los Angeles from 1962 to 1970, where he worked on the biochemistry of *Myxococcus xanthus*. Since 1970, he has been in the Department of Molecular Microbiology and Biotechnology, Tel Aviv University, as an associate professor (1970–1974), full professor (1975–2005), and professor emeritus (2006–present). He has held the Gol Chair in Applied and Environmental Microbiology since 1989. He is a member of the American Academy of Microbiology and European Academy of Microbiology. He has been awarded a Guggenheim Fellowship, a Fogarty International Scholar of the NIH, the Pan Lab Prize of the Society of Industrial Microbiology, the Proctor & Gamble Prize of the ASM, the Sakov Prize, the Landau Prize, and the Israel Prize for a "Beautiful Israel."

His research has focused on myxobacteriology; hydrocarbon microbiology; surface-active polymers from *Acinetobacter*; bioremediation; coral microbiology; and the role of symbiotic microorganisms in the adaptation, development, behavior, and evolution of animals and plants. He is the author of about 250 research papers and reviews, 9 books, and 16 patents.

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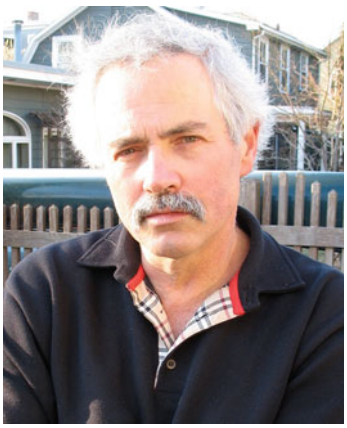
Edward DeLong received his bachelor of science in bacteriology at the University of California, Davis, and his Ph.D. in marine biology at Scripps Institute of Oceanography at the University of California, San Diego. He was a professor at the University of California, Santa Barbara, in the Department of Ecology for 7 years, before moving to the Monterey Bay Aquarium Research Institute where he was a senior scientist and chair of the science department, also for 7 years. He now serves as a professor at the Massachusetts Institute of Technology in the Department of Biological Engineering, where he holds the Morton and Claire Goulder Family Professorship in Environmental Systems. DeLong's scientific interests focus primarily on central questions in marine microbial genomics, biogeochemistry, ecology, and evolution. A large part of DeLong's efforts have been devoted to the study of microbes and microbial processes in the ocean, combining laboratory and field-based approaches. Development and application of genomic, biochemical, and metabolic approaches to study and exploit microbial communities and processes is his another area of interest. DeLong is a fellow in the American Academy of Arts and Science, the U.S. National Academy of Science, and the American Association for the Advancement of Science.



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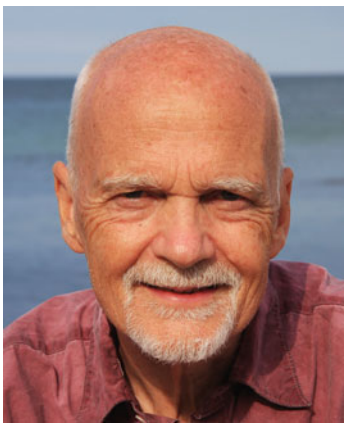
Stephen Lory received his Ph.D. degree in Microbiology from the University of California in Los Angeles in 1980. The topic of his doctoral thesis was the structure-activity relationships of bacterial exotoxins. He carried out his postdoctoral research on the basic mechanism of protein secretion by Gram-negative bacteria in the Bacterial Physiology Unit at Harvard Medical School. In 1984, he was appointed assistant professor in the Department of Microbiology at the University of Washington in Seattle, becoming full professor in 1995. While at the University of Washington, he developed an active research program in host-pathogen interactions including the role of bacterial adhesion to mammalian cells in virulence and regulation of gene expression by bacterial pathogens. In 2000, he returned to Harvard Medical School where he is currently a professor of microbiology and immunobiology. He is a regular reviewer of research projects on various scientific panels of governmental and private funding agencies and served for four years on the Scientific Council of Institute Pasteur in Paris. His current research interests include evolution of bacterial virulence, studies on post-translational regulation of gene expression in *Pseudomonas*, and the development of novel antibiotics targeting multi-drug-resistant opportunistic pathogens.



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Erko Stackebrandt holds a Ph.D. in microbiology from the Ludwig-Maximilians University Munich (1974). During his postdoctoral research, he worked at the German Culture Collection in Munich (1972–1977), 1978 with Carl Woese at the University of Illinois, Urbana Champaign, and from 1979 to 1983 he was a member of Karl Schleifer's research group at the Technical University, Munich. He habilitated in 1983 and was appointed head of the Departments of Microbiology at the University of Kiel (1984–1990), at the University of Queensland, Brisbane, Australia (1990–1993), and at the Technical University Braunschweig, where he also was the director of the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (1993–2009). He is involved in systematics, and molecular phylogeny and ecology of Archaea and Bacteria for more than 40 years. He has been involved in many research projects funded by the German Science Foundation, German Ministry for Science and Technology, and the European Union, working on pure cultures and microbial communities. His projects include work in soil and peat, Mediterranean coastal waters, North Sea and Baltic Sea, Antarctic Lakes, Australian soil and artesian wells, formation of stromatolites, as well as on giant ants, holothurians, rumen of cows, and the digestive tract of koalas. He has been involved in the description and taxonomic revision of more than 650 bacteria taxa of various ranks. He received a Heisenberg stipend (1982–1983) and his work has been awarded by the Academy of Science at Göttingen, Bergey's Trust (Bergey's Award and Bergey's Medal), the Technical University Munich, the Australian Society for Microbiology, and the American Society for Microbiology. He held teaching positions in Kunming, China; Budapest, Hungary; and Florence, Italy. He has published more than 600 papers in refereed journals and has written more than 80 book chapters. He is the editor of two Springer journals and served as an associate editor of several international journals and books as well as on national and international scientific and review panels of the German Research Council, European Science Foundation, European Space Agency, and the Organisation for Economic Co-Operation and Development.

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Fabiano Thompson became the director of research at the Institute of Biology, Federal University of Rio de Janeiro (UFRJ), in 2012. He was an oceanographer at the Federal University of Rio Grande (Brazil) in 1997. He received his Ph.D. in biochemistry from Ghent University (Belgium) in 2003, with emphasis on marine microbial taxonomy and biodiversity. Thompson was an associate researcher in the BCCM/LMG Bacteria Collection (Ghent University) in 2004; professor of genetics in 2006 at the Institute of Biology, UFRJ; and professor of marine biology in 2011 at the same university. He has been a representative of UFRJ in the National Institute of Metrology (INMETRO) since 2009. Thompson is the president of the subcommittee on the Systematics of Vibrionaceae–IUMS and an associate editor of *BMC Genomics* and *Microbial Ecology*. The Thompson Lab in Rio currently performs research on marine microbiology in the Blue Amazon, the realm in the southwestern Atlantic that encompasses a variety of systems, including deep sea, Cabo Frio upwelling area, Amazonia river-plume continuum, mesophotic reefs, Abrolhos coral reef bank, and Oceanic Islands (Fernando de Noronha, Saint Peter and Saint Paul, and Trindade).

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Bacteria in Human Health and Disease

1 The Gut Microbiota

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Abstract
The most densely populated microbial ecosystem that colonizes the human body is located in the gut and is commonly referred to as gut microbiota. This microbial community encompasses trillions of bacteria with an estimated biomass of 1.5 kg, a size that is similar to the liver, the largest organ in the body. It is tempting to consider the gut microbiota as an organ itself, composed of 1,000–1,200 cell types (species) that encode 150-fold more genes (microbiome) than we have in our own genome. The gut microbiota is highly dynamic and exhibits temporal (age) and spatial (along and across the length of the gut) variations. Furthermore, the intestinal microbial composition is responsive to host genetics, diet, ingested drugs, and a wide number of other environmental factors. The gut microbiota plays a fundamental role in human health, as it evolved specific functions that complement human metabolism and physiology. As an example, intestinal bacteria exhibit specific functions involved in fermentation of polysaccharides to bioavailable nutrients that may also act as signaling component. Moreover, intestinal bacteria take part in vitamin production, regulation of hormone synthesis, and maturation of the immune system. Hence, dysbiosis of the gut microbiota has been implicated in many human diseases such as inflammatory bowel disease, obesity, diabetes, and celiac disease.

Introduction

Microbes are the most abundant life form on Earth, and we are accordingly adapted to life in a microbial environment (Whitman et al. 1998). Evidence for the presence of microorganisms associated with the human body was provided at the end of the seventeenth century when Antonie van Leeuwenhoek observed that microorganisms, which he called "animalcules" (tiny animals), were associated with the mouth and feces of individuals in health and disease. While it is now well recognized that the human body provides many niches for a vast number of microbes, the importance of Leeuwenhoek's work remained unappreciated for a long time. The microorganisms, regularly found to colonize the different surface of the host, including skin, oral cavity, respiratory tract, urogenital, and gastrointestinal tract, and that peacefully coexist with their host, represent the so-called normal microbiota or microflora and their collective genomes – the gut microbiome, which is the major part of the metagenome (microbiome plus the human genome) (Dethlefsen et al. 2006). The largest collection of microorganisms is located in the gastrointestinal (GI) tract, which provides several functions for the host, including developmental, immunological, physiological, and nutritional functions, which may affect our life in health and disease (Drasar and Hill 1974; Guarner and Malagelada 2003; Nicholson et al. 2005).

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Microbial Diversity in the GI Tract

The human gut comprises members of the three domains of life on Earth – Bacteria, Archaea, and Eukarya (Finegold et al. 1983). Bacteria dominate this ecosystem where more than 90 % of the phylotypes are members of two of so far ten identified bacteria phyla (*Table 1.1*): the Bacteroidetes and the Firmicutes (Backhed et al. 2005; Turroni et al. 2008; Zoetendal et al. 2006). The Gram-positive Firmicutes include numerous different phylogenetic clusters of clostridia, with clusters IV (also known as *Clostridium leptum* group), IX, and XIVa (also referred to as *Clostridium coccoides* group) being the most abundant clusters (Collins et al. 1994). The predominant genera in these clusters are Clostridium, Eubacterium, Roseburia, and Ruminococcus. Two important groups of butyrate-producing bacteria are *Eubacterium rectale* and *Roseburia* species (members of Clostridium cluster XIVa), comprising 5–10 % of the total microbiota (Aminov et al. 2006; Scott et al. 2008), and *Faecalibacterium prausnitzii* (Clostridium cluster IV), comprising 5–15 % of the total microbiota (Eckburg et al. 2005; Hold et al. 2002; Scott et al. 2008). The Gram-negative genera Bacteroides and Prevotella represent the most well studied from the Bacteroidetes division. Furthermore, Actinobacteria, including the genera Bifidobacterium, Collinsella, and Atopobium, detected at high GC-content (guanine-cytosine content) Gram-positive bacteria, represent important members of the gut microbial community (Franks et al. 1998; Harmsen et al. 2002; Turroni et al. 2008; van der Waaij et al. 2005). Other members of the human gut microbiota, not recognized as dominant, are distributed between seven phyla – Cyanobacteria, Fusobacteria, Lentisphaerae, Proteobacteria, Spirochaetes, TM7, and Verrucomicrobia. The Verrucomicrobia were recently discovered and consist of a single species *Akkermansia muciniphila*, specialized in mucus degradation (Derrien et al. 2004). TM7 is also a newly identified phylum that is widely distributed in the environment and contained so far only uncultured bacteria (Hugenholtz et al. 2001). *Methanobrevibacter smithii* and *Methanobrevibacter stadtmanae* are the two methanogens that represent the Archaea domain in the gut microbial community and are highly prevalent in the GI tract of healthy individuals (Gill et al. 2006; Salonen et al. 2010).

Recent studies of the gut microbial ecosystem identified more than 1,000 species and possibly over 7,000 strains, of which the largest part (~80 %) remains uncultured (Backhed et al. 2005; Blaut and Clavel 2007; Rajilic-Stojanovic et al. 2007; Zoetendal et al. 2008). However, new approaches for culturing previously uncultured colonic microbes are being developed (Duncan et al. 2007; Ingham et al. 2007; Zoetendal et al. 2008). In addition, powerful tools for high-throughput sequencing of genomic DNA from minute quantities of sample and pyrosequencing of amplified microbial genes are providing new insights in the composition of the gut microbiota at high spatiotemporal resolution (Andersson et al. 2008; Marcy et al. 2007).

Host Factors That Affect the Distribution of the Gut Microbiota in the GI Tract

The mammalian GI tract is a compartmentalized system that consists of several distinct anatomical regions, ranging from the stomach to the rectum. Each of these anatomical sections is characterized by varying physicochemical features, such as transit rates of the luminal content, local pH, redox potential, availability of diet-derived compounds, and host secretions (e.g., hydrochloric acid, digestive enzymes, bile, and mucus). Hence, the composition and the abundance of the intestinal microbiota also vary throughout the different regions of the gut (*Fig. 1.1*). The upper GI tract consists of stomach, duodenum, and jejunum and contains a sparse microbiota, which concentration is less than 10^4 organisms per ml of digesta. The relatively low abundant endogenous microbial populations residing in this part of GI tract are affected firstly by the acid stress in the stomach and subsequently by bile acids and pancreatic enzymes released in the duodenum. In addition, the microbial colonization in this part of the small intestine is also impeded by the fast flow of food that causes a rapid wash out of the microbes. The microbial concentration increases toward the end of the small intestine and reaches densities of 10^7–10^8 bacterial cells per gram. The largest microbial concentration is located at the distal part of the GI tract where it reaches concentrations of 10^{12} bacteria per gram of stool, which likely is caused by reduced transit times and increased nutrient availability (*Fig. 1.1*). Thus, the gut microbiota of a single individual outnumbers the total human population of the world by a factor of 1,000 (Moore and Holdeman 1974).

Different microbial populations have been associated not only with the different anatomical regions of the GI tract but also with the latitudinal anatomical sites of the gut. The intestinal lumen forms a continuum with the external environment and is separated from the internal body environment by a single layer of intestinal cells, termed as epithelial surface, which is covered by a mucus layer. The composition of the microbiota associated with the mucus and epithelial crypts significantly differs from that present in the luminal content and the feces (Eckburg et al. 2005; Frank et al. 2007) (*Fig. 1.1*).

Establishment of Gut Microbiota: Succession and Colonization of the Infant GI Tract

The colonization of the intestinal lumen begins at birth when the sterile environment of the infant gut first is colonized by a simple microbial community which develops into a climax community at 2 years of life. The development of the newborn gut microbiota is a gradual and dynamic process that is determined by several factors such as mode of delivery, prematurity, maternal microbiota, type of feeding, illness and antibiotic therapy, and environmental hygiene (Wall et al. 2009). The initial colonization is a consequence of the contact and interaction with both the maternal vaginal and fecal microbes and the surrounding environment (Dominguez-Bello et al. 2010). Eventually, the

Table 1.1

Phylogenetic distribution of the human gastrointestinal microbial phylotypes

Phylum	Class	Order	Family/cluster
			Actinomycetaceae
		Actinomycetales	Corynebacteriaceae
Actinobacteria	Actinobacteria		Micrococcaceae
			Propionibacteriaceae
		Bifidobacteriales	Bifidobacterium
		Coriobacteriales	Corynebacteriaceae
			Rekenellaceae
			Bacteroidaceae
Bacteroidetes	Bacteroidetes	Bacteroidales	Prevotellaceae
			Porphyromonadaceae
			Unclassified
Cyanobacteria	Cyanobacteria	Chroococcales	Unclassified
	Asteroleplasma	Asteroleplasmatales	Asteroleplasmataceae
			Bacillaceae
	Bacilli	Bacillales	Staphylococcaceae
			Aerococcaceae
		Lactobacillales	Carnobacteriaceae
			Lactobacillaceae
			Leuconostocaceae
Firmicutes			Lactococcaceae
			Streptococcaceae
			Clostridium cluster I
			Clostridium cluster III
			Clostridium cluster IV
	Clostridia	Clostridiales	Clostridium cluster IX
			Clostridium cluster XI
			Clostridium cluster XIII
			Clostridium cluster XIVa
			Clostridium cluster XV
			Unclassified
			Clostridium cluster XVI
			Clostridium cluster XVII
	Mollicutes	Unclassified	Clostridium cluster XVIII
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae
Lentisphaerae	Lentisphaerae	Lentisphaerae	Lentisphaeraceae
		Rhizobiales	Unclassified
	α-Proteobacteria	Sphingomonadales	Unclassified
		Unclassified	Unclassified
			Alcaligenaceae
	β-Proteobacteria	Burkholderiales	Oxalobacteraceae
Proteobacteria			Burkholderiaceae
		Aeromonadales	Aeromonadaceae
			Succinivibrionaceae
		Enterobacteriales	Enterobacteriaceae
	γ-Proteobacteria	Pasteurellales	Pasteurellaceae

Table 1.1 (continued)

Phylum	Class	Order	Family/cluster
		Pseudomonadales	Moraxellaceae
			Pseudomonadaceae
		Vibrionales	Vibrionaceae
		Xanthomonadales	Xanthomonadaceae
	δ-Proteobacteria	Desulfovibrionales	Desulfovironaceae
			Campylobacteraceae
	ε-Proteobacteria	Campylobacterales	Helicobacteraceae
Spirochaetes	Spirochaetes	Spirochaetales	Serpulinaceae
TM7	TM7	Unclassified	Unclassified
Verrucomicrobia	Verrucomicrobia	Vericomicrobiales	Vericomicrobiaceae
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae

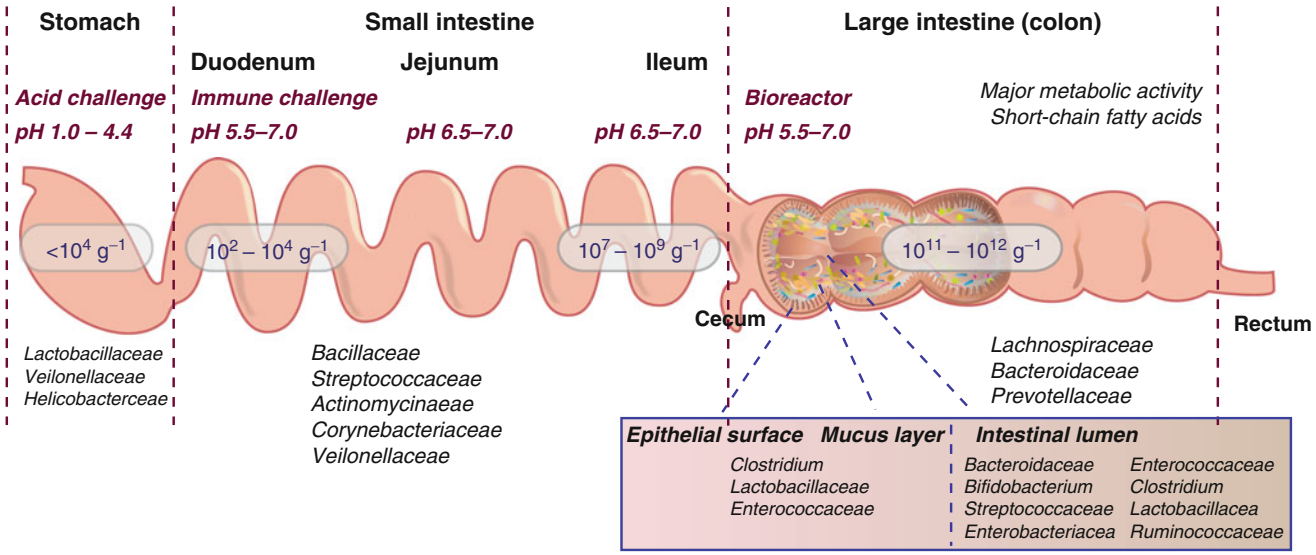

Fig. 1.1

Variations of the gut microbiota composition and numbers along the length of the GI tract. Major features that shape the gut microbiota into the different anatomical regions of the gut are indicated

newborn will be colonized only by those microbes to which it is exposed and that are capable of forming a permanent community in the neonatal GI tract. The succession of microbial populations in the infant gut starts with colonization by facultative anaerobes (*Escherichia coli* and *Streptococcus* spp.) due to the positive redox potential in the gut at birth. Microbial concentrations in the feces quickly reach levels of 10^8–10^{10} cells/g of feces within 1–2 days from birth. Gradually, the initial indigenous microbiota creates reducing conditions that are favorable for the proliferation of anaerobic bacteria. The anaerobic microbial community does not become established in the neonate's gut until the second month of life. Furthermore, the colonization process is strongly affected by the diet (i.e., breast milk and/or formula milk), which will be discussed further in this chapter. During the first month of life, bifidobacteria and *E. coli* are the predominant populations, followed by *Lactobacillus* spp., Bacteroidetes, and Gram-positive bacteria, all in similar quantities. During the first year of life, the microbial composition of the mammalian intestine is relatively simple and differs widely between individuals (Wall et al. 2009). Changes in the proportions of the dominant members of the neonates' gut microbiota appear after about one year of life, mainly as a result of the introduction of new food into the diet of the infant. *Lactobacillus* spp., *Bacteroides* spp., and clostridia numbers increase in this period of life, while bifidobacteria and *E. coli* decrease. Finally at the age of 2 years, the microbial community of the infant gut reaches a climax with a composition of microbes similar to that found in the adult intestine (Koenig et al. 2011). The colonization process of the infant gut, both in timing and composition, may play an important role in health and disease later in life. However, a number of factors, such as diet, host genotype (including the immune system), colonization history, aging,

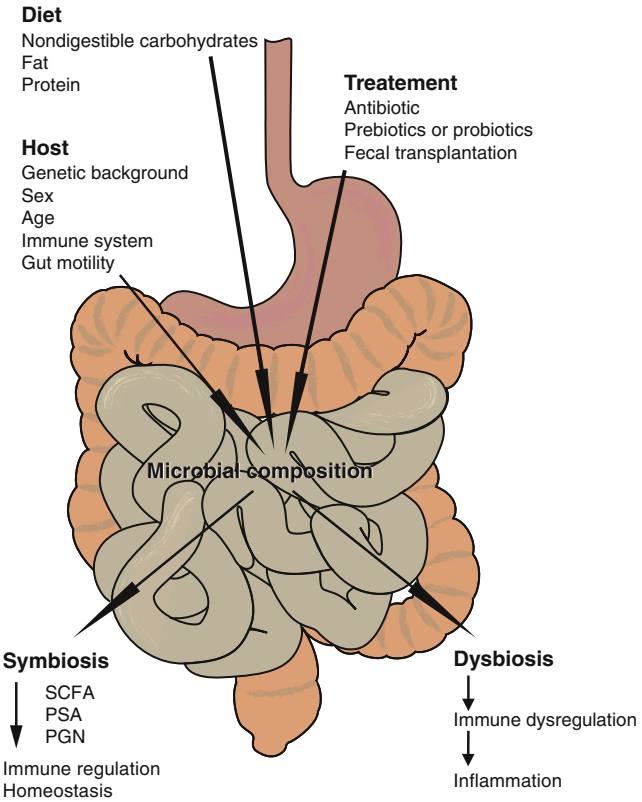

Fig. 1.2

Factors shaping gut microbiota composition. Abbreviations: *SCFA* short-chain fatty acids, *PSA* polysaccharide A, *PGN* peptidoglycan

disease, antibiotic treatment or medication, and stress, shape the final composition of the gut microbiota (Zoetendal et al. 2001) (*Fig. 1.2*).

Factors That Affect the Gut Microbiota Composition

Host Genetics

The genetic makeup of the host has an effect on the intestinal microbiota, as it has been shown in studies where related subjects, identical (monozygotic) and/or fraternal (dizygotic) twins, have been involved. In those culture-independent-based cohorts, high degree of similarity of the gut microbiota composition in monozygotic twins, higher than the similarity between random unrelated individuals, has been reported (Turnbaugh et al. 2009; Zoetendal et al. 2001). However, the study of Turnbaugh et al. reported that the microbiota of monozygotic twins overall was not significantly more similar than that of dizygotic twins suggesting that environment early in life may play an important role for the developing ecosystem. But among the different individuals, an extensive "core microbiome" at a functional (and metabolic) level has been stated, despite their different phylogenetic profiles (Turnbaugh et al. 2009). Furthermore, studies with germ-free (GF) hosts that receive interspecies gut microbiota transplants show that the mammalian hosts might be able to modulate their microbial lineages toward a composition that resembles the original one that is normally found in the conventional status (Rawls et al. 2006). Thus, the genetic makeup is likely to have a profound influence on the host microbiota composition and functionality, but more comprehensive studies are needed to elucidate the exact degree of dependence.

Diet

Diet, microbiota, and GI tract interactions in mammals are extremely complex and are the result of millions of years of coevolution between the higher vertebrates and their specific microbiota. As a consequence, any major change in lifestyle and diet is likely to place stress on the stability of these interactions and affect the entire GI tract ecophysiology. In the first stages of life, the effect of diet (breast vs. formula milk) dramatically influences the colonization pattern. Gut microbiota composition of breast-fed infant is dominated by bifidobacteria and lactic acid bacteria, while formula-feeding in most of the cases results in a more diverse community including bifidobacteria, Bacteroides, clostridia, and a number of facultative anaerobes such as staphylococci, streptococci, and Enterobacteriaceae (Palmer et al. 2007; Wall et al. 2009). Additionally, the number of pathogenic species, such as *E. coli*, *Clostridium difficile*, and some species of the *Bacteroides fragilis* group, is much lower in breast-fed infants, and incidence of *C. difficile* is higher in formula-fed babies. This selection of beneficial microbes in the gut microbiota of breast-fed infants is attributed to the composition of the human milk. The main compounds of the mother milk are oligosaccharides, which are known to act as substrates for fermentation in the distal gut and promote the growth of bifidobacteria (bifidogenic effect) (Wall et al. 2009). Bifidobacteria may also play an important role in the establishment of the microbial community that modulates the immune system (Bode 2009). Human milk is also a rich source of microbes, with numbers that reach up to 10^9 microbes per liter breast milk in a healthy mother, which also may affect microbial ecology in the infant gut (Moughan et al. 1992). Furthermore, introduction of solid food into the infant diet leads to a large compositional shift into the gut microbiota community (Koenig et al. 2011; Palmer et al. 2007).

Differences in human populations and geographic factors might also contribute to the variation of gut microbiota composition. In a recent study, the fecal microbiota of rural children from Burkina Faso (BF) and urban children in Italy was compared by means of 16S rDNA sequencing. During breast-feeding, no significant difference in the microbiota composition between the two geographically different cohorts was found. However, once solid food was introduced, significant enrichment of Bacteroidetes, of several microorganisms involved in polysaccharide degradation and depletion of Firmicutes, was observed in the BF children. These features were completely absent in the Italian children, and the selection of the microbes in the gut of BF children was attributed to the high level of plant polysaccharides

(fibers) present in the BF diet (De Filippo et al. 2010). Fiber fermentation by the gut microbiota leads to increase production of short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate (see p8 in this chapter), and the amounts of the produced SCFA will change quickly when there is a switch in the diet. In this respect, significantly less SCFA were measured in the feces of the European children in comparison with those in the African cohort (De Filippo et al. 2010).

Close relation between dietary habits and the human genome has been shown by Perry et al. when higher copy of salivary amylase gene (AMY) was associated with increased uptake of starch (Perry et al. 2007). Similar evolution has been observed in the gut microbiome, where porphyranase genes were only found in the microbiota of Japanese individuals. Those genes encode for the enzyme that digests porphyrans, a carbohydrate found only in seaweed and that is present in the Japanese diet. Hehemann et al. hypothesize that gut bacteria use horizontal gene transfer to acquire porphyranase genes from the ingested microbes present in the seaweed (Hehemann et al. 2010).

Antibiotics

Antibiotic therapy is another factor that perturbs the ecology of the gut microbiota. Commonly used to remove or prevent a bacterial colonization in the human body, antibiotic treatments cause changes into the gut microbiota, persisting for a long time after discontinuation of the treatment (Dethlefsen et al. 2008; Dethlefsen and Relman 2011; Jernberg et al. 2007; Palmer et al. 2007). A major impact of the use of antibiotics on the indigenous microbiota is a long-term decrease of overall community diversity.

Age

The human gut microbiota is influenced by aging. Medications, most noticeably antibiotic treatments, naturally decline physiological functions. Additionally, changes in the quality and quantity of foods also alter gut microbial ecology in elderly people. Almost similar to what happens at the early stage of our life, the elderly gut is characterized by lower microbial biodiversity, increase in opportunistic facultative aerobes, such as Staphylococci, Streptococci, and Enterobacteriaceae, and decrease in the anaerobic microbiota, particularly in members of Bacteroidetes and Clostridium cluster IV and XIVa. Differently from the infant gut microbiota, the elderly type has lower levels of bifidobacteria (Biagi et al. 2010, 2011; Claesson et al. 2009).

Gut Microbiota Functionality: Metabolic Roles of Gut Microbiota

In terms of functional diversity, recent metagenomic-based studies have indicated that the gut metagenome has a coding capacity that exceeds that of the human genome by at least 150-fold and encodes biochemical pathways that humans have not evolved (Backhed et al. 2005; Gill et al. 2006; Kurokawa et al. 2007; Ley et al. 2006; Qin et al. 2010; Turnbaugh et al. 2007). A plethora of important functions that define the physiology of the host have been assigned to the gut microbiota, including defense against pathogens, immune system maturation, development of the intestinal microvilli, and nutrition (*Table 1.2*). The nutritional function of the gut microbiota is linked to the fermentation of nondigestible dietary fiber and the anaerobic conversions of peptides and proteins, which result in recovery of metabolic energy for the host (Acheson and Luccioli 2004; Hooper et al. 1998; Xu and Gordon 2003). Particularly important is the ability of the gut microbiota to process otherwise indigestible components of our diet because this activity not only provides energy sources but also promotes the maintenance of gut health (Guarner and Malagelada 2003; Savage 1986; Xu and Gordon 2003).

Table 1.2
Beneficial contributions of intestinal microbiota to human health

Maintenance of gut homeostasis
Renewal of intestinal epithelial layer
Regulation of intestinal barrier integrity
Recovery of intestinal epithelial injury
Intestinal angiogenesis
Improved bowel motility
Maturation and education of the immune system
Protection against pathogens (colonization resistance)
Improved energy harvest through digestion of complex fibers in food (i.e., resistant starch and dietary fibers)
Production of nutrients (SCFA, amino acids)
Production of vitamins (vitamin K, vitamin B12, and folic acid)
Metabolism of xenobiotics and procarcinogens
Development of the nervous system
Regulation of appetite and behavior

Until recently, the colon was only considered as a storage place for undigested food components. Based on its biochemical (metabolic) potential, the gut microbiota has been suggested as a "metabolic" organ by itself with metabolic potential which is comparable to that of the liver (O'Hara and Shanahan 2006). These functions include utilization of nondigestible carbohydrates, host-derived glycoconjugates (e.g., mucin), deconjugation, and dehydroxylation of bile acids, cholesterol reduction, biosynthesis of vitamins (K and B group) and isoprenoids, and metabolism of amino acids and xenobiotics.

Microbial Fermentation

The host lacks enzymatic capacity to degrade complex carbohydrates, such as polysaccharides or nondigestible carbohydrates

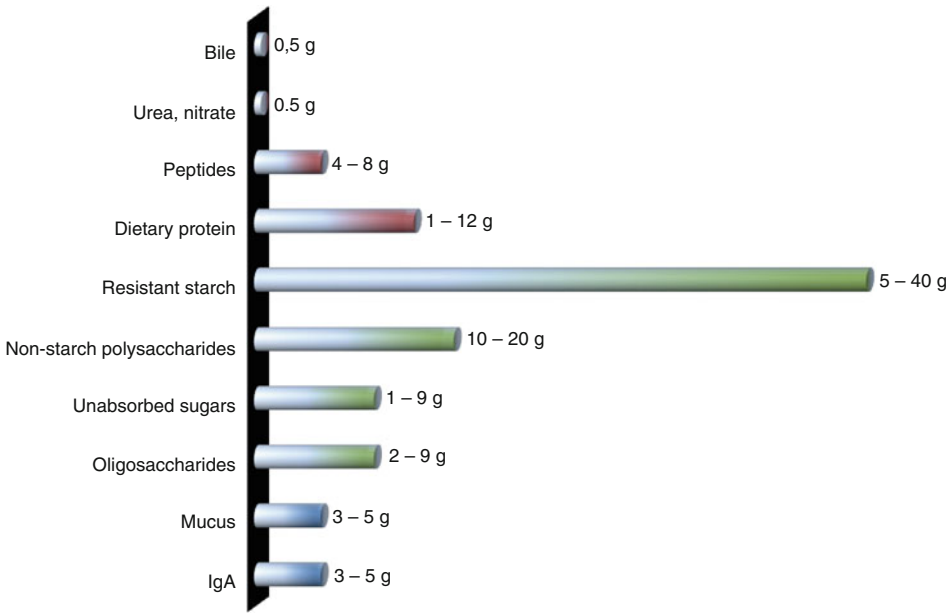

Fig. 1.3
Substrates available for fermentation in the human colon

(resistant starch, non-starch polysaccharides, and fibers of plant origin and nondigestible oligosaccharides), host-derived glycans (mucins, glycosphingolipids), and some proteins from the diet (Cummings and Englyst 1987). As a consequence, these food residues escape digestion in the small intestine and enter the colon where they are fermented by microorganisms (Ouwehand et al. 2005). The amount of dietary carbohydrates entering the colon varies in the range of 10–60 g/day, among which the most abundant carbohydrate is thought to be resistant starch (RS), which is starch recalcitrant to the activity of human amylases, followed by non-starch polysaccharide; unabsorbed sugars, such as lactose, raffinose, and stachyose; and oligosaccharides (*Fig. 1.3*) (Hughes et al. 2000; Macfarlane and Cummings 1991; Scott et al. 2008). These substrates consist of a complex assortment of macromolecules with diverse structures, and degradation requires an array of microbial hydrolytic enzymes produced by various members of the colonic community. Furthermore, during the multiphase conversions of dietary complex carbohydrates, cooperation via metabolic cross-feeding is an important process. Several classes of microbial activities can be observed to occur during such fermentations, and based on substrate utilization and metabolite formation, different functional groups in the colonic microbiota can be defined (*Table 1.3*). Nevertheless, there is still a lack of knowledge with respect to the key microbes involved in colonic fermentation of different relevant dietary carbohydrates. Firstly, enzymatic activities of the primary degraders of complex carbohydrates result in the release of large amounts of polysaccharides, which serve as substrates for the luminal microbiota. Furthermore, the colonic microbiota is involved in the breakdown of polysaccharides to oligosaccharides. Oligosaccharides are one of the most studied carbohydrates, and it has been shown that bacteria that primarily utilize oligosaccharides do not grow on polysaccharides (Rossi et al. 2005). During cross-feeding, the fermentation products released by one microorganism are utilized or serve as a growth factor for another population, often with an impact on the energy metabolism of one or both partners (Samuel and Gordon 2006; Wolin et al. 1997) (*Fig. 1.4*).

Few colonic bacteria, e.g., *Bacteroides* spp. and *Ruminococcus* spp., initiate the breakdown of insoluble substrates (Flint et al. 2008; Jindou et al. 2008; Rincon et al. 2005). In contrast, oligosaccharides, such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS), and the polysaccharide inulin are preferentially fermented by *Bifidobacterium* spp. and *Lactobacillus* spp. A number of bacteria belonging to Clostridium cluster XIVa form the group of butyrate producers, as they produce butyrate as major fermentation product (Barcenilla et al. 2000; Pryde et al. 2002). Different fermentation pathways could be involved in butyrate synthesis in the human colon. The most widespread pathway among butyrate producers of the Clostridium cluster XIVa is the CoA-transferase route (*Fig. 1.4*), whereas another pathway involving butyrate-kinase activity is less common (Louis et al. 2004). A third group of butyrate producers are found to utilize D- and L-lactate for butyrate production: this group includes species belonging to *Eubacterium hallii* and *Anaerostipes caccae* and *Clostridium indolis* (Duncan et al. 2004). There are three distinct pathways for propionate synthesis, namely, the succinate decarboxylase pathway, preferred by *Bacteroides* spp., the acrylate pathway utilized by members of Clostridium cluster IX group (Louis et al. 2007), and the recently described propanediol pathway reported for *Roseburia inulinivorans* (Scott et al. 2006) (*Fig. 1.4*).

The end products of microbial fermentation are gases such as hydrogen, carbon dioxide, and methane as well as short-chain fatty acids, which are the main products of the anaerobic

Table 1.3

Relevant metabolic features of members of the human gut microbiota

Microbial group	Species	Metabolic function	Produced metabolites
Bacteroidetes	*Bacteroidetes* spp. *Prevotella* spp. *B. thetaiotaomicron*	Polysaccharides breakdown; L-, S- Polysaccharides breakdown; L-, S- Polysaccharides breakdown, mucin degradation	Acetate, propionate, succinate Acetate, succinate, formate Acetate, propionate
Actinobacteria	*Bifidobacterium adolescentis* *B. longum* *B. bifidum* *Collinsella*	Carbohydrate metabolism Carbohydrate metabolism Carbohydrate metabolism Carbohydrate metabolism	Acetate, lactate Acetate, lactate, formate, ethanol Lactate, acetate, formate Lactate, acetate, formate
Clostridium cluster IV	*Ruminococcus bromii* *R. flavefaciens* *F. prausnitzii*	Carbohydrate metabolism Plant fibre breakdown Carbohydrate metabolism	Acetate, formate, H_2, ethanol Acetate Butyrate, formate, D-lactate
Clostridium cluster	*R. intestinalis* *R. inulinovarans* *E. hallii* *A. caccae* *E. rectale* *Coprococcus eutactus* *Dorea longicatena*	Carbohydrate metabolism Carbohydrate metabolism Carbohydrate metabolism; A-, L- Carbohydrate metabolism; A-, L- Carbohydrate metabolism; A- Carbohydrate metabolism; A- Carbohydrate metabolism	Butyrate, CO_2 Butyrate, propionate Butyrate Butyrate, CO_2 Butyrate, lactate, formate, H_2 Butyrate, formate, lactate Formate, acetate
Clostridium cluster IX	*Mitsoukella multiacida* *Megasphaera elsdenii*	Gluconic acid metabolism; A- Gluconic acid metabolism; A-, L-	Butyrate Butyrate, propionate
Proteobacteria	*Desulfovibrio* spp.: e.g., *Desulfovibrio piger* *Desulfovibrio desulfuricans*	SRB, L- SRB, L-	Acetate, H_2S
Verrucomicrobia/	*Akkermansia muciniphila* *Victivallis vadenis*	Mucin degradation Cellobiose degradation	Acetate, propionate Acetate
Archaea	*Methanobrevibacter* spp.	H_2-utilizer	CH_4

L lactate utilizer, *S* succinate utilizer, *A* acetate utilizer, *SRB* sulfate-reducing bacteria

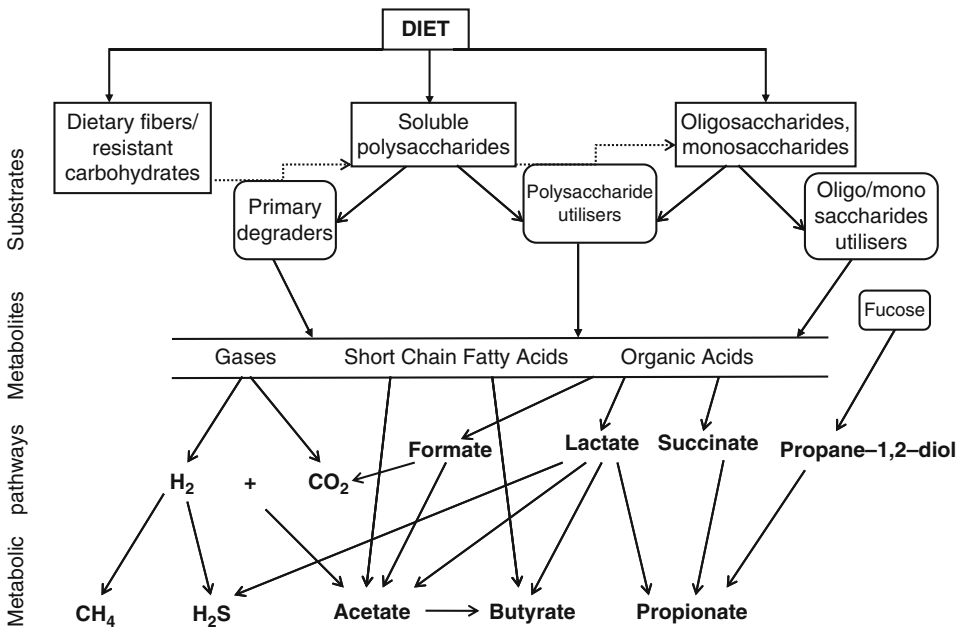

Fig. 1.4

Schematic representation of the conversions of dietary carbohydrates by the gut microbiota. Substrate utilization by different populations in the colon and metabolites produced as a result of the microbial anaerobic fermentations and cross-feeding interactions between primary carbohydrate degraders and other members of the gut microbiota

microbial fermentations occurring in the human colon. Particularly important are butyrate, propionate, and acetate, which have a fundamental role in host physiology (Mortensen and Clausen 1996; Scheppach 1994) (Fig. 1.4).

Physiologic Effects of Microbial Fermentation

SCFA with different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5), and caproate (C6)) are produced in varying amounts depending on the nutrient uptake and the composition of the host gut microbiota. The concentrations of SCFA, measured in autopsy samples from sudden death victims, varied from 137 to 197 mmol/kg chime in the proximal colon and 86–97 mmol/kg chime in the distal colon. The SCFA molar ratio for C2:C3:C4:C5:C6 was found to be approximately 54:20:21:4:1 (Cummings et al. 1987; Macfarlane et al. 1992). SCFA are rapidly absorbed, and only 5–10 % of the produced SCFA are being excreted in the feces (Wong et al. 2006). SCFA lead to lowering of the luminal pH, increase in bacterial biomass and fecal bulk, and modification of the microbial composition, especially by stimulating the growth of beneficial bacteria including bifidobacteria and lactobacilli (Le Leu et al. 2005).

SCFA stimulate mucosal cell proliferation, mucus production, and mucosal blood flow and also affect peripheral metabolism. Butyrate, one of the major SCFA, is also an important energy source for the colonic epithelium and for the prevention of colon cancer (Bauer-Marinovic et al. 2006; Cummings and Bingham 1987; Hamer et al. 2008; Sengupta et al. 2006). Recently, it has been also observed in healthy individuals that colonic butyrate application resulted in reduced visceral pain perception (Vanhoutvin et al. 2009). Acetate is used as a substrate for liver cholesterol and fatty acid synthesis; it also increases colonic blood flow and oxygen uptake and enhances ileal motility by affecting ileal contractions (Scheppach 1994). Propionate is better absorbed than acetate from the human colon and can act as a substrate for gluconeogenesis in the liver. Both acetate and propionate may protect against hepatic cancer, as well as other cancers known to metastasize in the liver, such as breast and colon cancer (Chambers et al. 2002). Formate, succinate, and lactate are also released into the lumen as a result of the carbohydrate conversions by the colonic microbiota. These metabolites also have been reported to affect the host physiology and gut microbiota functionality and composition (Bergman 1990; Cummings and Macfarlane 1991).

Gut Microbiota in the Protein Degradation in the Distal Colon

After anaerobic conversion in the proximal colon, the digesta moves through the colon transversum to the distal colon, and during this passage, carbohydrate availability decreases while proteins and amino acids become the main energy source for the gut microbiota, particularly in the distal colon (Macfarlane et al. 1992). The large intestine has been described as a site of intense protein turnover (Macfarlane and Macfarlane 1995). Important proteolytic species associated with the human colon include species belonging to *Bacteroides, Propionibacterium, Clostridium, Fusobacterium, Streptococcus*, and *Lactobacillus* (Macfarlane and Cummings 1991). Approximately 13 g of dietary proteins enter the colon daily (Fig. 1.3). Additionally, other sources of protein in the colon are provided by endogenous material, e.g., pancreatic enzymes, mucus, and exfoliated epithelial cells. In the colon, nitrogenous residues are initially depolymerized by a mixture of residual pancreatic endopeptidases and bacterial proteases and peptidases (Macfarlane and Cummings 1991), which form short peptides and release amino acids for fermentation. In addition to SCFA, hydrogen, and CO_2, which are formed during protein fermentation, branched-chain fatty acids such as isobutyrate, isovalerate, and 2-methylbutyrate and other organic acids are also produced. Other minor components resulting from protein fermentation are ammonia, amines, phenols, and indoles.

Colonic protein fermentation is associated with increased risk for developing colon cancer, probably due to the production of branched-chain fatty acids and potentially toxic metabolites (i.e., amines, ammonia, phenolic compounds, and thiols) (Bingham et al. 1996; Cummings et al. 1979). A link between protein fermentation and colon cancer is also provided by the fact that colon cancer mostly affects the distal end of the colon (Bufill 1990; Muir et al. 2004), where protein fermentation also occurs. Therefore, it is suggested that an intake of more slowly fermentable carbohydrates could result in prolongation of the potentially beneficial saccharolytic activity and reduce protein fermentation (Jacobasch et al. 1999; Topping and Clifton 2001; Wong et al. 2005).

The Activity of Methanogens and Sulfate-Reducing Bacteria in the Human Colon

During the anaerobic colonic fermentation of carbohydrates and proteins, molecular hydrogen (H_2) is generated, which is further removed from the system through the metabolism of the colonic microbiota (Fig. 1.4). Based on their activity, three functional groups can be defined (Table 1.3). H_2 can serve as growth regulator for colonic methanogens such as *M. smithii* and *M. stadtmanae*, which generate methane (CH_4) as end product of H_2 oxidation. In presence of accessible sulfate (Christl et al. 1992) and mucins (Gibson et al. 1988) in the colonic environment, the sulfate-reducing bacteria (SRB) will outcompete the methanogenic microbes and oxidize H_2 to produce H_2S (hydrogen sulfide). If the colonic sulfate pool is sufficient, the sulfate-reducing bacteria (SRB) will dominate in this niche, while during sulfate-limiting conditions, methanogenic and acetogenic populations will dominate. Acetogens dispose H_2 by reduction of CO_2 to acetate. H_2 that is not utilized by the colonic microbiota can be removed from the colon by passing through the gut wall into the blood stream, where it is

transported to the lungs and then excreted in breath. Measurement of breath H_2 is thus a good indicator for colonic microbial fermentations.

Strategies to Assess Microbial Diversity of the Human Gut

The vast majority of the human intestinal microbiota is strictly anaerobic (99 %) and hard to enumerate using culture-dependent methods. Accordingly, our current knowledge of the microbial composition of the intestinal ecosystem in health and disease is still limited. Development of anaerobic culturing methods such as introduction of the Hungate roll-tube technique and the development of the anaerobic glove box was a major advance in the characterization of gut microbes (Aranki et al. 1969). These strategies are still used as standard tools for the isolation and cultivation of intestinal microorganisms. However, it is generally accepted that only a minor fraction of this important community has been isolated in pure culture as of yet.

An alternative to characterize and enumerate the gut microbiota by culturing is to analyze different biomarkers by the application of molecular or culture-independent techniques. Several biological compounds can serve as biomarkers including metabolites, proteins, RNA, DNA, and single cells. However, the prime biomarker used for phylogenetic analysis of the complex gut microbiota, similarly to other complex environment, is the gene encoding 16S ribosomal RNA (16S rRNA) (Woese 1987). One consequence of the 16S rRNA-based techniques is that only a minor fraction of the gut microbiota has been isolated in pure culture.

The culture-independent techniques that have been successfully applied to study GI tract samples include cloning and subsequent sequencing of the 16S rRNA genes and fingerprinting techniques, such as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), and terminal restriction length polymorphisms (T-RFLP) analysis. Conventional clone library analyses by Sanger sequencing are costly and time-consuming, preventing their application for the analysis of a larger number of samples in order to provide information of microbial composition at sufficient spatiotemporal resolution. TGGE/DGGE and T-RFLP are semiquantitative approaches that allow rapid profiling of the total microbiota. These fingerprinting techniques are mostly used to compare microbial communities and monitor their dynamics. Quantitative 16S rRNA gene-based techniques are fluorescence in situ hybridization (FISH) combined with flow cytometry and quantitative PCR (qPCR).

Phylogenetic microarrays are high-throughput analytical tools, which can be used to measure diversity and abundance of the human gut microbiota. Recently, such a DNA microarray, the Human Intestinal Tract Chip (HITChip), was developed, combining the power of fingerprinting and phylogenetic and quantitative community analysis (Rajilic-Stojanovic et al. 2009). In a recent study, the potential of the HITChip approach was further explored by performing a comparative analysis of the phylogenetic array and pyrosequencing technologies. This study confirmed the high capacity of the HITChip for in-depth profiling of complex microbial communities (Claesson et al. 2009). One limitation of the microarray technology is that these chips target only known sequences. The most recent approaches to enumerate the gut microbiota are based on next-generation sequencing technologies that generate larger amounts of sequences to a reduced price compared with Sanger sequencing. The Roche GS FLX systems developed by 454 life sciences (454 pyrosequencing) represent a sequencing platform that generates around one million DNA sequence reads of up to 450 base pairs each. Application of 454 pyrosequencing of hypervariable regions of the 16S rRNA gene has revealed that the taxonomic richness of the gut microbiota exceeds any previously reported estimates (Andersson et al. 2008; Dethlefsen et al. 2008). Additionally, 454 pyrosequencing analyses have opened new frontiers for the understanding of the role of the gut microbiota in health and disease. Recently, much effort has been put into using shotgun sequencing of the entire metagenome, which in addition to provide phylogenetic information also provide information about the functional capacity.

All together, the introduction of molecular techniques, especially 16S rRNA-based approaches, in microbial ecology over the last 10 years has emphasized the extreme diversity of the human colonic microbiota and has indicated the limitations of culture-based approaches. However, rRNA-based techniques are limited by the fact that they do not provide a direct link to the physiology and metabolic capacities of the intestinal microbiota. In this respect, functional studies are required.

Strategies to Assess Microbial Functionality of the Human Gut

To understand the complex changes in the gut microbiota composition that may predispose toward intestinal disorders or promote human health, techniques that can assay and link metabolic activity to the diversity of intestinal bacteria are needed.

Recently explored metagenomic approaches allow the comprehensive study of phylogenetic, physical, and functional properties of complex microbial communities, providing a full picture of microbiota dynamics (Handelsman 2004). Because metagenomic analyses allow the study of phylogenetic diversity and provide inventories of potential functions of the gut microbiota, they can be used as a tool to link diversity to functionality. Additionally, metagenomic strategies can be divided into functional and sequence-based analyses of collective microbial genomes in complex environments (Gabor et al. 2007). Sequence-based metagenomic investigations have started to reveal core metabolic functions of the gut microbiota. An early metagenomic study on two healthy adults showed that their fecal microbial metagenomes were enriched in genes involved in energy metabolism, which include also the production of SCFA as pivotal energy supply for the intestine (Gill et al. 2006). A recent study, where metaproteomic analyses were

applied to study the distal gut microbiota of a healthy twin pair, indicated more than 50 % of the detected proteins to be involved in translation, energy production, and carbohydrate metabolism (Verberkmoes et al. 2009). Comparison of metagenomic (Gill et al. 2006) and metaproteomic data (Verberkmoes et al. 2009) indicated matches in the fucose and butyrate colonic fermentation pathways. Large-scale comparative metagenomic analyses demonstrated a clear effect of diet and age on the gut microbiome (Kurokawa et al. 2007). A gene catalogue of all prevalent genes of the gut microbiome was generated by illumina-based metagenomic sequencing from the fecal material of 124 European individuals (Qin et al. 2010). This analysis provided a broad overview of functions crucial for the bacteria in the human gut but also pointed out the existence of a bacterial core for the different individuals (Qin et al. 2010). Metabonomics, quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification, is another omics strategy used to assay metabolic functions of the host as well as the gut microbiota (Nicholson 2006). It is estimated that >90 % of the metabolites found human urine are microbial derived. While metagenomics and metatranscriptomics aim to describe the metabolic potential of the gut microbiome, the metabonomics has "the capacity to measure the metabolic kinetic or flux of metabolites through an ecosystem at a particular point in time or over a time course" (Tuohy et al. 2009).

However, an ongoing challenge for microbiologists is to be able to identify which microbes in the human gut carry out a specific metabolic conversion, the products of which may promote intestinal disorders and/or gut health. A strategy, that offers a great potential to identify microbes that are involved in the metabolism of specific substrates, is the so-called stable-isotope probing (SIP). SIP-based approaches involve the usage of commercially prepared substrates highly enriched in a stable isotope (e.g., ^{13}C) or radioisotope (e.g., ^{14}C), which is added to an environmental sample. Endogenous microbes that metabolize the labeled substrate will incorporate the isotope into components of the microbial cells, thus providing phylogenetic information (Radajewski et al. 2000). SIP methodologies vary in the use of biomarkers but also in the means by which biomarkers are analyzed for isotopic and phylogenetic content. To explore the capacity of the gut microbiota in the fermentation of relevant dietary carbohydrates, we applied RNA-based SIP, which in combination with molecular identification tools, provides a direct link between the structure of a microbial community and the function of its members (Kovatcheva-Datchary et al. 2009). This approach is an effective strategy to clarify the functionality of the gut microbiota and to elucidate the role of individual species within the community in their natural environment. Moreover, RNA-based SIP allowed us to identify the primary degraders of tested substrates, and a phylotype related to *R. bromii* was reported to be primary degrader of potato starch, corroborating results that were reported in other in vivo and in vitro experiments (Abell et al. 2008; Leitch et al. 2007). Furthermore, more information on the functionality of the different microbes, found to be active in the colon ecosystem, could be generated after combining the data from the SIP technique with NMR and liquid-chromatography-mass spectrometry. Based on the detection of (partially) labeled metabolites, this allowed the identification of active metabolic pathways and delineation of food webs that may influence human health (de Graaf et al. 2007; Egert et al. 2007; Kovatcheva-Datchary et al. 2009). Integration of the molecular and metabolite data suggested metabolic cross-feeding where populations related to *R. bromii* were the primary starch degraders, while other members of the community related to *Prevotella* spp., *Bifidobacterium adolescentis*, and *E. rectale* were likely involved in this trophic web.

Protein-based stable-isotope probing (Protein-SIP) is a novel approach, which analyzes specific metabolic activity of a single bacterial species within a community that incorporates the labeled substrate in the cellular protein fraction (Jehmlich et al. 2008, 2010). The most important advantage of protein-based analysis is the direct connection to physiological function, as proteins are known to catalyze the biochemical reactions. Thus, proteins are source of phylogenetic and functional information, making them ideal biomarkers for monitoring community structure and function. Furthermore, SIP techniques are suitable for obtaining qualitative and quantitative information about metabolic fluxes in the colon. Isotopically labeled compounds enable the selective study of that part of the microbial or host metabolism that involves the isotopic tracer. NMR and gas- or liquid-chromatography can be used to measure the labeled compounds and further identify active metabolic pathways (Bacher et al. 1998; Egert et al. 2007).

The comprehensive understanding of the metabolic activity of the gut microbiota will enable the development of direct strategies to treat or prevent intestinal disorders caused by microorganisms in humans. Identification of the prime functions of the human gut microbiota in maintaining human health requires better understanding of its diversity and functionality, which can facilitate its manipulation. Most intestinal microbes have not been cultured, and the in situ functions of distinct groups of the gut microbiota are largely unknown but pivotal to understand their role in health and disease.

Relevance of Gut Microbiota for Human Disease

Many of the bacteria that stably inhabit the human gut establish a mutualistic relationship with the host and provide beneficial functions. Besides beneficial bacteria, the human GI tract is associated with several commensals that do not confer specific advantage or detriment. Finally, the gut microbiota also contains potentially harmful species, which are likely to be in closer contact with the intestinal epithelium and have been defined as pathobionts (Round and Mazmanian 2009; Sansonetti 2011). The sum of all symbiotic interactions between the host and its microbiota, and between different microbial species in the community, results in eubiosis, a balance condition that is essential for the maintenance of intestinal, immune, and metabolic

homeostasis (Table 1.2). The condition opposed to eubiosis is defined as dysbiosis and is characterized by qualitative and quantitative changes in composition, spatial distribution, and function of the symbiotic microbial community.

The composition of the gut microbiota in healthy individuals is stable at the phylum level, with Bacteroidetes and Firmicutes being the two dominant phyla. The prevalence of only two indicates that the gut microbiota is a highly adapted system that has coevolved with its host (Dethlefsen et al. 2007). However, enumeration of bacterial species revealed considerable variation among individuals and the abundance of shared species varies up to thousand folds (Qin et al. 2010). The variability at low taxonomical level indicates that the gut microbiota is endowed with functional redundancy and resilience to environmental stress, meaning that the composition of the microbial community may vary without alterations to its overall structure and function (Turnbaugh et al. 2009). However, if the perturbation is deep and/or repeated, the changes introduced eventually compromise the functionality of the community (Dethlefsen et al. 2008; Dethlefsen and Relman 2011). Given the broad contribution of the gut microbiota to human health, dysbiosis is bound to affect not only the intestine but also other, and far, organ systems (Fig. 1.5). In this section, the contribution of the gut microbiota to human disease will be discussed.

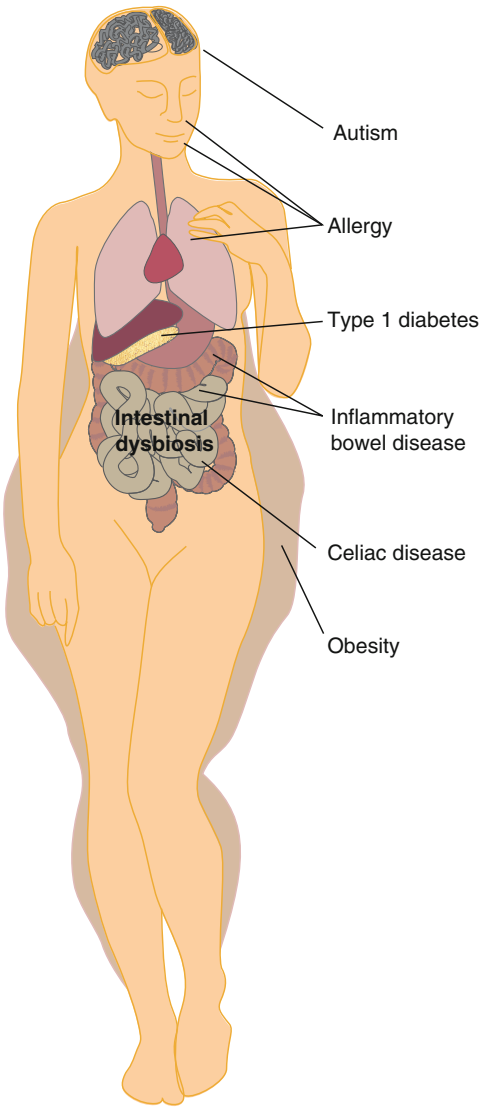

Fig. 1.5
Diseases that have been suggested to be affected by the gut microbiota

Inflammatory Bowel Disease (IBD)

The intestinal mucosa confronts the task of coexisting with symbiotic microbes while preventing microbial overgrowth and pathogen invasion. In health, the mucosal immune system recognizes microbial signals deriving from both symbionts and pathogens and mounts an adequate response that is not detrimental either locally or systemically. The induction of a proper response may be achieved by several mechanisms, such as (1) physical separation of the microbiota from the epithelial surface by the mucus layer or compartmentalization of microbial sensors (i.e., TLRs) to the basolateral membrane of intestinal epithelial cells, (2) evolution of less immunogenic antigens in commensal microbes, and (3) induction of tolerogenic immune responses by the gut microbiota. The microbiota-immune system bidirectional interaction results in the induction of innate immune responses, recruitment of lymphocytes, and activation of both pro-inflammatory (Th1 and Th17 lymphocytes) and anti-inflammatory (Treg cells) adaptive responses that, while preventing deleterious activation of the immune system against innocuous bacteria, also keep the mucosal immune system in a poised state against invading microbes. This condition has been defined as "physiological intestinal inflammation" and is fundamental for maintaining homeostasis at the mucosal interface (Fiocchi 2008; Sansonetti 2011).

Inflammatory bowel disease (IBD) refers to a group of disorders characterized by severe chronic and relapsing inflammation of the GI tract. The two most common forms of IBD in humans are Crohn's disease (CD), which targets the whole length of the GI tract inducing discontinuous inflammation, and ulcerative colitis (UC), which specifically affects the colon and is associated with continuous inflammation. The etiology of IBD is uncertain but strong evidence points to the involvement of an aberrant mucosal immune response to the intestinal microbiota in genetically predisposed subjects (Sartor 2008; Xavier and Podolsky 2007). In particular, IBD has been associated to abnormal activation of pro-inflammatory T cells that release pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF) and interferon-γ (IFNγ)) and to the lack of immunoregulatory cytokines (e.g., interleukin 10 (IL10) and transforming growth factor-β (TGF-β)) that are produced by Treg cells. Susceptibility alleles are present in the population at high frequency but only confer a moderate increase in risk of IBD development. Furthermore, familial aggregation has been observed in IBD, the concordance of CD in monozygotic twins is 60 % while only 20 % for UC (Sun et al. 2011). Finally, increasing incidence of

the disease has been observed in countries with historically low rates of IBD. Taken together, these observations indicate that environmental factors are strongly involved in the pathogenesis of IBD and the gut microbiota could be considered as one.

Evidence for the involvement of gut microbiota in IBD has been found in the observations that antibiotic treatments ameliorate IBD symptoms and microbes or microbial molecules can be isolated from intestinal lesions. Moreover, genetically susceptible animals, if kept germ-free, do not develop the disease. Other environmental triggers of IBD could be the diet, the use of antibiotics, infections, smoking, and stress, which have also been shown to alter the gut microbiota. Recent studies aimed at the global profiling of the gut microbiota showed that both CD and UC are associated with intestinal dysbiosis of both the fecal and the mucosa-associated microbiota (Peterson et al. 2008). One consistent feature detected by several studies is the increased representation of facultative anaerobes (e.g., Proteobacteria, particularly Enterobacteriaceae) in IBD-associated gut microbiota. Increased numbers of these microorganisms have been proposed to result from the aberrant intestinal inflammation that characterizes IBD patients and that could provide a colonization advantage to facultative anaerobes. The gut microbiota of IBD patients is also found to be less diverse and depleted of specific members of the Firmicutes, of the *Clostridium leptum* group, and *F. prausnitzii* in particular (Manichanh et al. 2006; Sokol et al. 2008). The decrease of *F. prausnitzii* was observed in several studies and was associated with an increased risk of postoperative recurrence of ileal CD. This microbe was found to have anti-inflammatory properties due to the releases of a soluble factor that mitigated inflammation in a mouse model of colitis (Sokol et al. 2008). Besides the decrease in clostridia, a decrease in bifidobacteria was also reported both in the feces and in the mucosa-associated microbiota (Hansen et al. 2010; Joossens et al. 2011). As described in the previous sections, clostridia and bifidobacteria are important for the production of butyrate that, besides having a role for colonic cell nutrition, also induces intestinal anti-inflammatory responses (Maslowski et al. 2009). In agreement with these results, fecal extracts of IBD patients contain decreased amounts of butyrate and other SCFA (Marchesi et al. 2007) and increased levels of H_2S (Fava and Danese 2011; Sartor 2008). One recent metagenomic study has also shown that the fecal microbiota of IBD patients contains fewer genes than the microbiota of healthy individuals (Qin et al. 2010). Thus, dysbiosis characterized at the phylogenetic level also bears consequences for the functional diversity and the metabolic activity of the community.

The intestinal barrier also plays an important role in the pathogenesis of IBD, as defects in MUC2 (the major mucin in the human intestine) secretion from goblet cells are observed in UC patients. Several gut microbes secrete glycosidases that can remove the mucins' terminal sugars and expose the chain oligosaccharides and protein core to the action of other gut microbes, thus mediating further degradation of the mucin molecule. A recent study has shown that the mucosa of IBD patients, both in the presence and absence of inflammation, is more efficiently colonized by gut microbes in comparison with mucosa of healthy subjects (Png et al. 2010). The authors found that mucolytic bacteria are normally associated with the mucus of healthy individuals and argue that this mucolytic activity could be important for mucus turnover and stability of the community at the mucosal niche. However, *A. muciniphila*, the major described mucolytic microbe in healthy humans, was reduced in IBD patients, while other mucolytic bacteria, such as *Ruminococcus gnavus* and *R. torques*, were dominant in IBD non-inflamed patients, thus indicating a shift in the mucolytic community as a consequence of altered host factors but independent of intestinal inflammation. The general increase of mucosa-associated bacteria in IBD may be explained by aberrant mucolytic activity, which may increase substrate availability for other microbes, such as *B. vulgatus*, and promote their growth (Png et al. 2010). Additionally, less efficient killing of commensal microbes by the host immune response could also contribute to increase the number of mucosa-associated microbes, which in turn may contribute to increased mucosal inflammation and exacerbate the disease.

While IBD has been associated with alterations in the gut microbiota, the causal relationship between intestinal dysbiosis and disease development has not yet been proven, but recent data indicates that a colitogenic microbiota can be both a cause and a consequence of intestinal inflammation (Sekirov et al. 2010). T-bet deficient RAG2−/− mice lacking adaptive immunity and the ability to regulate inflammatory responses develop spontaneous inflammation that resembles UC. Treatment with antibiotics cures the diseases, indicating that the gut microbiota of these animals contains colitogenic microbes. In this model, colitogenic microbes seem to be selected by the host's predisposition to inflammation. However, transferring of gut microbiota from colitic T-bet deficient RAG2−/− mice to healthy RAG2−/− mice equipped with T-bet or to wild type (WT) animals was sufficient to induce colitis, thus showing that a dysbiotic microbiota can initiate disease (Garrett et al. 2007). The gut microbiota of T-bet deficient RAG2−/− mice was observed to contain higher levels of two Enterobacteriaceae, *Proteus mirabilis* and *Klebsiella pneumonia* (Garrett et al. 2010). Although these bacteria were not sufficient to induce the disease in susceptible mice, they were able to colonize the gut of WT mice and induce colonic inflammation if administered together with a specific pathogen-free microbiota (Garrett et al. 2010). So, selected members of the gut microbiota may be colitogenic, but their ability to induce the disease depends on host's genotype and intestinal inflammation. The nature of host-microbe interactions, which affect the eubiotic/dysbiotic composition of the gut flora, might be decisive for the development of IBD.

Autoimmunity and T1D

Autoimmune diseases are characterized by immune reactivity against self-antigens due to reduced self-tolerance. In health, self-tolerance is attained by elimination of autoreactive lymphocytes during their development in the bone marrow and in the thymus (central tolerance) as well as killing or inactivation of

mature self-reactive lymphocytes in peripheral organs (peripheral tolerance). In genetically predisposed individuals, exposure to environmental triggers may overcome the body's tolerogenic mechanisms and initiate autodestructive inflammatory responses. As mentioned below in this chapter, the gut microbiota has the potential to shape the development of systemic immune responses (Kranich et al. 2011), and in so doing, it may influence the development of autoimmune diseases, such as type 1 diabetes (T1D).

T1D is caused by the progressive T cell-mediated destruction of insulin-producing β-cells in the pancreas, which results in insulin deficiency and high blood glucose concentrations. This disease affects genetically predisposed children and young adults, and approximately 50 % of the genetic risk for T1D is linked to mutations in the human leukocyte antigen (HLA) genes, which encode for molecules that bind and present antigens to T cells. The incidence of T1D in affluent countries has increased during the past decades, and environmental factors have been claimed to have augmented the penetrance of risk genes (Harrison et al. 2008). Interestingly, an inverse association between infectious diseases and autoimmune diseases has also been noted (Bach 2002), similarly to the trend observed for allergic diseases. Clinical and experimental evidence indicates that the onset of T1D may be linked to the gut and the interaction between intestinal permeability, mucosal immune system, and microbiota. Indeed, humans at risk for T1D also display abnormal gut permeability, lack of intestinal Treg activation, aberrant responses to wheat and cow milk (Vaarala et al. 2008), and altered gut microbiota (Giongo et al. 2011). Profiling of the fecal microbiota in children at high risk for T1D revealed increased levels of Firmicutes and reduced levels of Bacteroidetes in children that progress toward diabetes while the opposite trend (i.e., decrease in Firmicutes and increase in Bacteroidetes) is observed for controls.

Evidence for the role of the gut microbiota in T1D can also be found in experimental studies. The incidence of the disease depends on housing conditions and microbial status of animal facilities, with specific pathogen-free (SPF) facilities displaying higher rates of disease (Bach 2002). Besides, the lack of a gut microbiota promotes development of T1D in non-obese diabetic (NOD) mice (Rossini et al. 1979). These results suggest that the gut microbiota exerts a protective role against the development of T1D. A recent study showed that NOD mice that lacked the toll-like receptor (TLR) adapter molecule MyD88, a mutation that altered the intestinal microbiota, were protected from T1D. However, the protection was lost when the mice were rederived as GF or if the gut microbiota was altered through antibiotic treatment (Wen et al. 2008), thus indicating that alterations in an individual's microbiota may play a role in the induction of T1D.

Celiac Disease

In healthy individuals, the presentation of food antigens to the gut immune system results in systemic unresponsiveness to the same antigens, a phenomenon known as oral tolerance. Perturbation of the homeostatic mechanisms that govern oral tolerance can lead to abnormal activation of the gut immune system and damage to the intestinal mucosa, with negative consequences for body's digestive functions and nutrition. Celiac disease is a chronic inflammatory disorder that primarily affects the upper small intestine and is caused by an abnormal immune response to gluten in genetically predisposed subjects who have specific HLA-DQ alleles (i.e., HLA-DQ2/8 alleles, which occur in 30–40 % of the general population but their presence is not sufficient for developing the disease). This enteropathy is prevalent in children, estimated to affect as many as 1 % of the European and North American population, and the therapy for the disease is adherence to a gluten-free diet. Gluten is the principal storage protein in wheat, barley, and rye, but it is poorly digested in the human upper GI tract. The partial digestion of gluten produces toxic polypeptides rich in proline and glutamine that, after deamidation by the enzyme tissue transglutaminase, can bind to the molecules HLA-DQ2 (present in 95 % of the patients) or HLA-DQ8 (present in most of the remaining patients) on the surface of antigen-presenting cells (APCs). The interaction of loaded APCs with reactive T cells leads to the release of pro-inflammatory cytokines and tissue damage.

It has been noted in recent years that environmental factors associated to a decreased or increased risk of celiac disease (i.e., milk-feeding type, breast-feeding, and viral infections) also influence the composition of the intestinal microbiota, and a number of studies have shown that dysbiosis of the gut microbiota is linked to celiac disease (Sanz et al. 2011). The composition of the fecal microbiota of children with active celiac disease was characterized by a decrease in Bifidobacterium, *Clostridium histolyticum*, *C. lituseburense*, and *F. prausnitzii*, while there was an increase in the levels of bacteria belonging to the Bacteroides-Prevotella group (De Palma et al. 2010). The composition of both fecal and mucosa-associated duodenal communities showed a decrease in Bifidobacterium in children with active and non-active disease, while the fecal community of children in the active phase was enriched in Staphylococcus and *E. coli*. However, the count of these two microorganisms was normalized after treatment with a gluten-free diet (Collado et al. 2008a, 2009). The comparison of the mucosa-associated microbiota of children with active disease and in remission, after a 9-month treatment with a gluten-free diet, also revealed that the total diversity of the microbial community was higher in patients in comparison to healthy controls. However, the communities of celiac disease patients were less stable than the healthy ones (Schippa et al. 2010). Rod-shaped bacteria, likely *Clostridium* sp., *Prevotella* sp., and *Actinomyces graevenitzii*, were found to be associated with the epithelium of celiac disease pediatric patients, both active and on a gluten-free diet, during the so-called Swedish epidemics (Forsberg et al. 2004; Ou et al. 2009). In that period, a fourfold increase in celiac disease was observed in 2-year-old children that followed a twofold increase in gluten consumption. Besides the alterations in the gut microbiota, the composition of the fecal SCFA pools of pediatric patients and first-degree relatives was shown to be significantly altered when

compared to healthy controls (Tjellstrom et al. 2005, 2007). The pool of SCFA also differed in asymptomatic children in comparison to healthy controls and was found to resemble the fecal SCFA profile of symptomatic patients (Tjellstrom et al. 2010). These results indicate that the gut microbiota of celiac disease patients also has specific metabolic properties that are different from the microbiota of healthy subjects.

Allergy

Allergic diseases result from the induction of abnormal antibody-mediated immune responses to innocuous environmental antigens. Although genetics play a major role in a person's predisposition to allergy, the increased incidence of allergies in affluent countries since the 1950s is hard to explain solely in terms of genetic factors. As an example, the prevalence of asthma was found to be higher in West Germany in comparison to former East Germany, despite the common genetic background of the population (Bach 2002). Many aspects of everyday life have changed as a result of improved socioeconomic conditions in several countries, and theories have been proposed to explain the increase of allergic as well as other diseases. A couple of decades ago, an inverse correlation between the prevalence of allergic rhinitis and family size was observed by David Strachan (Strachan 1989), which resulted in the formulation of the "hygiene hypothesis." This theory suggests that infections early in childhood could have a protective role against allergic diseases by driving the neonate's immune response, which is Th2-skewed, toward a Th1 response. Indeed, healthy infants exhibit a decrease in Th2 responses after birth while infants that develop allergic diseases show an opposite pattern (Isolauri et al. 2009).

More recently, several authors have proposed that the "hygiene hypothesis" should be modified in order to include a role for the gut microbiota (Bjorksten 2009; Shreiner et al. 2008). The newer hypotheses not only explain the increase in allergy but also the increase of other diseases, such as IBD, T1D (a disease with a Th1-biased immune response), and obesity (Isolauri et al. 2009). The immune system of newborn infants is immature, and the cross talk between the gut microbiota and the immune system is critical for the development toward tolerance or allergy, as shown by the observation that GF mice cannot generate oral tolerance (Lewis et al. 2006; Maeda et al. 2001; Shreiner et al. 2008). Ultimately, the sequential and regulated colonization of the human gut during the first years of life shapes the adult physiology and may have lifelong influences on the organism's well-being. The "microbiota hypothesis" (Shreiner et al. 2008) and the "microbial deprivation hypothesis" (Bjorksten 2009) suggest that continuous exposure to a eubiotic gut microbiota along with its postnatal ecological succession in the intestine, more than sporadic infections, are important factors for the prevention of allergy and other diseases linked to the gut microbiota.

The composition of the gut microbiota is altered between allergic and nonallergic children, as revealed by several studies applying both culture-dependent and culture-independent techniques. Reduced diversity in the fecal microbiota of infants that develop allergy is observed early in life (i.e., 18 months) (Wang et al. 2008), and studies in children up to 5 years of age have shown that differences in gut microbiota composition could be detected before the onset of the disease (Kalliomaki et al. 2001; Sjogren et al. 2009). In particular, decreased Lactobacillus and Bifidobacterium levels and early colonization with *Clostridium* spp. were often positively associated with the development of allergy. In a study comparing the fecal microbiota of Estonian and Swedish children, Björksten and colleagues observed increased incidence of allergy in Sweden in comparison to Estonia. However, the fecal microbiota of allergic children from either country had a more similar composition that differed from the nonallergic population. These findings suggested that intestinal dysbiosis may be a common feature in allergy, independent of geographical and other differences possibly existing between the two countries (Bjorksten et al. 1999; Voor et al. 2005). Although a causative relationship between intestinal dysbiosis and allergy has not been established thus far, experimental evidence shows that alteration of the intestinal microbiota by antibiotic treatment and oral exposure to *Candida albicans* can mediate a break in airway tolerance to fungal allergens (Noverr et al. 2005). In summary, the lack of early exposure of infants to specific microorganisms and/or the elimination of beneficial microbes from the intestinal environment due to modern lifestyle in affluent countries may explain the increased incidence of immunologically mediated diseases in those countries.

Autism Spectrum Disorders (ASD)

The gut microbiota has been suggested to affect organs other than the intestine and as far away as the brain (Heijtz et al. 2011). Autism spectrum disorders (ASD) include a number of related neurodevelopmental conditions that can be detected early in childhood and are characterized by communicative, social, and behavioral problems. The prevalence of ASD in the general population is close to 1 %, with an increasing trend in the last few decades, which is partly explained by the greater attention reserved to autism in the media and the improved detection and/or diagnosis of the disease. Little is known about the etiology of ASD, but genetics and environmental triggers have been equally implied (Bailey et al. 1996; Herbert et al. 2006). Autistic children experience a range of dietary and gastrointestinal problems, and the associated symptoms have been connected to intestinal dysbiosis and overgrowth of potentially pathogenic and neurotoxin-producing microbes, such as clostridia (Bolte 1998; Parracho et al. 2005). Oral administration of vancomycin, an antibiotic active against Gram-positive bacteria, has been shown to improve ASD, but the benefits were limited to the period of treatment, and the condition regressed within 2 weeks from the discontinuation of the antibiotic (Sandler et al. 2000). As vancomycin is only minimally absorbed in the intestine, its effects are likely to be mediated by its activity on the gut microbiota. A circumstantial explanation of these results was given by

hypothesizing a reduction in neurotoxin-producing clostridia following vancomycin treatment while recolonization of the intestinal environment by clostridial spores would cause the relapse of the disease. It is also interesting that ASD patients often have a history of multiple exposures to antibiotics, which are known to alter the structure of the gut microbiota. Treatment with gluten-free and casein-free diets has been associated with improved gastrointestinal symptoms and behavior in some children (Knivsberg et al. 2002; Whiteley et al. 2010), thus providing support for the hypothesis that the gut microbiota may play a role in ASD (Finegold 2008). By using culture-based techniques, the fecal microbiota of ASD patients was found to contain ten times more clostridia than healthy controls, with selected species only present in autistic individuals (Finegold et al. 2002). In another study, increased levels of *C. histolyticum* were detected (Parracho et al. 2005). Finally, a recent profiling of the fecal microbiota by pyrosequencing of the 16S rRNA gene revealed a variation in the diversity of the gut microbiota associated with ASD: in particular, an increase in Bacteroidetes and a decrease in Bifidobacterium were observed in severely autistic children compared with healthy controls (Finegold et al. 2010).

Microbial metabolism has also been studied in the context of ASD. A recent ^{1}H-NMR metabolomic profiling of urine from ASD patients, unaffected siblings, and healthy controls has revealed decreased levels of the mammalian-microbial cometabolites hippurate and phenylacetylglutamine (Yap et al. 2010). These aromatic compounds are produced by the gut microbiota as a result of benzoate metabolism, and the decrease in their levels in individuals with ASD was suggested to derive from diminished production of benzoic acid by the gut microbiota. The authors of this study also notice that depletion of hippurate and phenylacetylglutamine was observed after oral treatment with vancomycin, thus providing a link for the functional role of the gut microbiota in ASD.

Obesity and Type 2 Diabetes

The prevalence of obesity has been dramatically increasing during the last 30 years, and the latest estimate says that today as many as 1.5 billion people are overweight and least 300 million of them obese. Obesity is recognized as a chronic disease associated with a low-grade systemic inflammation and is a major risk factor for type 2 diabetes mellitus (T2D), hypertension, and cardiovascular disease. Altogether, these diseases are known as the metabolic syndrome and are one of the major public health concerns. Particularly alarming is also the hypothesis that obesity could be transmissible, as maternal obesity seems to be a risk factor for adulthood obesity (Lawlor et al. 2007). Development of obesity depends on the interplay of genetic and nongenetic factors (i.e., age, diet, and lifestyle) governing the balance between energy intake and expenditure. The range of these factors goes beyond individual nutritional habits and amount of physical activity: complex regulatory mechanisms, link digestion of food, and absorption of nutrients in the intestine with hypothalamic regulation of energy balance and feeding behavior.

The intestinal microbiota acts at the interface between the food that we ingest and the nutrients that we absorb, and consequently, it plays a fundamental role in nutrition. During the coevolution of the human species and its gut microbiota, a metabolic partnership has evolved and the microbial symbionts that inhabit the gut have provided the genes for the degradation of dietary fibers and production of amino acids and vitamins that are not encoded by the human genome. Research conducted on GF mice has proven that the gut microbiota is an environmental factor that affects energy homeostasis. In these studies, the gut microbiota has been shown to (1) promote monosaccharide absorption in the intestine, (2) increase the amount of calories that can be extracted from the food through fermentation of nondigestible fiber, (3) increase the storage of these calorie in fat tissue, and (4) decrease fatty acid oxidation in muscle (Bäckhed et al. 2004). Additionally, microbial production of SCFA also influences the hormonal regulation of glucose homeostasis, intestinal motility, satiety, and feeding behavior by affecting the levels of ghrelin, peptide YY (PYY), and glucagon-like peptide-1 (GLP-1) (Cani and Delzenne 2009).

Recent studies in humans have shown that obesity is associated with intestinal dysbiosis. The first human studies on the impact of the gut microbial community in obesity showed that the fecal microbiota of obese individuals differed greatly from that of lean controls at the phylum level: the obese microbiota was enriched in Firmicutes and depleted in Bacteroidetes, so displaying a high Firmicutes/Bacteroidetes ratio. This ratio was observed to decrease upon weight loss after either low-carbohydrate or low-fat diets as a consequence of increased Bacteroidetes levels (Ley et al. 2006). In later studies, the obese microbiota was shown to be characterized by decreased phylogenetic diversity and enrichment in gene coding for enzymes involved in energy harvesting (Turnbaugh et al. 2009). Although an increased Firmicutes/Bacteroidetes ratio in obesity was shown also in other studies (Santacruz et al. 2010; Turnbaugh et al. 2009), several reports did not confirm this initial observation (Collado et al. 2008b, Schwiertz et al. 2010), and it was suggested that increased concentrations of fecal SCFA could be more relevant for obesity than the variation in the composition of the gut microbiota (Schwiertz et al. 2010). However, differences at lower taxonomical levels than phylum level are detected in a large number of studies. Early fecal samples of infants that developed obesity by the age of 7 had high Staphylococcus counts while corresponding samples of infants that remained lean were dominated by Bifidobacterium, *B. longum*, and *B. breve* in particular (Kalliomaki et al. 2008). Additionally, weight loss in obese adolescents subjected to a low-calorie diet was associated to differences in the gut microbiota (Santacruz et al. 2010): high weight loss was observed in subjects whose intestinal community before the beginning of the intervention was enriched in *B. fragilis*, *C. leptum*, and *B. catenulatum*, while containing lower numbers of *C. coccoides*, *B. breve*, and *B. bifidum*. Finally, intestinal colonization by methanogens, such as *M. smithii*, has been reported as an additional factor that may affect the development of obesity (Million et al. 2011; Schwiertz et al. 2010). Hence, although there is no consensus for

a clear compositional profile of the obese microbiota, dysbiosis and differences in SCFA are two features consistently associated with obesity.

Obesity and T2D are characterized by a low-grade systemic inflammation, which has been suggested to derive from the infiltration of immune cells (macrophages) in adipose, liver, and muscle tissue. Infiltrating macrophages are proposed to release pro-inflammatory factors that alter the host's homeostatic metabolic signaling and cause resistance to insulin. Nevertheless, the role of macrophages and the identity of the inflammatory triggers are not clarified. Recent data indicate that the gut microbiota might contribute to the development of the low-grade inflammation associated with insulin resistance in metabolic diseases. In this context, lipopolysaccharide (LPS) has been suggested as a microbial trigger of inflammation. Increased plasma levels of LPS were originally observed in mice fed a high-fat diet and then associated with increased gut permeability (Cani et al. 2007). Infusion of physiologic concentrations of LPS and high-fat feeding were observed to induce inflammation, insulin resistance, and increased fat mass deposition in mice. In these obese mice, high-fat feeding was also shown to reduce fecal levels of Bifidobacterium and Bacteroides. Similarly, the interplay between metabolic endotoxemia (i.e., high LPS level in plasma), consumption of high-fat diet, and development of obesity and T2D was confirmed in humans, and recent data indicate that visceral adiposity correlates with increased gut permeability in overweight women (Gummesson et al. 2011). A few studies have analyzed the correlation between gut microbiota, T2D, and inflammation and have shown that *F. prausnitzii* is depleted in T2D patients (Furet et al. 2010). Additionally, the abundance of Firmicutes seems to decrease while the abundance of β-Proteobacteria increases in T2D in comparison to healthy controls. In this context, the ratios Bacteroidetes/Firmicutes and Bacteroides-Prevotella/*C. coccoides-E. rectale* were observed to be correlated with plasma levels of glucose (Larsen et al. 2010).

Therapeutic Modulation of the Gut Microbiota

The observation that intestinal dysbiosis is associated with human diseases opens to the possibility of manipulating the intestinal microbiota in order to prevent or cure pathological conditions. To achieve this goal, it is necessary to provide a better understanding of the intrinsic features that characterize a "healthy gut microbiota" as opposed to a disease-associated gut microbiota. For example, it needs to be confirmed that a "core human microbiome" exists at the species level (Qin et al. 2010) and at the genetic level (Turnbaugh et al. 2009). At the same time, the mechanisms and microbial players involved in the etiology of specific diseases need to be characterized, and, besides the phylogenetic composition and the genetic ability of the community, the functionality of the microbiota needs to be assessed. The possibility of developing therapeutic measures based on the modulation of the gut microbiota is complicated by the observation that each individual is endowed with a unique microbiota. Hence, individuality in the gut microbiota might also require a personalized therapy based on the knowledge of subjective microbial requirements.

Ilya Mechnikov, who was awarded a Nobel Prize in 1908 together with Paul Ehrlich for their work on immunity, was the first to hypothesize that the use of live bacteria could be associated to health-promoting effects. At that time, he drove his conclusion by observing that a population of elderly healthy Bulgarians consumed fermented milk, to which he attribute beneficial effects. Today, several approaches are being developed for the manipulation of the gut microbiota, namely, the use of probiotics, prebiotics, synbiotics, and, recently, the transplantation of intestinal microbiota.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit for the host" (FAO/WHO 2002). Prebiotics are "non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host" (Roberfroid 1998). It is implicit in the last definition that, in order for the prebiotic to produce health benefits, the gut microbiota needs to contain the microorganism(s) able to degrade the fibers that are supplemented. Therefore, synbiotics are developed and consist in mixtures of probiotic(s) and prebiotic(s). In the mixture, the prebiotic part is selected in order to promote the growth and/or function of the associated probiotic(s), which commonly is able to ferment only a selected range of fibers. As an example, amylase-resistant starch selectively increases the numbers of bifidobacteria and lactobacilli while decreases the levels of Enterobacteriaceae. Probiotics and prebiotics will be discussed further in dedicated chapters of The Prokaryotes.

Another possible therapeutic intervention that has recently received renewed attention is transplantation of fecal microbiota from healthy donors. The first report on fecal transplantation dates back to 1958 and describes the treatment of four patients affected by pseudomembranous colitis, a pathology caused by opportunistic infection of *Clostridium difficile* (CDI). Treatment of pseudomembranous colitis is clinically challenging as this pathology is characterized by fulminant disease and recurrent infections. Up to now, the treatment of 200 cases of refractory CDI has been described, with a success rate of 90 % and no ill effects reported (Khoruts and Sadowsky 2011). More recently, a clinical study was initiated that showed that the transplanted microbiota quickly colonizes the gut of the receivers and represents the dominant microbiota for at least 1 month (Khoruts and Sadowsky 2011). For CDI, this stable association results in the resolution of symptoms, thus indicating that the patients' dysbiotic microbiota is likely to cause the disease.

Recent findings have suggested that fecal transplantation may be a feasible approach for the treatment of metabolic diseases. In a preliminary report, Koote and colleagues have shown that transplantation of fecal microbiota from healthy donors to newly diagnosed metabolic syndrome patients (i.e., obese subjects with impaired fasting glucose), but not

autologous transplantation, significantly improved glucose metabolism after 6 weeks although no effect was observed on body weight (Kootte et al. 2011). Besides the relevance of this approach for the development of new therapeutic strategies, transplantation of human microbiota in animal models will add to the current knowledge of the mechanisms of human disease and might be useful for testing the applicability of microbial interventions for the treatment of human diseases associated with intestinal dysbiosis.

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2 Prebiotics

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Abstract
The human large intestine is an intensively colonized area containing bacteria that are health promoting as well as pathogenic. This has led to functional food developments that fortify the former at the expense of the latter. Probiotics have a long history of use in humans as live microbial feed additions. In contrast, a prebiotic is a nondigestible food ingredient that beneficially affects the host by targeting indigenous components thought to be positive. Dietary carbohydrates, such as fibers, are candidate prebiotics, but most promise has been realized with oligosaccharides. As prebiotics exploit nonviable food ingredients, their applicability in diets is wide ranging.

Main prebiotic targets at the moment are bifidobacteria and lactobacilli (although this may change as our knowledge of the microbiota diversity and functionality expands). Any dietary component that reaches the colon intact is a potential prebiotic; however, much of the interest in the development of prebiotics is aimed at nondigestible oligosaccharides such as inulin-type fructooligosaccharides (FOS) and trans-galactooligosaccharides (TOS). In Europe, FOS and TOS have been shown to be prebiotics, through numerous volunteer trials, as evidence by their ability to positively change the gut flora composition after a short feeding period. Other prebiotics are emerging. Some prebiotics occur naturally in several foods such as leek, asparagus, chicory, Jerusalem artichoke, garlic, artichoke, onion, wheat, banana, and oats. However, these foods contain only trace levels, so developments have taken the approach of removing the active ingredients from such sources and adding them to more frequently consumed products in order to attain levels whereby a prebiotic effect may occur, for example, cereals, confectionery, biscuits, infant feeds, yogurts, table spreads, bread, sauces, drinks, etc.

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As gastrointestinal disorders are prevalent in terms of human health, both probiotics and prebiotics serve an important role in the prophylactic management of various acute and chronic gut-derived conditions. Examples include protection from gastroenteritis and some inflammatory conditions.

History of Prebiotics

There continues to be an exponential rise in the cost of pharmaceutical applications for human use. Moreover, the indiscriminate use of antibiotics has led to a reduction in their potency and efficiency. As such, attention has turned toward food materials that may offer improved health bonuses. So-called functional foods have a high profile, and many new products exist or are being developed. Early approaches involved vitamin and mineral supplementation to the diet. Dietary modulation of the human gut microbiota can be of great benefit to health, and in recent years, the functional food concept has moved away from mineral and vitamin supplementation toward the situation whereby improved gut function is a major driving force (Gibson and Williams 2000; Saarela 2011).

Breast-fed infants develop a different gut microbiota to those on formula feeds. The former has bifidobacteria as the numerically predominant genus (Roger et al. 2010), whereas formula feeds give rise to a, more complex, adult-like gut microbiota with clostridia, bacteroides, bifidobacteria, and streptococci all as prevalent genera (Harmsen et al. 1999, 2000). Breast-fed infants generally have less gastrointestinal problems than their formula-fed counterparts, and this may, at least partly, be attributed to the powerful anti-pathogen effects exerted by bifidobacteria. The final phase of microbiota acquisition occurs at weaning when a complex microbiota develops.

The gut microbiota ferment substances that cannot be digested by the host in the small gut; these include resistant starch, nondigestible carbohydrates, oligosaccharides, proteins, and mucins (Gibson and Macfarlane 1995), (*Table 2.1*). The two main types of fermentation that are carried out in the gut are saccharolytic and proteolytic. Saccharolytic fermentation is more favorable than proteolytic fermentation due to the end products that are formed. The main end products of saccharolytic fermentation are the short-chain fatty acids, acetate, propionate, and butyrate. All contribute toward the host's daily energy requirements. Acetate is metabolized in systemic areas like muscle, while propionate is transported to the liver and used to generate ATP. Butyrate is an important source of energy for the colonocytes and has antitumor properties (Cummings et al. 1989). The end products of proteolytic fermentation on the other hand include nitrogenous metabolites (such as phenolic compounds, amines, and ammonia), some of which are carcinogens. The proximal gut (right side) is essentially a site of saccharolytic fermentation, whereas the more distal (left side) is an area of higher proteolytic fermentation than the proximal bowel (Macfarlane et al. 1992). This is probably one reason why many gastrointestinal disorders (including colon cancer, ulcerative colitis) occur distally. The bacterial

Table 2.1
Type of substrates available for bacterial growth in the human large intestine

Substrate	Estimated quantity (g/day)
Resistant starch	8–40
Non-starch polysaccharides	8–18
Unabsorbed sugars	2–10
Oligosaccharides	2–8
Chitins, amino sugars, synthetic carbohydrates, food additives	?
Dietary protein	3–9
Mucins	?
Bacterial recycling	?
Sloughed epithelial cells	?

? Unknown

microbiota in the human large intestine is thought to compromise the majority of total cells in the body, representing 10^{12} cells/g dry weight feces. The vast majority reside in the large intestine, where the slow transit time, availability of nutrients, and pH are favorable for microbial growth. Through the activities of the resident microbiota, the colon plays a major role in host nutrition and welfare (Cummings and Macfarlane 1991).

Colonic bacteria can be categorized as being either beneficial or potentially pathogenic due to their metabolic activities and fermentation end products. Health-promoting effects of the microbiota may include immunostimulation, improved digestion and absorption, vitamin synthesis, inhibition of the growth of potential pathogens, cholesterol reduction, and lowering of gas distension (Gibson and Roberfroid 1995, 2008). Harmful effects include carcinogen production, intestinal putrefaction, toxin production, diarrhea/constipation, liver damage, and intestinal infections. Bifidobacteria and lactobacilli are considered to be examples of health-promoting constituents of the microbiota. Lactobacilli may aid digestion of lactose in lactose-intolerant individuals, reduce constipation and infantile diarrhea, help resist infections such as salmonellae, prevent traveller's diarrhea, and help in irritable bowel syndrome (IBS) (Salminen et al. 1993). Bifidobacteria are thought to stimulate the immune system, produce B vitamins, inhibit pathogen growth, reduce blood ammonia and blood cholesterol levels, and help to restore the normal microbiota after antibiotic therapy (Gibson and Roberfroid 1995). Both probiotics and prebiotics are functional foods that fortify the lactate-producing microbiota of the human or animal gut (Hamilton-Miller 2001; Reid et al. 2001).

The approach of using diet to prophylactically manage disorder is both user friendly and attractive to the consumer. Current marketing strategies target improved resistance to infections, irritable bowel syndrome, chronic gut disorder (inflammatory bowel disease, colon cancer), lactose intolerance, coronary heart disease, recurrent vaginal thrush, skin problems, food allergy, and mineral bioavailability. One popular approach

is to involve live microbial additions into the diet (probiotics). Many different products exist, and new developments are continuing at a rapid pace.

The use of probiotics has been widely supported (Fuller 1997; Versalovic and Wilson 2008). In this case, foodstuffs such as fermented milk products containing viable cultures perceived as beneficial (e.g., lactobacilli, bifidobacteria) are used to proliferate populations in the colon. Probiotics are defined as live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance. To be effective, probiotics must be capable of being prepared in a viable manner and on large scale (e.g., for industrial purposes), while during use and under storage, the probiotic should remain viable and stable, be able to survive in the intestinal ecosystem, and the host animal should gain beneficially from harboring the probiotic. However, these live microorganisms may reach the lower gut in a compromised state due to the bile and stomach acid secretions in the upper gastrointestinal tract. For the microorganisms to be fully effective, they will then have to compete with an established colonic microbiota for nutritional sources and attachment sites (Collins and Gibson 1999).

Main positive effects associated with probiotics include protection against gastroenteritis, pathogen inhibition, improved lactose tolerance, and stimulation of the immune system through nonpathogenic means (Fuller and Gibson 1997). This also has implications for disorders thought to be mediated by gut bacteria. To this end, irritable bowel syndrome, inflammatory bowel diseases, and colorectal cancer have all been researched. For systemic benefits, reduced cholesterol and/or triglyceride levels, protection from atopic reactions, and better absorption of minerals are suggested. The following references summarize these health aspects: Nobaek et al. (2000), Wullt et al. (2003), Yamano et al. (2006), D'Sousa et al. (2002), Marteau et al. (2001), de Vrese et al. (2001), Fuller (1992, 1997), Tannock (1999), (2002), Naidu et al. (1999), Sanders et al. (2007), Versalovic and Wilson (2008), Saulnier et al. (2009), and Kolida and Gibson (2011).

Desired characteristics of a good probiotic are:

- Exerts a beneficial effect when consumed
- Nonpathogenic and nontoxic
- Contains a large number of viable cells
- Has the capacity to survive and metabolize in the gut
- Retains its viability during storage and use
- If incorporated into a food, should have good sensory qualities (Bezkorovainy 2001; Dunne et al. 2001)

Prebiotics have been developed as an alternative concept for gut microbiota modulation. These are nonviable food components (carbohydrates) that have a selective metabolism in the human or animal gut. They serve to induce beneficial changes by fortifying indigenous levels of probiotics already within the gut microbiota.

Oligosaccharides have long been used to impart health bonuses, principally in Asia, where they are used for dietary fiber-type effects. However, the term prebiotic as directed toward gut microbiota modulation through diet was not coined until 1995. This was driven by the health values seen to occur with probiotics, but with a view toward fortification of the indigenous microbiota, rather than the ingestion of exogenous species. This first definition was: *non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon* (Gibson and Roberfroid 1995). Thus, the prebiotic approach advocated administration of nonviable food ingredients that transferred to the colon and had a selective metabolism therein. This is in contrast to most dietary fibers where a specific fermentation does not occur. The prebiotic concept considers that many probiotic-type microorgansims, such as bifidobacteria and lactobacilli, are already present in the human colon – and was derived to specifically increase them. Diet is a large factor controlling the intestinal microbiota, so in altering intake it is possible to modulate the microbiota composition. A prebiotic substrate is selectively utilized by bifidobacteria and/or lactobacilli but does not promote growth of potential pathogens

This original definition was updated in 2004 and defined prebiotics as *selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health* (Gibson et al. 2004). The latter definition does not consider only the microbiota changes in the colonic ecosystem of humans, but in the whole gastrointestinal tract, and as such extrapolates the definition into other areas that may benefit from a selective targeting of particular microorganisms. As mentioned, the target genera are lactobacilli and bifidobacteria. However, prebiotic success has predominantly been with the latter, probably because they are usually present in higher numbers than lactobacilli.

As such, the prebiotic concept owes much to the science of probiotics and the desire to stimulate microorganisms seen to be of value to the host. As the knowledge of gut microbiota diversity has expanded, there may be other target genera such as *Roseburia, Eubacterium, and Faecalibacterium.* However, it is the case that more physiological understanding of these groups is required and their definitive health bonuses need to be more thoroughly understood before they can be advocated as prebiotic responders.

Any dietary material that is nondigestible and enters the large intestine is a candidate prebiotic. This includes polysaccharide-type carbohydrates such as resistant starch and dietary fiber as well as proteins and lipids. However, current prebiotics are confined to nondigestible oligosaccharides, many of which seem to confer the degree of fermentation selectivity that is required (toward bifidobacteria). Some prebiotics occur naturally in several foods such as leek, asparagus, chicory, Jerusalem artichoke, garlic, artichoke, onion, wheat, and oat as well as soybean. However, the overall intake from these sources within a normal, in particular, Western-type, diet is small. An effective route to achieve a health-promoting intake is the fortification of more frequently eaten foodstuffs with prebiotic ingredients. Prebiotics are thus a subcategory of functional food ingredients.

They are added to many foods including yogurts, cereals, breads, biscuits, milk desserts, ice creams, spreads, drinks as well as animal feeds and supplements. Some prebiotics can be obtained by extraction from crops, for example, inulin from chicory or agave. Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides (e.g., oligofructose from inulin) or through catabolic enzymatic reactions from lower molecular weight sugars, for example, short-chain fructooligosaccharides (scFOS) from sucrose or galactooligosaccharides from lactose. The review by Crittenden and Playne (1996) gives an overview of various aspects of the production and properties of food-grade oligosaccharides.

The three criteria required for a prebiotic effect are (Gibson et al. 2004):

- Resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption
- Fermentation by intestinal microbiota
- Selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being

As it stands, the prebiotic field is dominated by gastrointestinal events. However, in the future, it may be the case that other mixed microbial ecosystems may be modulated by a prebiotic approach, such as the oral cavity, skin, and urogenital tract. A decision was taken therefore to build upon current prebiotic status and define a niche for "dietary prebiotics": *A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health* (Gibson et al. 2010).

Major Dietary Prebiotics and Candidate Prebiotics

Prebiotics are largely confined to a class of molecules termed nondigestible oligosaccharides. These are chains of carbohydrates typically consisting of three to nine units, although the number of moieties contained within prebiotic chains (referred to as degree of polymerization (DP)) varies. For example, the synthetic prebiotic lactulose is a disaccharide consisting of one galactose unit and one fructose unit, while naturally occurring inulin may comprise up to 60 linked fructose molecules. Prebiotic chain lengths are not consistent for each type but are instead a mixture of chains of varying polymerization. The indigestible property of prebiotic oligosaccharides derives from the β-glycosidic bonds between the sugar units. Human digestive enzymes are specific for α-glycosidic bonds and as such cannot hydrolyze linkages in the β-configuration. This evasion of digestion allows these oligosaccharides to escape the upper gut and reach the colon relatively intact (Roberfroid 1999). In the colon, oligosaccharides serve as fermentation substrates for the indigenous microbiota, and it is here where they can be distinguished from other fibers by selectively stimulating the growth of beneficial bacteria such as the bifidobacteria and lactobacilli. The fermentation rate of these oligosaccharides is governed by factors such as monosaccharide composition, glycosidic linkages, degree of polymerization, and the overall complexity of the molecule. The end products of prebiotic fermentation include short-chain fatty acids (e.g., acetate, butyrate, propionate), lactic acid, succinate, ethanol, propionate, and gases (CO_2, H_2, CH_4, and H_2S). This fermentation process and resultant products acidify the colonic content and modulate the microbiota toward a favorable composition, for primarily bifidobacteria and lactobacilli. Oligosaccharide chain length influences the colonic region where fermentation occurs, with shorter oligosaccharides being metabolized in the proximal colon while longer chains persist to the more distal colonic regions. It is this characteristic indigestibility and selective fermentable potential that allows oligosaccharides to fulfill the established criteria outlined for prebiotic classification.

Several nondigestible oligosaccharides exist including inulin-type fructans, galactooligosaccharides, lactulose, isomalto-oligosaccharides, xylo-oligosaccharides, and soybean oligosaccharides. However, there are currently only three carbohydrates considered to fulfill the prebiotic criteria. These are inulin-type fructans, galactooligosaccharides, and lactulose.

Inulin-type Fructans

Inulin-type fructans (ITF) are nondigestible carbohydrates that occur naturally in plants as oligo- and polysaccharides. They are members of a larger group of compounds called "fructans" which include all plant oligo- and polysaccharides where the majority of the glycosidic bonds are fructosyl-fructose linkages. Hence, fructans are predominantly polymers of fructose units. The prebiotic effects of ITF are well established, and as reflected in their production volume, they represent the major class of prebiotic compounds. This group of prebiotics includes inulin, oligofructose, and fructooligosaccharides (FOS).

Chemically, ITF are linear chains (oligomers or polymers) of fructose joined by β(2–1) fructosyl-fructose linkages. The linear chain may have a terminal glucose molecule (α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-β-D-fructofuranoside) ($G_{py}F_n$) or no glucose molecule (β-D-fructopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-β-D-fructofuranoside) ($F_{py}F_n$). Here, F_n refers to the number of repeating fructose units occurring in the fructan (equivalent to the degree of polymerization). Inulin and inulin-type fructans are generic terms used to describe all β(2–1) linear fructan molecules. The majority of commercially available inulin and oligofructose is derived from the roots of the chicory plant. Chicory inulin is a mixture of oligosaccharides and polysaccharides in which the DP varies between 2 and 70 (average value (DP_{av}) of 12) and can be of both the G_{py} F_n and $F_{py}F_n$ variety. Oligofructose and fructooligosaccharides (FOS) are often used synonymously to describe the mixture of small inulin oligomers with a maximum DP value of 10 (DP_{max} <10). These shorter fructan chains can be obtained through the enzymatic hydrolysis of inulin using an endoinulinase to produce a mixture of both G_{py} F_n and $F_{py}F_n$ with a DP range of 2–7 (DP_{av} = 4). Alternatively, these oligosaccharides can be synthesized *via* the transfructosylation of sucrose using the fungal

enzyme β-fructosidase from *Aspergillus niger*. This synthetic oligofructose is exclusively of the $G_{py}F_n$ type with a DP range of 2–4 (DP_{av} = 3.6). Strictly speaking, the term oligofructose should be used to describe the oligosaccharides obtained naturally from the hydrolysis of inulin, while FOS is the term adhered to the synthetic products generated on a commercial scale from sucrose.

Numerous in vitro and in vivo studies, including several human trials, have demonstrated the ability of inulin to selectively stimulate the growth of beneficial bacteria. Inulin, oligofructose, and FOS have been shown to be bifidogenic in infants (Moro et al. 2002), adults (Gibson et al. 1995; Kruse et al. 1999, Menne et al. 2000; Tuohy et al. 2001), and the elderly (Kleessen et al. 1997; Bouhnik et al. 2007). Functional modifications have also been noted with elevations in butyrate production following ITF supplementation (Levrat et al. 1991). Additional evidence suggests that this class of prebiotics modulates other members of the gut microbiota such as the promotion of lactobacilli and inhibition of the clostridia, although studies yield inconsistent results (Gibson et al. 1995).

Trans-galactooligosaccharides

The predominance of bifidobacteria in breast-fed infants is considered to arise, at least in part, from the presence of oligomers in human milk (Sela et al. 2008; Saito et al. 1987). It is the potential for commercially produced TOS to replicate the bifidogenic qualities of breast milk that has led to TOS becoming a major class of prebiotics.

TOS consist of between two and eight saccharide units, with one being a terminal glucose and the remainder being galactose. As such, these compounds consist of a number of β(1–6)-linked and β(1–4)-galactopyranosyl units linked to a terminal glucopyranosyl residue through an α(1–4) glycosidic bond. TOS are produced from lactose by enzymatic transglycosylation, whereby the enzyme β-galactosidase transfers the galactose moiety of a β-galactoside to an accepting hydroxyl group. Disaccharides are predominantly formed, but depending upon the source of the enzyme and the reaction conditions, different synthetic product mixtures are generated with trisaccharides and larger oligomers also produced in smaller amounts. In general, TOS is protected from the action of the digestive enzymes by the β-configuration of the glycosidic linkages. There is potential for TOS to be broken down by the β-galactosidases located in the brush border of the small intestine, but the activity of such enzymes is weak, and there is sufficient evidence to suggest that TOS reaches the colon relatively intact. Data collected from human studies support the prebiotic effects of TOS, especially in infants (*Table 2.1*). TOS is selectively fermented by *Bifidobacterium* spp. and to a lesser extent by *Lactobacillus* spp. in the colonic microbiota (Ito et al. 1993; Bouhnik et al. 1997; Depeint et al. 2008). Functional outcomes have also been noted post prebiotic feeding with elevations in short-chain fatty acids; fecal pH decreases and stool characteristics improve (frequency and consistency).

Lactulose

The third class of carbohydrates accepted as prebiotic molecules is lactulose (galactosyl β(1–4) fructose). This is a disaccharide of galactose linked β(1–4) to fructose and is produced from the isomerization of lactose. Lactulose is commonly prescribed as a laxative, but when consumed at sub-laxative doses, it has been shown to exert a bifidogenic effect (Terada et al. 1993; Ballongue et al. 1997). This disaccharide has proved to be resistant to digestion by both human and calf intestinal β-galactosidases.

Candidate Prebiotics

Isomalto-oligosaccharides, xylo-oligosaccharides, and soybean oligosaccharides are oligosaccharides that show prebiotic promise, but sufficient evidence does not yet exist to justify their classification as prebiotics. In particular, there is a lack of data from human trials.

Isomalto-oligosaccharides

Isomalto-oligosaccharides (IMO) are mixtures of α-1–6-glucosides such as isomaltose, isomaltotriose, and isomaltotetraose. IMO are starch derivatives obtained by a two-step enzymatic process. Firstly, starch is hydrolyzed by the action of α-amylase and then treated with β-amylase and α-glucosidase. β-amylase converts the liquefied starch into maltose, and then the hydrolytic and glucotransferase activity of α-glucosidase converts the α(1–4)-linked maltooligosaccharides into a mixture of α(1–6)-linked IMO. These candidate prebiotics do not fully meet the primary criterion for prebiotic classification as they can undergo digestion in the jejunum by isomaltase. Extending the DP of the oligosaccharide has been suggested to confer resistance to digestion enabling the molecule to survive gut transit and enter the colon intact. Human studies have demonstrated that IMO are bifidogenic (Kohmoto et al. 1988, 1991).

Xylo-oligosaccharides

The xylo-oligosaccharides (XOS) typically used in commercial products are disaccharides of the pentose sugar xylose connected through a β(1–4) linkage. These disaccharides, termed xylobiose, are obtained from the enzymatic hydrolysis of xylan, a naturally occurring polysaccharide found in corncobs. Endo-1,4-xylanse is the enzyme used to facilitate this hydrolysis. Xylan is recognized as a nondigestible dietary fiber because the mammalian genome does not encode the β-xylosidase required to break the linkages between the xylose monomers. Although xylobiose constitutes the major proportion of commercially used XOS, low amounts of higher oligosaccharides are also present. Experimental data shows that XOS can stimulate the bifidobacterial growth (Howard et al. 1995).

Table 2.2
Studies that have led to selective effects following the incorporation of prebiotics into foodstuffs

Prebiotic	Foodstuff	Prebiotic dose/day	Results	Bacterial enumeration technique	References
FOS	Biscuits	6.6 g	Bifidogenic	FISH	Tuohy et al. (2001)
Inulin	Chocolate drink	5 g/8 g	Bifidogenic	FISH	Kolida et al. (2007)
FOS	Jelly	5 g	Bifidogenic	Selective plating	Mitsou et al. (2009)
Inulin	Fruit/vegetable shots	5 g	Bifidogenic, lactobacilli increase	FISH	Ramnani et al. (2010)
TOS	Orange juice	8 g	Bifidogenic	FISH	Walton et al. (2011)

Soybean Oligosaccharides

Galactosyl-sucrose oligosaccharides are a major component of soybeans and are found throughout the plant kingdom. These oligosaccharides comprise a chain of α(1–6)-linked galactose units joined to a terminal sucrose through an α(1–6) bond. These oligosaccharides include the trisaccharide raffinose and tetrasaccharide stachyose. They are obtained from soybean whey, a waste product of soy protein production. Humans lack the enzyme α-galactosidase and are unable to digest these molecules in the upper gut, allowing safe transit into the colon where they are able to elicit a prebiotic effect. Studies suggest SOS are capable of inducing a bifidogenic effect (Hayakawa et al. 1990).

Food Vehicles

Existing prebiotics have a range of physiological properties that enable them to be incorporated into many different food matrices. Many products exist on the market using the bulking structures of oligosaccharides to enhance the end products or even to create low-calorie alternatives.

Inulin and FOS are heat stable and have been used as sugar replacements and to improve mouthfeel in a variety of food products. Both have been used in baked goods and have been reported to improve texture and taste (Coussement and Franck 1998); furthermore, inulin has been used in low-fat foods as it has been observed to have gelling characteristics and can stabilize an emulsion while improving foam stability and enabling a spreadable texture to be achieved.

TOS are stable over time in acidic conditions (6 months, pH3); thus, use within fruit beverages is an option. Furthermore, high-temperature treatments do not denture the structure of TOS (160 °C for 10 min; 100 for 10 min at pH2) (Sako et al. 1999), rendering the possibility of baking TOS into a variety of food products. The low calorific value and sweet taste means that TOS is potentially useful as a sweetener. The main use of TOS in the food marketplace is within infant formula.

Currently, some infant formulae are fortified with TOS, FOS, or a combination of both these prebiotics to offer an alternative way of altering the microbiota and bringing about the associated benefits to infants that are not breast fed (Boehm et al. 2004), such as enhancing secretory IgA levels (Bakker-Zierikzee et al. 2006). Indeed more realistic formula milks have been developed over recent years enabling more similarities to the microbiota of breast-fed infants to be observed in formula-fed infants (Hascoët et al. 2011).

Currently on the market, many prebiotic-containing products exist. These include spreads, breads, cereals, fruit juices, bottled water, rye-based snacks, fruit bars, frozen desserts, and chocolate spread. Although when incorporated into these matrices, the prebiotic dose and effectiveness in populations have not always been confirmed. Within an adult population, human intervention studies that have led to selective effects following the incorporation of prebiotics into foodstuffs are summarized in *Table 2.2*. In addition to this, other products have demonstrated prebiotic effects, such as wheat- and maize-based breakfast cereals (Costabile et al. 2008; Carvalho-Wells et al. 2010), cocoa-based beverages, and fortified coffee beverages (Tzounis et al. 2011; Walton et al. 2010).

Microbial Aspects

The main target for bacterial increases is those with proven beneficial effects to the host. Subsequently, the targets of prebiotic intervention are frequently the host's own bifidobacteria and lactobacilli. Currently, prebiotics used normally are more stimulatory to bifidobacteria; this is possibly due to a fermentative advantage to the selected prebiotic substrate; however, completion between the complex microbiota is still likely to occur.

Lactobacilli are usually classified as beneficial or benign organisms that are tolerant of acid conditions and therefore often used in the food industry (Harmsen et al. 1999). Within the large intestine, however, lactobacilli are an important component of the microbiota and, for example, have been seen to stimulate the immune system, to aid against antibiotic-associated diarrhea (Cimperman et al. 2011) and lactose intolerance (Beausoleil et al. 2007). Different species of *Lactobacillus* have been involved in reducing the actions of harmful enzymes in the colon, thus promoting a beneficial environment. Furthermore,

lactobacilli have also been observed to possess antimicrobial activities, enabling them to exhibit activities against potentially pathogenic microorganisms (Cheikhyoussef et al. 2008).

Bifidobacterium spp. were first isolated by Tissier in 1900. Bifidobacteria typically occupy about 3 % of the adult fecal microbiota (Franks et al. 1998). The importance of bifidobacteria within the human large intestine has been linked largely to health status of the host. From the beginning of life, the levels of bifidobacteria in the large intestine have been observed to aid with the resistance to infection of the growing infant (Brück et al. 2003; Bakker-Zierikzee et al. 2006) and reducing the growth of potential pathogens (Knol et al. 2005).

Beyond infancy, bifidobacteria are still shown to be important members of the gut microbiota. Their effects include antibacterial activity against foodborne pathogens (Roopashri and Varadaraj 2009), outcompeting less desirable microorganisms (Gibson and Wang 1994), reducing cholesterol levels (Pereira and Gibson 2002), enhancing the immune response (Young et al. 2004), and showing potential actions against irritable bowel syndrome (Silk et al. 2009). *Bifidobacterium* spp. are a saccharolytic genus that produce acetate, ethanol, formate, and lactate following fermentation. These end products of fermentation lower the colonic pH, making the colonic environment less favorable to pathogenic bacteria (Campbell et al. 1997). Furthermore, some of the effects of bifidobacteria have been attributed to the production of antimicrobial peptides (Cheikhyoussef et al. 2008). These antimicrobial peptides have been observed to act against potentially harmful microorganisms and to prevent pathogenic bacteria from adhering to epithelial cell receptors in in vitro studies of the intestine (Bernet et al. 1993).

Saccharolytic Metabolism

Saccharolytic metabolism is the digestion of carbohydrates by bacteria for energy, and a source of carbon, it is the main metabolic process of the colonic bacteria. The end products of saccharolytic metabolism can be considered either benign or to exert positive effects at the cellular and systemic levels (Gibson 1999). Hitherto described prebiotics are fermentable carbohydrates.

The end products of saccharolysis are organic acids, the major ones being acetate, propionate, and butyrate. They are involved in supporting the growth of colonic epithelial cells and reducing absorption of toxic products. The major pathway of saccharolytic fermentation within the colon is the Embden-Meyerhof-Parnas pathway (EMP) which is the breakdown of glucose to the intermediate pyruvate. Pyruvate is then further broken down into SCFA and to the organic acid lactate. Many organisms of the genus *Lactobacillus* are homofermentative, in that they convert hexoses solely to lactate using NADH as the cofactor and the enzyme lactate dehydrogenase, via the EMP pathway. Heterolactic members of the *Lactobacillus* utilize a different pathway, known as the 6-phosphogluconate/phosphoketolase pathway. This yields production of lactate, CO_2, and ethanol. The heterofermentative bacteria of this genus can use both.

Bifidobacteria lack the enzymes glucose-6-phosphate dehydrogenase and fructose-1,6-diphosphate aldose; therefore, glucose metabolism is undergone by a different pathway, known as the bifidus pathway. This pathway generally yields 3 M acetate per 2 M lactate – as the pyruvate is not only used for the formation of lactate but also ethanol and formate.

Health Outcomes

Short-Chain Fatty Acid Production

Fermentation of prebiotics by beneficial members of the gut microbiota (e.g., bifidobacteria and lactobacilli) generates the principal end products, short-chain fatty acids (SCFA). This class of metabolites includes acetate, butyrate, and propionate and underlies many of the benefits associated with prebiotic consumption. One major benefit is the provision of energy to various tissues throughout the body. However, bifidobacteria and lactobacilli only manufacture acetate of these SCFA. Through a cross-feeding pathway, other members of the microbiota may produce butyrate from acetate and lactate (Belenguer et al. 2006). Other emerging prebiotics may generate propionate or butyrate. Butyrate is a key energy substrate for the colonic epithelium, while propionate can be utilized in gluconeogenesis in the liver, and acetate is metabolized in peripheral tissues such as the brain, kidney, heart, and muscle. Butyrate is considered an important SCFA produced by the gut microbiota and is capable of regulating cell growth and differentiation. Indeed, through stimulation of apoptosis, butyrate has been proposed to reduce the risk of colon cancer (Kruh et al. 1995). Butyrate is also capable of enhancing cellular detoxification and antioxidant defenses by upregulating the expression of glutathione S-transferase and catalase in the colonocytes (Pool-Zobel et al. 2005; Sauer et al. 2007). Other health benefits associated with SCFA include restriction of pathogenic proliferation in the colon by acidifying the luminal contents (Blaut 2002) and modulating intestinal motility (Dass et al. 2007).

Modifications to Colonic Physiology

Prebiotics have been shown to modify the architecture of the gut wall. The absorptive surface of the gut, particularly the cecum, was significantly increased following prebiotic application (Raschka and Daniel 2005). Increases in the length, width, and depth of colonic crypts have been observed in rats post prebiotic supplementation, and the number of epithelial cells and the intensity of their secretory functions were also increased (Mineo et al. 2006). Such effects were not witnessed in germfree animals receiving prebiotics indicating the need for microbial participation in the manifestation of these structural modifications. Microbial metabolites may underlie these morphological alterations, particularly butyrate and lactic acid, which have been shown to stimulate epithelial cell proliferation in the cecum (Ichikawa and Sakata 1997; Blottiere et al. 2003).

Improvements in Colonic Transit Time

Stimulation of favorable microbial growth increases fecal microbial mass and gas production. This increases fecal bulk and stimulates passage through the colon, reducing transit time and consequently lowering water absorption. Collectively, this results in more frequent heavier stools with an improved consistency. Constipation is a common clinical complaint, and prebiotics may represent an effective strategy for alleviating this widespread ailment. Indeed, various studies have shown a beneficial laxative effect of prebiotics in constipated humans, increasing stool frequency, weight, and improving composition (Kleessen et al. 1997; Schumann 2002; Tateyama et al. 2005).

Mineral Absorption and Prebiotics

Prebiotics are recognized as useful health interventions to improve the retention of minerals. Various prebiotics and candidate prebiotics have been shown to improve mineral absorption, especially calcium and magnesium (Brommage et al. 1993; Chonan et al. 1995; Kashimura et al. 1996; Beynen et al. 2002). In a 1-year intervention trial by Abrams et al. in young adolescents, ITF was found to significantly increase calcium absorption, improve whole body bone mineral content, and increase bone mineral density (Abrams et al. 2005). Reductions in colonic pH due to increased SCFA production has been proposed as a potential mechanism. Acidification of the luminal environment is thought to improve mineral solubility and enhance passive diffusion from the lumen.

Prebiotic Regulation of Food Intake

Several studies have shown that gut microbial fermentation of prebiotics promotes satiety, reduces hunger, and also lowers energy intake. A decrease in fat mass, largely attributed to a reduction in food/energy intake, has been observed in various rodent models provided with ITF. Substitution of the ITF with non-fermentable dietary fibers does not yield the same results, indicating that colonic fermentation has some contribution to this observation.

Glucagon-like peptide (GLP)-1, ghrelin, peptide Y Y (PYY), and oxyntomodulin are peptides with an important role in food intake and energy expenditure. Endocrine cells located in the intestinal mucosa secrete these peptides, and their secretion helps to regulate energy homeostasis. Prebiotic studies in mice and rats have demonstrated that following ITF supplementation, the anorexigenic (promotes satiety) peptides, GLP-1 and PYY, are elevated in the portal plasma, and in some cases, the orexigenic (increases appetite) peptide, ghrelin, is reduced (Cani et al. 2004; Delzenne et al. 2005; Urias-Silvas et al. 2008). Similar effects were demonstrated in humans following 16 g/day of ITF feeding where satiety was increased following breakfast and dinner while hunger was reduced after dinner. These modulations in appetite resulted in a 5 % daily decrease in total energy intake (Cani et al. 2006). Raised bacterial fermentation has been associated with increased plasma gut peptide concentrations and subsequent appetite regulation (Cani et al. 2009).

Reduction of Glucose and Insulin Following Prebiotic Intake

The beneficial effects of prebiotics on glycemia and insulinemia have been shown in animals, but current human data appears to be inconsistent. A significant decrease in insulin was witnessed in healthy adults following 4 weeks of inulin feeding (Jackson et al. 1999), and a significant reduction in blood glucose was observed in non-insulin-dependent diabetics after 4 weeks of ITF supplementation. A study by Luo et al. (1996) found the provision of ITF to healthy adults for 4 weeks reduced hepatic glucose output but had no impact on fasting plasma glucose or insulin levels. Another study by Luo et al. (2000) feeding short-chain FOS to type II diabetics for 4 weeks had no impact on either plasma glucose or insulin or hepatic glucose production. The prebiotic effect on blood glucose and insulin appears to be largely governed by physiological (fasted or fed state) or disease (diabetes) state.

SCFA production may underpin these prebiotic effects. Propionate can stimulate hepatic glycolysis (glucose breakdown) and inhibit hepatic gluconeogenesis (glucose synthesis). Indirectly, propionate can also reduce plasma fatty acids, which consequently reduces gluconeogenesis. Butyrate can promote the secretion of GLP-1, which can reduce or delay gastric emptying, gastric secretion, and intestinal motility. These manipulations decrease transit time in the small intestine lowering the absorption of micronutrients following a meal.

Effect of Prebiotics on Lipid Metabolism

Bifidobacteria are inversely correlated with the development of fat mass, and bifidogenic prebiotics have been demonstrated to counteract high-fat-diet-induced obesity and related metabolic modulations (Cani et al. 2007; Dewulf et al. 2011). Numerous animal studies have shown the ability of prebiotics to lower blood lipid and cholesterol levels and reduce hepatic lipid accumulation (steatosis). For example, ITF reduced hepatic and serum triglycerides in several animal models receiving a high-carbohydrate or high-fat diet (Diez et al. 1997; Kok et al. 1998; Delzenne and Kok 2001). Reductions in steatosis and fat mass development were observed in obese rats when fed oligofructose (Daubioul et al. 2000). A prolonged study feeding oligofructose to rats observed a moderate reduction in circulating cholesterol (Fiordaliso et al. 1995), and ITF have also been demonstrated to ameliorate the hypercholesterolemic effects of a high-fat diet in rats and hamsters (Levrat et al. 1994; Trautwein et al. 1998).

The primary mechanism through which prebiotics reduce blood triglycerides is considered to be diminished hepatic de novo lipogenesis. Fructans have been shown to lower the activity

of lipogenic enzymes and downregulate the gene expression of several key enzymes involved in fatty acid synthesis including acetyl-CoA carboxylase, ATP citrate lyase, fatty acid synthase, and glucose-6-phosphate dehydrogenase (Kok et al. 1996). Reduced fat synthesis lowers the secretion of very low-density lipoprotein (VLDL) triglycerides from the liver into the serum. The bacterial fermentation products, acetate and propionate, are implicated in this mechanism. Acetate can stimulate lipid and cholesterol synthesis by entering lipogenesis and cholesterogenesis, while propionate can inhibit the entry of acetate into the hepatocyte and thus restrict these pathways. Additionally, propionate can inhibit hydroxymethylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis (Demigne et al. 1995). Fermentation patterns of prebiotics (relative proportion of propionate to acetate) can therefore determine their lipid-lowering properties.

An indirect route through which prebiotics can influence host lipid metabolism is by decreasing blood glucose and insulin concentrations. Both glucose and insulin can upregulate the expression of lipogenic enzymes, and so, reductions in these factors can attenuate this induction.

Another hypothesis for the cholesterol-lowering effects of prebiotics is modification of bile acid metabolism. Prebiotics can modulate the functional capacity of the microbiome toward an enhanced ability to deconjugate bile acids. Deconjugation facilitates the excretion of bile acids in the feces, and so, this enhanced functionality may increase the excretion rate of bile acids. To compensate, further bile acid synthesis is required, which leads to greater cholesterol utilization in the liver (Levrat et al. 1994). Given the implication of serum triglyceride and cholesterol levels as risk factors in coronary heart disease (Williams 1997), prebiotics may represent a useful tool in managing this risk.

Prebiotic Effects and the Immune System

Human intervention and animal studies demonstrate the ability of prebiotics to modulate both the local (gut) and systemic immune system of the host. The gut microbiota plays an integral role in the development of the immune system, both structurally and functionally. This is particularly true for the gut-associated lymphoid tissue (GALT). The mechanisms through which prebiotics modulate the immune system can be direct or indirect. An indirect effect is through the perturbation of the colonic bacterial ecosystem. Stimulation or inhibition of a particular microbial genus or species can alter the cumulative microbial signal being relayed to the GALT with potential to induce differences in GALT expression. Lactic acid bacteria can modulate both nonspecific host defense mechanisms and elements of the specific immune response. Such factors include upregulated GALT expression of secretory IgA (SIgA) and increased phagocytic activity.

Prebiotics can also improve resistance to pathogens through the fortification of indigenous bifidobacteria and lactobacilli. Growth of health-promoting bacteria increases competition for colonization sites preventing pathogenic adhesion and raises competition for nutrients. Furthermore, beneficial bacteria are able to excrete antimicrobial substances to create an inhospitable environment for pathogens (Manning and Gibson 2004). Microbial products such as SCFA lower colonic pH, making the colonic environment less favorable to pathogenic bacteria (Campbell et al. 1997), and bifidobacteria can produce antimicrobial peptides that act against potentially harmful microorganisms (Cheikhyoussef et al. 2008; Bernet et al. 1993). In addition, prebiotics can increase villous height, mucous release, and improve mucosal biofilm composition. Collectively, these factors enhance the integrity of the intestinal mucosa and improve colonization resistance.

Direct products of prebiotic fermentation, SCFA have been implicated with a role in immunomodulation. Leukocytes in the human colon, in addition to enterocytes and enteroendocrine cells, possess G-coupled protein receptors (GPR) for SCFA (Brown et al. 2003). Through binding to these receptors, SCFA can modulate chemokine expression in intestinal epithelial cells. Butyrate can regulate the proliferation and apoptosis of lymphocytes and monocytes (Millard et al. 2002; Kurita-Ochiai et al. 2003) and can influence colonic inflammatory responses by inhibiting NF-κB activity (Inan et al. 2000). SCFA, whose intestinal production is increased by prebiotics, may therefore impact on immune cells in the GALT.

Carbohydrate motifs in prebiotic molecules can also serve as ligands for pattern recognition receptors on immune cells and have potential to modify immune function.

Minor subsets of T and B lymphocytes, phagocytic cells, and NK cells express receptors for carbohydrates, but evidence is currently limited regarding receptors specific for prebiotic structures on immune cells.

Target Populations

Gastrointestinal problems are ubiquitous. It can therefore be argued that prebiotic-based fortification of positive gut bacteria can be applied to anyone. However, the early and later stages of life are thought to be especially relevant.

Infants

The gastrointestinal tract is considered a sterile environment until birth, and bacterial colonization begins during the delivery process (from the maternal fecal or vaginal microbiota and/or the environment). Initial bacteria to colonize the large gut are facultatively anaerobic strains such as *Escherichia coli* and streptococci. These first colonizers metabolize any traces of oxygen in the gut, thereby reducing the environment into one of strong anaerobic conditions. The bacteria that then further colonize depend largely upon the feeding profile of the infant. Human milk, apart from being a nutritious complete food for infants, also induces marked changes in probiotic levels in the infant gut. Factors such as the microbiota of the female genital tract, sanitary conditions, obstetric techniques, vaginal or

Table 2.3
Studies with prebiotics in infants

Test oligosaccharide	Study design	Prebiotic effect	References
TOS and polydextrose, lactulose (4 or 8 g/L)	Healthy formula-fed term infants	Normal growth and stool characteristics similar to breast fed	Ziegler et al. (2007)
TOS and long-chain FOS (inulin) (4 g/L)	Healthy bottle-fed infants	Significant decrease in clostridia, trend of increased bifidobacteria	Costalos et al. (2008)
TOS and FOS (inulin) (10 g/L)	Preterm infants on enteral nutrition, 14d supplementation	Significant reduction in gastrointestinal transit time and stool frequency	Mihatsch et al. (2006)
TOS and FOS (inulin) (8 g/L)	Formula-fed infants with colic	Significant reduction in crying episodes as compared to standard formula	Savino et al. (2006)
TOS and FOS (inulin) (4.5 g/L)	Formula-fed infants in weaning	Significant increase in bifidobacteria	Scholtens et al. (2006)
TOS and FOS (inulin) (8 g/L)	Healthy, formula-fed infants	Similar microbial composition between formula- and breast-fed infants	Haarman and Knol (2006)
TOS and FOS (6 g/L)	Healthy, formula-fed infants, breast-fed comparisons	Similar metabolic activity of the microbiota in TOS/FOS group as breast fed	Bakker-Zierikzee et al. (2005)
TOS and FOS (8 g/L)	Healthy formula-fed infants	Significantly higher bifidobacteria with prebiotic compared to control	Moro et al. (2005)
TOS (2.4 g/L)	Healthy term infants fed TOS	Significant increases in bifidobacteria, lactobacilli, and stool frequency	Ben et al. (2004)
TOS and FOS (inulin) (10 g/L)	Preterm infants	Significantly higher bifidobacteria compared to placebo group, similar to breast-fed group	Boehm et al. (2002)
Inulin (0.25 g/kg/day)	Formula-fed healthy infants	Inulin significantly increased lactobacilli and bifidobacteria	Kim et al. (2007)

FOS fructooligosaccharides, *TOS* trans-galactooligosaccharide

caesarean mode of delivery, and type of feeding have an immediate effect on the level and frequency that various species colonize the infant gut (Sherman et al. 2009). Infant fecal microbiota appears to more or less stable at 4 weeks of age and until weaning when the introduction of solid foods takes place. At this time, the fecal microbiota of breast-fed infants undergoes a more dramatic change than in formula-fed counterparts, and this could lead more easily toward gastrointestinal infections in the former. Formula-fed infants appear to develop a complex microbiota with facultative anaerobes, bacteroides, and clostridia at higher levels and frequency than in breast-fed infants. Bifidobacteria usually thought to be, by far, the predominant microorganisms not only in numbers (cfu/g wet feces) but also in frequency in breast-fed infants. Because of their various beneficial roles and use as probiotics, it is suggested that this can confer improved health status on the breast-fed infant. The final phase of microbiota acquisition occurs at weaning when a complex microbiota develops (Sherman et al. 2009). The classical prebiotic therefore is human breast milk. One approach to fortify the microbiological role of formula feeds has been to use prebiotics as stimulants for bifidobacteria, and thereby the aim is to improve the gut microbiota composition (to better resemble that seen with breast feeding). *Table 2.3* details prebiotic intervention studies, where the infant microbiota has been assessed following daily consumption of prebiotic formulae milk.

Elderly Populations

Above the age 60–65 years, there is a large decrease in the levels of gut microbial probiotics (bifidobacteria and lactobacilli). These are known to inhibit foodborne pathogens such as campylobacter, listeria, *E. coli*, and salmonella. Concurrent with this is an increased susceptibility to such infections in the elderly. It is not known why beneficial bacteria decrease as we get older, but it may be connected with altering diets and/or changes in their receptor (attachment) sites in the gut itself. Nevertheless, the trend does seem to occur in both Western and Eastern populations (Macfarlane and McBain 1999). Food-poisoning outbreaks and other forms of gastroenteritis are destructive for anyone, but the elderly are especially prone. Probiotics like bifidobacteria and lactobacilli have very powerful anti-pathogen effects related to their competitive influences, acid production, stimulation of the immune response, and excretion of antibiotics, including types that kill off pathogens like *E. coli*. Higher levels of probiotics in the gut of elderly persons may therefore offer improved protection against such common causes of gut illness (Bengmark 1998).

In light of the importance of the gut microbiota to health, changes in the composition of the gut microbiota with age could be of major significance. Reduced microbial protection in the gut of ageing persons opens up the possibility of reversing such trends by administration of particular dietary ingredients.

In view of this, dietary modulation of the gut microbiota in the elderly can greatly impact on gastrointestinal health in this disease susceptible, but health conscious, population group. The elderly are also particularly susceptible to very chronic gut diseases like colon cancer. In the ageing population, this impacts greatly on national medical expenditure.

Aside from this, there may be situations where prebiotic use may benefit the clinical situation. Examples include the following.

Acute Gastroenteritis

This is something that probably affects everyone at one time or another. However, it may be that certain populations have a higher risk than others. These could include patients taking antimicrobials (especially broad-spectrum forms), frequent travellers, individuals in highly stressful occupations (e.g., military, high performance athletes, firefighters, police), and the developing world. Gastroenteritis involves the ingestion of food or water contaminated with pathogenic microorganisms and/or their toxins. The economic costs and medical aspects are huge – with food safety incidence still increasing in most civilizations. Typical causative agents include shigellae, salmonellae, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli*, *Vibrio cholerae*, and *Clostridium perfringens*. Pathogens may either colonize and grow within the gastrointestinal tract and then invade host tissue or they may secrete exotoxin-contaminating food prior to its ingestion. Such enterotoxins disrupt the function of the intestinal mucosa, causing nausea, vomiting, and diarrhea. The principle human intestinal bacterial pathogens can be characterized according to the virulence factors that enable them to overcome host defenses. These include invasion that enables bacterial multiplication within enterocytes or colonocytes, for example, *Escherichia coli*, *Shigella* spp., salmonella, and yersinia. Cytotoxic bacteria, which include enteropathogenic and enterohemorrhagic strains of *Escherichia coli* as well as some shigellae, are able to produce substances which directly cause cell injury. Toxigenic bacteria such as *Vibrio cholerae* and some shigella are capable of producing enterotoxins, which affect salt and water secretion in the host. Lastly, enteroaggregative *Escherichia coli* has the ability to tightly adhere to the colonic mucosa. Such mechanisms enable potentially pathogenic bacteria to establish infections in the gastrointestinal tract, evade the immune system, and surmount colonization resistance afforded by the indigenous gut microbiota.

It is our belief that improved microbiota composition through diet can induce powerful effects against foodborne pathogens that are of relatively short duration (e.g., *E. coli*, salmonellae, campylobacter, rotavirus). This is because the target organisms for prebiotic ingestion can induce a number of barrier mechanisms against such pathogens. In terms of consumer relevance, this form of prophylactic management of gut disorder will be of large impact. This is because acute gastroenteritis:

- Affects most people and has a huge medicinal and economic impact
- Is currently of high profile (given various current concerns with food safety and the fact that foodborne pathogenesis is still on the increase)
- Will have more of a consumer impact than reference to chronic conditions like colon cancer, inflammatory bowel disease, and coronary heart disease (i.e., a person would accept that they will suffer from food poisoning but perhaps be less concerned by longer term problems)

Cancer

As a disease, cancer is probably as old as humans. Early medical writings dating back to Ancient Egypt and Greece describe diseases that are likely to have been cancers. However, incidence rates and the patterns of occurrence of different types were unknown until relatively modern times. Cancer is a major cause of death throughout the world and, in the developed world, is exceeded only by cardiovascular disease. In the case of colon cancer, bacterial fermentation is of considerable importance and may also be involved in protection against cancer. Products of bacterial fermentation SCFA, principally acetate, propionate, and butyrate, decrease colonic pH, and this has been associated with decreased risk of cancer. Butyrate has been shown to inhibit DNA synthesis and reduce cell proliferation (Kruh et al. 1995). Also, increased fermentation reduces the concentration of ammonia in feces, and the rate of the breakdown of lignans to an unconjugated active form is also increased (Bingham 1993). Various lignans together with isoflavonoids appear to have anticarcinogenic activity, and evidence suggests that they might be involved in reducing the biological effect of sex hormones that are important in prostate and breast cancers (Aldercreutz 1993).

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a collective term describing two main conditions: ulcerative colitis (UC) and Crohn's disease (CD). It affects between 0.1 % and 0.2 % of the population in developed countries (Shanahan 2000). UC is a relapsing and remitting disease characterized by acute noninfectious inflammation of the colorectal mucosa and submucosa, usually associated with diarrhea and rectal bleeding with an excess production of mucus. CD has similar clinical manifestations to UC, but in UC, the site of disease is confined to the large intestine, whereas in CD, any region of the gastrointestinal tract can be affected. The disease exhibits a north–south geographical gradient distribution risk.

Evidence has slowly, but surely, accumulated for a microbiological factor in both main forms. For Crohn's disease, this has mainly involved the mycobacteria. However, as Crohn's disease can involve any area of the digestive tract, including fairly sterile regions, the evidence for a definitive microbial factor is poor. On the contrary, for ulcerative colitis implications from

studies with germfree animals and the fact that the disease is confined to the colon, which is the most heavily colonized region of the human body, has led to the assumption that bacteria are involved. The feeding of sulfated polymers to conventional laboratory animals results in colitis-like lesions, while this is not the case for germfree counterparts (Marcus and Watt 1969). Our previous studies have shown that bacteria are present in the human gut that are capable of reducing sulfate to sulfide, a toxic metabolite (Gibson et al. 1991, 1993). We therefore hypothesize that sulfate-reducing bacteria (SRB) are etiological "triggers" for ulcerative colitis in humans. This has been supported by epidemiological data showing a high incidence and activity of SRB in fecal specimens from patients with colitis (Gibson et al. 1991). Over 90 % of the total SRB detected in the diseased stools belonged to the *Desulfovibrio* genus, and our latest data have used molecular-based principles to help confirm the hypothesis. Two recent reviews (Watanabe et al. 2007; Rowan et al. 2009) have surveyed the literature and reignited the debate on SRB having an involvement in the disorder. New prebiotics to help combat SRB are being developed.

Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a common disorder of the intestines that affects 8–22 % of the general population (Parker et al. 1995). Symptoms include bloating, abdominal pain, gas, and changes in bowel habits. Some IBS sufferers have constipation, while others have diarrhea, and some people experience both. Should specific etiological agents be involved, then it should be feasible to advocate prebiotics to manage this disorder. Indeed, in a study by Silk et al. (2009), the consumption of TOS was seen to reduce symptoms of IBS, improve stool consistency, and reduce flatulence while increasing fecal bifidobacteria levels.

Evaluating the Effects of Prebiotics

Prebiotics affect their host by altering the bacteria or activities of bacteria within the intestinal tract of the host. The human colonic microbiota is largely inaccessible for the study of different regions; subsequently the colonic microbiota is mostly studied through the use of feces. This is not ideal as microbial populations, pH, and substrate availability vary throughout different intestinal regions of the colon (Macfarlane et al. 1998). However, other samples are not so readily available, and variations in the fecal microbiota in response to dietary fibers/putative prebiotics can give an indication of what happens to major bacterial groups following consumption.

In Vitro Models

In vitro models of the gut are often used to screen effects that different prebiotics, synbiotics, probiotics, and dietary fibers may exert on the colonic microbiota. Such models range from simple and rapid approaches to more sophisticated and costly approaches. These models do not always provide an accurate model of what occurs in vivo as they lack mucosa and colonocytes, absorptive capacities, and host immunological interactions. However, they enable changes in colonic microbiota, in terms of numbers and metabolism attributable to the substrate to be initially assessed.

Batch Cultures

Batch cultures are closed systems whereby nutrient availability becomes depleted over time; thus, the bacteria follow a typical growth pattern. As such, these systems are only applicable over short time periods. Anaerobic batch culture systems can be used to assess how different substrates may affect the colonic microbiota. Static batch cultures are generally used with small volumes (e.g., 50 mL total); these are non-pH controlled, so generally used for an initial screening process (Macfarlane et al. 1990). pH controlled, stirred batch cultures systems can be used as a more detailed approach to assess what happens to the colonic microbiota in a steady pH range. Both systems are limited due to substrate availability and buildup of end products within the vessels; however, these models can be used for rapid assessment of potential fermentation end products and bacteriological changes.

Three-Stage Continuous Culture System

Chemostat models are continuously fed through a medium inlet; therefore, bacteria are growing in constantly replenished medium. A three-stage, continuous culture system (used to model the colon) consists of three vessels linked to each other to sequentially feed from a medium supply. The pH within the three vessels feeds from 5.5 to 6.2 then 6.8 with an exit to a waste vessel. This medium becomes more nutrient depleted between vessels, thus analogous to the proximal, transverse, and distal colonic regions. The system is conducted under anaerobic conditions, held at 37 °C, and is inoculated with feces. The bacteria within the model are allowed to equilibrate before the addition of test products (this would take a minimum of 320 h if the retention time was at 40 h); a second equilibrium stage has to be reached to enable assessment of changes attributable to the test product. The continuous culture system has been validated against the colonic contents of sudden death victims and hence has been confirmed to provide an appropriate microbiological method for testing substances of interest (Macfarlane et al. 1998). Medium is pumped through at a specific rate to mimic intestinal transit. Three-stage continuous culture systems are time consuming, so generally used following preliminary screening in batch culture systems. There are limitations to this model in that there is no way to assess the mucosal microbiota, which provides a niche for many different bacteria. Secondly, host absorption is not incorporated into this model; therefore,

the system effluent remains as a liquid and fermentation end products build up. Nevertheless, the model provides a validated bacterial model of the luminal colonic microbiota.

TNO Intestinal Model (TIM)

TIM is a model of the intestine made by TNO in the Netherlands. TIM-1 system consists of 4 compartments, modeling the stomach and the small intestine sections (Minekus et al. 1995), while TIM-2 models of the proximal colon region. These computer-controlled models mimic peristalsis and absorption through the use of a dialysis membrane. The TIM-1 model simulates intestinal secretions and pH gradients. Substrates are dosed into the system at predetermined intervals. The provision of gastric juices, enzymes, bile, and pancreatic juices enables a predigestion to be achieved. Hollow fiber membranes enable absorption of digestive materials in the jejunum and ileal regions. The end product of TIM-1 may then be used in the TIM-2 model, which is a colonic simulator. For the proximal model after fecal inoculation, the system is left to adapt for 16 h; then the test is conducted over 48 h, with medium fed at a typical rate of 4.6 mL/h. The model can be sampled to assess microbial and metabolite changes. Through the use of the TIM-2 model, there is no steady-state period; however, the model has been found to have similar microbial population ranges and SCFA ratios to sudden death victims (Minekus et al. 1999). Within TIM-2, the removal of metabolites associated with in vitro models is avoided. The TNO system has been used by industries during product development to provide information on bioaccessibility, biofunctionality, metabolites produced, and changes in microbiota composition.

Enteromix Colon Simulator

The enteromix colon simulator is a four vessel, semicontinuous computer-operated model of the human colon. The model, designed by Mäkivuokko et al. (2005), mimics pH and transit times of the large intestine. The model is held under anaerobic conditions before sequential inoculation with fecal slurry. The system is allowed to stabilize for 3 h, after which media is pumped into the first vessel, for fermentation over 3 h before being transferred sequentially every 3 h. Simulation is conducted for 48 h, after which the samples are collected. The system has been observed to give rise to similar data to in vivo studies.

Simulator of Human *Intestinal* Microbial Ecosystem (SHIME)

This model consists of five vessels which sequentially simulate the stomach, small intestine, and the large intestine. The first two vessels are used in a semicontinuous manner, whereas the final three vessels act as a continuous culture system. The system is typically allowed to stabilize over 3 weeks with a standard feed. A 2-week baseline period in which only the feed is dosed to the system is followed by a 2-week supplementation period to the ascending colon compartment. Finally, a postinoculation period of standard SHIME feed is undertaken. During the different phases, samples from the colon reactors can be taken at various time intervals. The system has been observed to lead to similar fermentative endpoints when compared with eight human volunteers (Molly et al. 1994).

In vitro models have the benefit of enabling the products of fermentation to be measured. For example, organic acids produced by the microbiota would normally be absorbed. In vitro the fermentation products can be monitored at times during the fermentation. Furthermore, with the multivessel systems, more information about the site and completeness of fermentation and changes throughout the intestine can be predicted.

In Vivo Models

The use of animal experiments enables control of diet, thus reducing variation in environmental factors often associated with human intervention. Animal models have been used extensively to study effects of dietary substances, on the colonic microbiota and on biomarkers. However, differences in physiology should be considered.

Rats and mice are frequently used in studies of the large intestine. The relatively small amounts of food required and small housing facilities make for an appealing option. Care should be taken however when interpreting results from such experiments. For example, a rat does not always respond in the same way that a human would. In rats, cancers occur much quicker, that is, after exposure to one carcinogen, and while this allows rapid studying of effects, it means that effects cannot be directly extrapolated to humans. Rats associated with a fecal with human microbiota have provided a model of the mature human colonic microbiota (Rumney and Rowland 1992). In such a model rat, colonic microbiota does respond to fiber supplementation in the diet, in a similar manner to humans (Mallett et al. 1987).

Pigs have a much larger stomach capacity than humans (6–8 L vs. 1–1.5 L); additionally the small and large intestine are also approximately three times that of a human; however, proportionally to the length of the intestine, the different regions are of a similar ratio (*Table 2.4*). Furthermore, the fecal pH of

Table 2.4
Relative intestinal tract of animals typically used in vivo

Animal	Relative length (%)			
	Small intestine	Cecum	Large intestine	Fecal pH
Pig	78	1	21	7.1
Rat	89	4	7	6.9
Human	78	2.5	19	7.0

a pig is similar to that of a human (Smith 1965). Despite the similarities, pigs are not always the chosen animal due to their large appetites and their expensive housing requirements.

While a human trial is the ultimate way to investigate effects of the colonic microbiota, initial screening is of course helpful to establish prebiotic potential and also to obtain information of the possible consequence before dietary intervention is an option. A range of in vitro and in vivo models means that much information can be gathered before the necessity of human intervention.

Human Intervention Studies

Following preliminary studies, human intervention trials are ultimately used to assess the role that prebiotics may have on the fecal microbiota, fecal metabolites, and also on various clinical outcomes or biomarkers of health. Volunteers are recruited to match the requirements of the study – for example, as various pharmaceuticals, such as antibiotics, will alter the microbiota, studies usually indicate that participants should not have been consuming these for 3–6 months prior to the start of the intervention. Human intervention studies can use healthy individuals to assess microbial changes within the feces following consumption of prebiotics. Other studies looking at more targeted use of prebiotics may recruit volunteers accordingly, for example, the effect of TOS on patients with irritable bowel syndrome (IBS) was assessed using only volunteers with Rome II positive IBS (Silk et al. 2009).

Prebiotic intervention studies typically collect feces to assess fecal metabolites, enzymes, other biomarkers, and the microbiota itself. Blood samples can often be collected to assess changes in immune parameters, cholesterol, glucose, or insulin levels. Urine is a tool for assessing changes in metabolites and also used in assessing whether saccharolytic or proteolytic fermentation is affected.

Human intervention studies typically take the form of a double-blind, randomized, placebo-controlled, crossover design. In such a study, every volunteer acts as their own control by consuming both the potential prebiotic and a matched placebo product. The products are consumed in either order, and in a double-blind design, neither the investigator nor the participant will know which treatment is being consumed at the different stages of the study. To observe a shift in the fecal microbiota, typically the intervention periods can be as short as 3 weeks (Costabile et al. 2008; Kolida et al. 2007). Studies looking at different health outcomes may however choose a longer intervention period (Silk et al. 2009; Jackson et al. 1999; Vulevic et al. 2008).

A parallel study design may be chosen when studying the effects of prebiotics. Such a study design is more frequently employed as a pilot study or when many different treatments or doses are being tested. A crossover study of many treatments would take a long time, while in a parallel study, volunteers need only consume a test product. In order to assess changes in such a study, the outcomes of volunteers in different groups can be compared (Ramnani et al. 2010). To generate a statistically highly powered parallel study, it is necessary to use more volunteers than within a crossover design study. To avoid issues of volunteers not acting as their own controls, some parallel study uses a crossover design, whereby all volunteers take a placebo product and one of the treatment products (Silk et al. 2009).

Human intervention studies are the only way to investigate the actions of prebiotics on humans. Therefore, the study design is important, as are the chosen outcomes.

Methods for Detecting Changes in the Gut Microbiota

Microbial enumeration using molecular techniques has transformed microbiology over the last two decades. 16S rRNA contains regions with different degrees of sequence variation. There are conserved regions, where the DNA sequence is similar even between distantly related bacteria, and variable, or even hypervariable regions, where the DNA sequence is unique to a particular bacterial species or group of closely related types. Hence, 16S rRNA is a useful molecule for looking at evolutionary relationships and for distinguishing between different unknown bacterial species. Utilizing the unique sequences of 16S rRNA between different bacterial groups means that probe libraries can be created; such probes are highly specific and bind selectively to their target sequences (Langendijd et al. 1995; Vaughan et al. 2000).

Fluorescence In Situ Hybridization (FISH)

The method of fluorescent in situ hybridization (FISH) utilizes the high abundance of target 16S rRNA molecules, within actively growing cells. Fluorescently labeled probes are hybridized to the test samples and the presence of target species or groups identified by fluorescence microscopy or flow cytometry. FISH is an accurate technique that does not require the culturing of bacteria. Probes have been created with varying specificities; some can be used to identify bacteria at species level, whereas other may be more genus specific. There are currently a set of probes used widely for enumeration of the fecal microbiota. The FISH technique is subject to a detection limit of 1×10^6, enabling bacteria to be accurately enumerated above this point (Harmsen et al. 2002)

Quantitative Polymerase Chain Reaction (qPCR)

qPCR is a method that can also be used for enumeration of the colonic microbiota. This involves the denaturation of the test DNA through heating to 90 °C to yield single-stranded DNA. The temperature is lowered to around 60 °C, and in the presence of the DNA of interest, the complementary primer sequence will anneal. DNA polymerase will then be used to extend the primer, thus doubling the amount of DNA of interest present per cycle.

A probe with a reporter and a quencher dye anneals downstream of the primer; the DNA extension by the polymerase enables separation of the quencher and the reporter, thus enabling fluorescence. The fluorescence is proportional to the amount of DNA in the sample. 40 cycles are typically used in total and the point at which the fluorescence becomes detectable is proportional to the amount of target DNA within a sample. This amount is determined using a calibration curve of known quantities of the DNA of interest. This technique for bacterial enumeration is more sensitive than FISH, with a detection limit of just 10,000 cells. However, different numbers of ribosomal operons within the cell can lead to confusion in results when using group-specific probes (Apajalahti et al. 2003).

Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE)

DGGE and TGGE are two molecular techniques used for comparing genetic diversity and population dynamics of a microbial community. Nucleic acids are extracted from the samples of interest; PCR is then used to amplify genes encoding the relevant 16S rRNA region. Separation of nucleic acid fragments in DGGE and TGGE is based on the degrees of electrophoretic motility of the different nucleic acid sequences in polyacrylamide gels. The polyacrylamide gels contain either a gradient of DNA denaturants (DGGE) or a temperature (TGGE) gradient. This then enables the population diversity of a specific group, or of total bacteria, to be observed (Muyzer et al. 1993). Fragments may then be identified by sequencing following extraction from the gel. This technique, while being qualitative rather that quantitative, does enable non-culturable microorganisms to be characterized.

Human Intestinal Tract Chip Technology

The use of microarray technology to assess microbial ecosystems within the intestinal tract has recently been developed. The technique uses fluorescently labeled probes based on the small subunit ribosomal RNA (SSU rRNA) gene from the sequences of intestinal communities (4,600 probes) which are attached on a tiling array. A reproducible high-throughput approach can then be used to assess the microbial diversity through the presence of genes within the samples (Rajilic-Stojanovic et al. 2009). For species/strain-level resolution, however longer ribosomal sequences or additional experiments are required (Claesson et al. 2009). This technique enables thousands of intestinal species to be assessed within a microbial sample at once, offering a fast and reproducible technique to study gut diversity in a relatively quantitative manner.

Modern molecular techniques for determining microbial communities are enabling a clearer approach for assessing the efficacy of different prebiotic products. Recent advances mean that more information can be obtained from intestinal studies and human intervention trials.

Concluding Remarks

Prebiotics are now incorporated into a wide range of food ingredients and therefore are becoming a big part of everyday diets. Currently, the main prebiotic targets are bifidobacteria and lactobacilli; however, more genera may be soon included in this with more research in the field and improved knowledge of the microbiota diversity and functionality.

The use of carefully planned human intervention studies and a growing range of prebiotic substrates can help this area expand. As gastrointestinal disorders are prevalent worldwide, prebiotics can provide an important role in the prophylactic management of various acute and chronic gut-derived conditions.

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3 Probiotics

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Abstract
The rapid and widespread growth in interest in probiotics has developed for several reasons. There has been a relatively recent appreciation that humans are made up of vastly more microbes than their own cells, and these microorganisms are involved in almost every facet of life. The ability to utilize microbes for human health dates back to paleolithic times with fermentation of food. However, only with the advent of modern day molecular biology and high-throughput sequencing have we been able to probe the human microbiome and its relationship to health. Defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host," probiotic applications have ranged from oral care to skin, gastrointestinal, and urogenital health, and in the future to cardiovascular, brain, and other areas, as well as in programming of the fetus and newborns. However, too many products are untested and should be banned from using the term probiotic until sufficient evidence has been accumulated on their effectiveness and safety. In order to manipulate the microbiota, strains and products must be carefully selected, documented, and administered appropriately. Studies have explored mechanisms of action, but few have linked these directly with outcomes in humans. Modulation of immunity, alterations in metabolism of microbes and host cells present in the niche to which probiotics are applied, and an ability to displace pathogens are likely important factors. But as studies uncover how microbes affect obesity, diabetes, and mental health, new applications of probiotics will undoubtedly emerge.

The Definition Is Everything

The definition of probiotics is "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). The manner in which this definition evolved from previous ones has been reviewed by others (Hoffman et al. 2008) as has the meaning of the definition (Reid 2005), and will not be repeated here. Suffice to say, it has been widely accepted by scientists, governments, and the leading science and industry organizations including the International Scientific Association for Probiotics and Prebiotics (ISAPP), the International Probiotics Association (IPA), and the Yogurt and Live Fermented Milks Association (YLFA).

The definition absolutely necessitates that probiotics be live microorganisms. Thus, there is no such entity as a dead probiotic, or a probiotic component such as a cell wall. Such substances may well confer health benefits, but they are not probiotics. The wording allows for all forms of microbes including genetically engineered strains to be designated as probiotic as long as they are shown to be safe and effective. The mere manipulation of the genome does not necessarily place the organism in a separate category. Notably, the amount of evidence required to show safety will depend upon the organism, the mode of use, the intended host recipient, and the regulatory authorities of the country in which the organisms is to be administered.

Of note, the use of the plural "microorganisms" implies that a single living bacterium is insufficient to provide a benefit, but the organism can be a single species and strain. If more than one species of microbes is to be administered, the benefit accrued should be determined in a manner by which more than one strain is shown to confer a benefit that exceeds the use of a single strain. This latter point is rarely proven in probiotics currently on the market, with the exception of *Lactobacillus rhamnosus* GR-1 combined with *Lactobacillus reuteri* RC-14 where the latter strain was added because the GR-1 strain was not sufficient on its own to confer benefits to the vagina (Reid et al. 1995).

Producers of multi-strain probiotics such as VSL#3 might argue that it is unnecessary to show an added effect of 2–8 strains over 1 strain, if the totality of the 8 strain product provides a physiological effect in pouchitis (Gionchetti et al. 2003). As long as each batch of product contains the same proportion of bacteria as shown to provide a benefit in the clinical studies, it is hard to counter this argument. Nevertheless, it would still be preferable to prove that the combination used was critical to its success.

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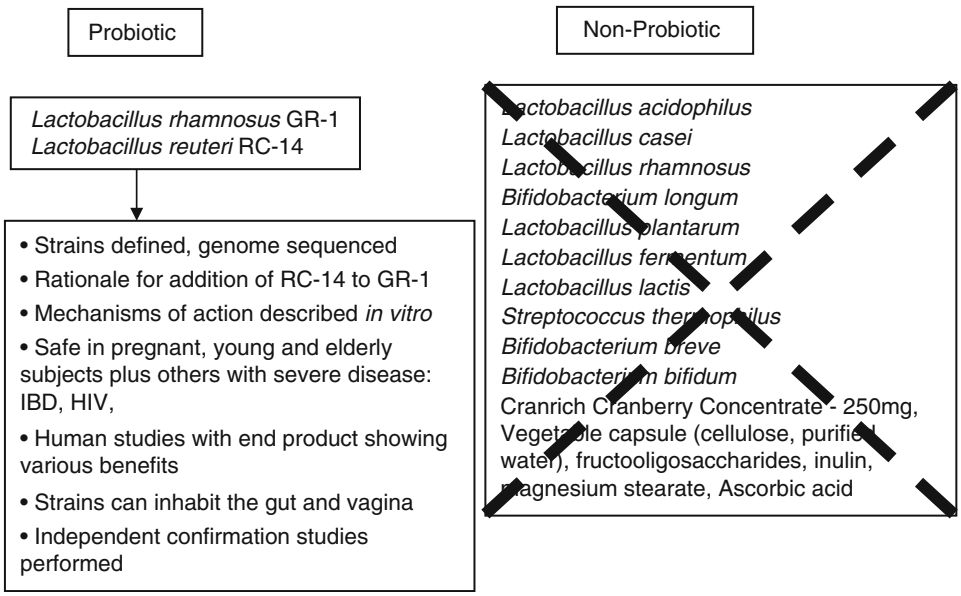

Fig. 3.1

The product on the right is not a probiotic. Its strains are not designated; the proof that more than one strain is needed is lacking, as is clinical efficacy; the effect of other products (e.g., inulin) on strain viability and growth is unclear; the benefits of adding these other compounds are untested. No peer-reviewed reports on safety, effectiveness, comparison with standard therapy, or other outcomes in humans

The term "administered" reflects the many ways in which probiotics can be delivered. This transcends the concept that probiotics are only for intestinal health. Rather, oral, vaginal, skin, and bladder applications have been studied, along with distant site effects in the brain, respiratory tract, and vascular system following oral administration.

The use of "adequate amounts" was included to emphasize that the manufacturer or retailer of a probiotic must decide through trial and error what the dose of probiotics should be to reach an efficacious result for the host. In order to prove efficacy and that a "health benefit" is conferred upon the host, human studies must be carried out and the results published in peer-reviewed journals. This too is critically important. It is not sufficient to simply do a study; the results have to show a benefit above and beyond placebo, or equivalent to a well-studied conventional therapy. Likewise, it is not acceptable to assess healthy subjects and show some benefit, if the intent of sales and health claims is to treat or prevent a specific disease. Thus, a probiotic for irritable bowel syndrome (IBS) should be shown to benefit IBS patients.

The latter point raises the question of can you call a strain proven in capsule form to alleviate IBS a probiotic if it is to be used in lozenge form for oral care? The answer is no, but this standard is almost never applied in practice. The argument is that the strain met the definition by conferring a health benefit, and thus the site of action for each usage does not need to be stipulated. This is strictly speaking correct, but Guidelines for the Evaluation of Probiotics in Food published 1 year later in 2002 (FAO/WHO 2002) implied a preference for testing each application. The document stated: "The principal outcome of efficacy studies on probiotics should be proven benefits in human trials, such as statistically and biologically significant improvement in condition, symptoms, signs, well-being or quality of life; reduced risk of disease or longer time to next occurrence; or faster recovery from illness. Each should have a proven correlation with the probiotic tested." On reflection, it would have been clearer to have stated that a probiotic shown for one application needs to be appropriately tested before being referred to as probiotic for treating, preventing, or affecting a biologically distinct condition at a different body site.

Likewise, the definition and Guidelines did not stipulate that the end product in which the microbes are delivered should be tested, rather than just the microbes themselves. Nevertheless, this was implied. The main reason for testing a product is that the carrier vehicle, whether food, tablet, capsule, or other, alters the availability of the strain(s), the speed and effectiveness with which it grows, replicates, and produces by-products that may help confer the health benefits. In the case of foods, if the strain was active during preparation, the metabolic by-products may already be present and be a vital part of the effect that occurs; and if the product's organisms are delivered in a dried form, this effect may not arise. In terms of testing every possible combination of formula, such as different fruits in probiotic yogurt, this is debatable and must rely upon some evidence that different flavors, excipients, and contents do not significantly affect microbial viability. *Figure 3.1* illustrates the difference between a probiotic and a product called probiotic but that should not be permitted this designation.

The definition did not create subcategories of probiotics, although a case may be made for this to occur in future, to align products and improve regulatory pathways and help

consumers understand the different products (Reid 2012). Thus, under the current FDA setup, a probiotic could be a drug, food, dietary supplement, medical device, or cosmetic, depending on how it is delivered and what claims are sought. It has been suggested in some quarters that probiotics that are designed to treat and prevent disease should be referred to as biotherapeutic. In my view, this is not needed and only results from inadequacies within the FDA system and an inability to accept that a probiotic food can, and in many cases does, have biotherapeutic effects. The term "biotherapeutic" should be reserved for other product forms, such as dead organisms, or metabolic by-products of microbes. In the case where a probiotic effect is shown to be due solely by a molecule, such as a bacteriocin that acts upon *Listeria monocytogenes* (Corr et al. 2007), the molecule would be the biotherapeutic substance produced by a probiotic organism. Just as in war, it is the army (probiotic) that wins, but the armamentarium (biotherapeutic) is the reason they won.

Human Evolution and Microbes

The basic concept of probiotics came from a belief that replenishment of certain microbes was necessary and would be beneficial. The "necessary" part comes from mainly two sides: (1) many organisms are excreted, for example, half the content of feces, and thus replenishment provides a way to restore those excreted and (2) the diet of modern-day humans has been depleted of beneficial bacteria through food processing, consumption of long shelf life products and excessive use of antimicrobial agents in foods and cleaning agents. The correlation between long life and ingestion of fermented foods observed by Metchnikoff (1907) is a frequently cited example of the benefits of ingesting lactic acid bacteria, and studies of the paleolithic diet further support a correlation between these organisms being a part of human evolution (Rook 2010). Thus, lactobacilli are members of the Firmicutes, and their abundance would be expected to be higher in people who eat fermented dairy products, such as the French (Tap et al. 2009). But, early humans ate and drank fruit and vegetables that had undergone fermentation, deliberately or not. As such, other fermenting organisms, for example, *Prevotella* and *Xylanibacter* genera which are members of the *Bacteroidetes* family could also have propagated, as appears to be the case in children from a rural village in Burkina Faso, where the diet and high fiber content is similar to that of early human settlements at the time of the birth of agriculture (De Filippo et al. 2010). This led to the hypothesis that these bacteria coevolved to maximize energy intake from fibers.

An epidemiological transition is when a long-term shift occurs in health and disease patterns. An early transition of humans took place when hunter-gatherers began primary food production. A later epidemiological transition occurred in modern times with the industrial revolution, during which shifts in social and economic patterns with the introduction of refrigeration, hygiene, vaccination, water filtration, deworming, and antimicrobials (Manton 1988) changed dietary consumption of fats, alcohol, smoking, physical activity, and the gut microbiota.

Of course, it would be foolhardy to suggest that ancient diets were always optimal for health, and recent evidence of Egyptian Mummies having ischemic heart disease recognizes this (Allam et al. 2011). It is also difficult to correlate long life with food types; for example, Native Africans particularly in rural areas ate fermented food products, plants, and plant roots high in prebiotic fructooligosaccharides that stimulate growth of lactic acid bacteria and this may have correlated with longer life. However, political strife, wars, malnutrition, and diseases such as human immunodeficiency virus (HIV) infection, tuberculosis, and malaria significantly impair modern-day Africans' longevity (Anukam and Reid 2009), making it impossible to compare the two eras.

Another method of assessing the value of ingesting lactic acid bacteria and fermented foods is through longitudinal studies. A 12 year study of 1,000 men and women in Sweden showed a lower risk of developing a first myocardial infarction with intake of fermented milk (Warensjö et al. 2010). Another study of over 900 women showed a negative association with breast cancer risk and intake of fermented milk products (trend, $P = 0.003$) (Wirfält et al. 2005). The largest study was undertaken on 82,002 Swedish women and men followed for 9.4 years. There was a statistically significant inverse association with intake of cultured milk (sour milk and yogurt) and risk of bladder cancer (Larsson et al. 2008).

A good test of this concept was performed in 69 Swedish children (<2 years old) living an anthroposophic lifestyle, which comprises a diet of vegetables spontaneously fermented by lactobacilli, and a restrictive use of antibiotics, antipyretics, and vaccinations. Compared to 59 infants of a similar age with a traditional lifestyle, the anthroposophic children had more lactic acid bacteria in their gut, which the authors concluded might contribute to the lower prevalence of atopic disease previously observed in children from anthroposophic families (Alm et al. 2002).

Before Birth: Health of Mother and Fetus

This latter example has led to studies in which pregnant women at risk of having children with severe atopic dermatitis (AD), and their newborns, were administered probiotics with the aim of modulating immunity and preventing the condition. In a double-blind, randomized placebo-controlled trial in which *Lactobacillus rhamnosus* GG or placebo was given prenatally to 159 mothers who had at least one first-degree relative (or partner) with atopic eczema, allergic rhinitis, or asthma, and postnatally for 6 months to their infants, the frequency of atopic eczema was half in the probiotic group (Kalliomäki et al. 2001). In another randomized, double-blind trial 415 women received probiotic milk or placebo from 36 weeks of gestation to 3 months postnatally during breastfeeding. The probiotic milk contained *L. rhamnosus* GG, *L. acidophilus* La-5, and *Bifidobacterium animalis* subsp. *lactis* Bb-12. Children with an itchy rash for more than 4 weeks were assessed for AD. The odds ratio (OR) for the cumulative incidence of AD was 0.51 in the probiotic

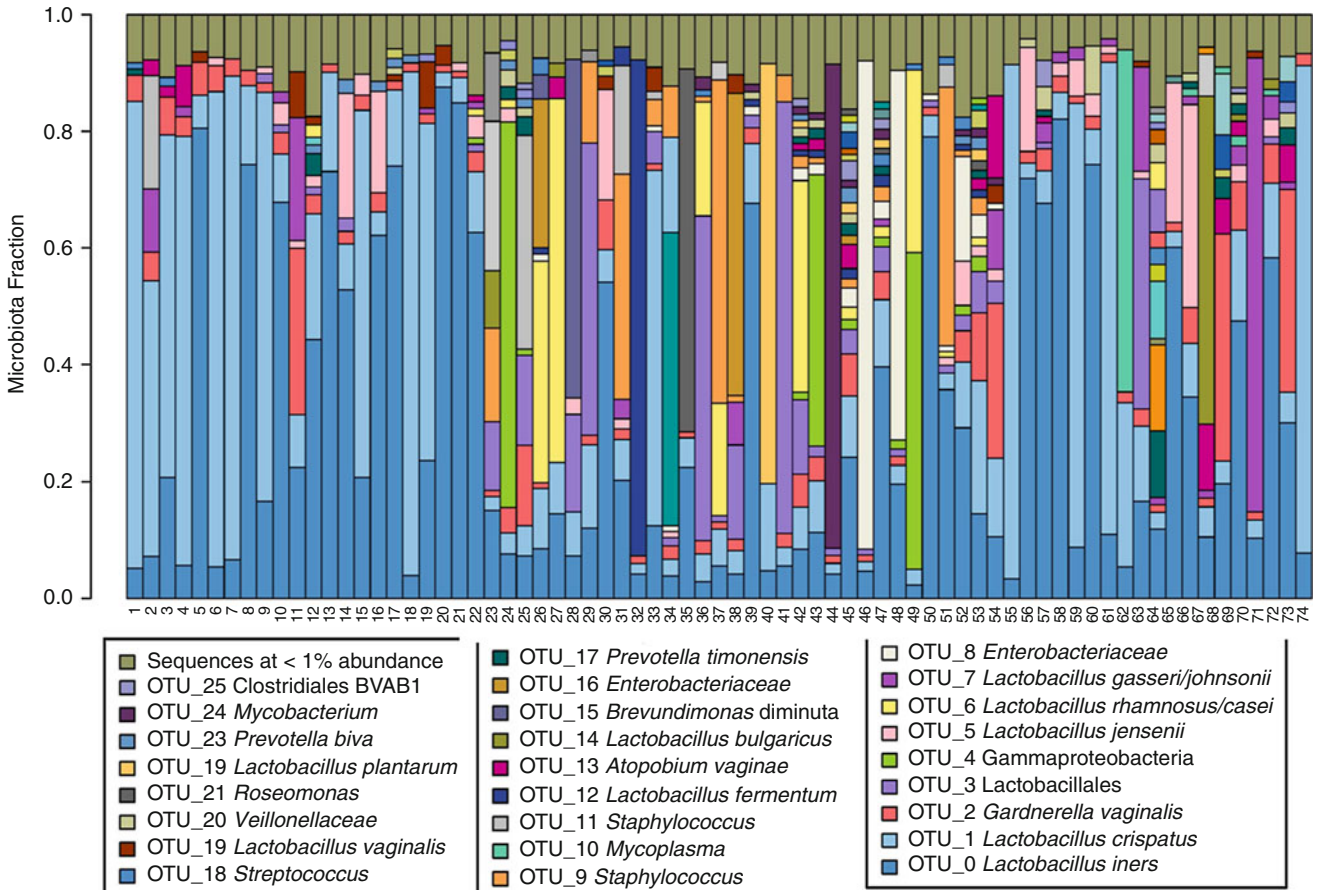

Fig. 3.2
Sampling of the vaginal microbiota of pregnant women (Thanks to M. Yeganegi, A. Bocking, J. Macklaim and G. Gloor)

group compared with the placebo [95 % confidence interval (CI) 0.30–0.87; P = 0.013], but there were no significant effects on asthma or atopic sensitization (Dotterud et al. 2010). Another study showed that administration of probiotic *L. rhamnosus* HN001 but not *B. animalis* subsp *lactis* HN019 taken daily from 35 weeks gestation until 6 months of breast-feeding, resulted in significantly (P =.01) reduced risk of eczema (hazard ratio [HR], 0.51; 95 % CI, 0.30–0.85) compared with placebo. There was no significant effect of *L rhamnosus* or *B animalis* subsp *lactis* on atopy (Wickens et al. 2008). In a somewhat radical approach, babies in the Czech Republic administered a probiotic strain of *E. coli* orally after birth had a reduced incidence of allergies after 10 and 20 years (12 % and 16 % in the colonized groups and 33 % and 32 % in controls), suggesting that immune modulation is important early in life (Lodinová-Zádníková et al. 2004).

However, not all studies have been positive. A double-blind, placebo-controlled prospective trial of 105 pregnant women from families with one or more members (mother, father, or child) with an atopic disease who were randomly given *L. rhamnosus* GG or placebo 4–6 weeks before expected delivery, followed by a postnatal period of 6 months resulted in similar rates and severity of atopic dermatitis (Kopp et al. 2008). Of interest, probiotic failure was evident if the newborns and not the mothers were treated with *L. rhamnosus* GG (Taylor et al. 2007), raising the questions: Does bacterial programming of the immune system take place during fetal growth? If so, which bacteria, if any, play a role?

The microbiota of the vagina has recently been thoroughly interrogated by sequencing methodologies. Over 250 microbial species have been identified, but *Lactobacillus iners* is consistently the most common detected followed by *L. crispatus*, *L. jensenii*, and *L. gasseri* (Hummelen et al. 2010; Ling et al. 2010; Srinivasan et al. 2010; Zhou et al. 2010; Ravel et al. 2011). There are some changes over the menstrual cycle (Keane et al. 1997; Srinivasan et al. 2010) and at different locations within the vagina and cervix, but preliminary analysis of pregnant women has found an unusual propensity for Gram-positive cocci not seen in nonpregnant cohorts (Yeganegi et al. unpublished) (*Fig. 3.2*). This is all the more interesting for three reasons: (1) Group B streptococci in the vagina are problematic for the newborn (Koenig and Keenan 2009); (2) Streptococci and staphylococci are known to reach the mammary ducts at lactation (Martín et al. 2007a, b), presumably from the gut but perhaps from the vagina; and (3) Mastitis is common in breast-feeding mothers (Spencer 2008), and could be due to transfer by nipple contamination or bloodstream translocation from the vagina and domination in the mammary areas.

The application of probiotics for vaginal and bladder health has been studied by orally and vaginally administered approaches. Only a few probiotics exist for these applications, with the most documented by far being *L. rhamnosus* GR-1 in combination with *L. reuteri* (formerly fermentum) RC-14. These are not species commonly found in the vagina, but the strains were selected for properties that interfere with pathogenesis: bacteriocins, biosurfactants, hydrogen peroxide, and signaling molecules (McGroarty and Reid 1988; Velraeds et al. 1996; Li et al. 2011), plus the ability to stimulate mucus production, and enhance immunity (Kirjavainen et al. 2008a). Both strains reach the vagina in a portion of women following oral ingestion and natural transfer along the perineum from the rectum (Reid et al. 2001; Morelli et al. 2004), and perhaps more importantly reduce pathogen transfer (Reid et al. 2003), and encourage indigenous strains to proliferate (Burton et al. 2003). Studies have shown that *L. rhamnosus* GR-1 has the capacity to influence the inflammatory cascade that leads to preterm labor (Yeganegi et al. 2009, 2011). As an estimated third of preterm labor is associated with bacterial vaginosis (BV), the ability of these strains to normalize the vaginal microbiota (Reid et al. 2003; Petricevic et al. 2008) and pH (Oleszczuk et al. 2006) may be key to a healthy pregnancy and fetus.

The influence of vaginal and cervical bacteria on fetal development has mainly been examined with respect to pathogens, with the exception that a dominant lactobacilli presence has been shown to correlate with conception and successful pregnancy in women undergoing in vitro fertilization (Eckert et al. 2003). The precise reason is not known, and may simply be due to reduced inflammation allowing the fertilization process to proceed. Unlike the presence of *Enterobacteriaceae* and *Staphylococcus* that prevent pregnancy, the presence of lactobacilli need not (Selman et al. 2007). If pathogens are present in the placental parenchyma, fetal development of the brain can be compromised (O'Shea et al. 2009). It would be interesting to find out if species like *Lactobacillus* play any active role in fetal development and programming. In the latter case, it seems unlikely that the baby enters the world naïve to all microbes. Rather, a more likely scenario is that the fetus is exposed to bacteria and their by-products, and thus non-pathogenic bacteria could be important for that purpose. If so, this opens up a whole new area to contemplate probiotic applications. As with any interventions on the fetus, such approaches would have to undergo rigorous ethical discussion.

The probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 administered vaginally has been shown in a small study to cure BV (Anukam et al. 2006), and this effect was also found for a different probiotic containing *L. brevis* CD2, *L. salivarius* subsp. *salicinius* FV2, and *L. plantarum* FV9 (Mastromarino et al. 2009). Nothing is known about the properties of the CD2, FV2, and FV9 strains, but presumably they are able to displace BV pathogens and/or kill them in order to cure the infection. This is the case with GR-1 and RC-14, where penetration of BV biofilms (McMillan et al. 2010) and stronger adhesion than pathogens (Younes et al. 2012) along with biosurfactant production appear to be important. Oral administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 has also been shown to prevent UTI (Beerepoot et al. 2009) as has a strain of *L. acidophilus* (Lee et al. 2007). The latter is important for children, as early onset bladder infection can lead to pyelonephritis and long-term renal damage. The mechanism is likely reduction of pathogen load seeding the bladder, and modulation of immunity to improve antimicrobial defenses. In patients with recurrent UTI, an aberrant vaginal microbiota appears to damage innate immunity and potentially lead to more infections (Kirjavainen et al. 2008b).

Upon Birth and the First Years of Life

Three infectious complications are particularly harmful to the newborn. Group B streptococci transferred from the vagina at birth can cause sepsis, meningitis, and death. Invasive phenotypes are particularly harmful. Probiotics have not yet been tested clinically, but the aim would be to prevent the streptococcal colonization and transfer at time of birth.

Transfer of viruses such as HIV and herpes simplex is difficult to control as it can occur in utero as well as at the time of birth. Treatment mostly comprises use of antiretroviral therapy. If probiotics were to be used to reduce infection in the newborn, data would have to be accumulated to show that probiotics can reduce viral shedding in the mother.

Premature infants are at risk of necrotizing enterocolitis (NEC), a potentially lethal infection induced by microbial disturbances in the intestine. The breadth of evidence showing that different types of probiotics can prevent NEC and mortality has led to a call for the treatment to become mandatory in neonatal intensive care units (Tarnow-Mordi et al. 2010). The mechanism likely involves competition with pathogens for space and nutrients, and the lactic acid bacteria modulating immunity, upregulating gut barrier function, and stabilizing pH.

While the prevention of allergies has focused on the mother and newborn, the treatment option has also been explored using probiotics. A 2008 literature review concluded that more controlled trials were needed before probiotics could be deemed useful for treatment of atopic dermatitis (Betsi et al. 2008). Still, some people clearly have responded to treatment for atopy, such as a group given twice daily *L. acidophilus* DDS-1, *B. lactis* UABLA-12, and fructo-oligosaccharide for 8 weeks (Gerasimov et al. 2010). Likewise, responses have been reported for other allergic conditions. A study of over 100 school children suffering from asthma and allergic rhinitis, showed that treatment with *Lactobacillus gasseri* A5 for 8 weeks improve the clinical symptoms and immunoregulatory changes (Chen et al. 2010). The explanation given was a significant reduction in the TNF-α, IFN-γ, IL-12, and IL-13 production by the peripheral blood mononuclear cells.

An additional effect found in breastfed babies was relief from colic following administration of *L. reuteri* DSM 17 938 (Savino et al. 2010). The reduction in fecal *E. coli* and ammonia was believed to be involved in the outcome.

Oral Care

The application of probiotics for oral care makes sense not so much from the perspective of a need to replenish beneficial microbes, more with a view to compete with an ever-present range of bacteria responsible for caries and plaque. Two approaches have been investigated. In the first, because some probiotics are taken in food form, such as fermented milk and yogurt, researchers investigated whether the exposure of the "beneficial" organisms resulted in any net reduction in disease. The use of milk with *L. rhamnosus* GG for 5 days a week in a study of 594 children showed a significant reduction in dental caries ($P = 0.01$)(Näse et al. 2001). The reason for the effect is presumably temporary interference with pathogen colonization and possibly their displacement. Only one strain specifically chosen for oral health has been commercialized. *Streptococcus salivarius* BLIS K12™ was selected from a range of streptococci isolated from healthy New Zealand subjects. Shown to produce a bacteriocin against a number of oral pathogens, daily use of the strain has been shown to lower volatile sulfur compounds and treat halitosis (Burton et al. 2006).

Another *Streptococcus salivarius* strain 9 has been identified with potential to treat and/or prevent *S. pyogenes* throat infections (Wescombe et al. 2011). This ability of indigenous oral bacteria to prevent infection dates back to the work of Sanders and Sprunt almost 40 years ago (Crowe et al. 1973; Sprunt et al. 1980). The use of alpha hemolytic streptococci to colonize babies was shown to help prevent infection (Sprunt et al. 1980). A more recent application of this concept was undertaken in patients with voice box prosthesis which become dysfunctional through pathogenic biofilms. Daily ingestion of an *L. casei* Shirota fermented milk drink significantly increased the lifetime of the prosthesis by a factor of 3.76 (Schwandt et al. 2005). This same drink consumed for 4 weeks reduced the effects of plaque-induced gingival inflammation in otherwise healthy individuals with a higher plaque score (Slawik et al. 2011). Other strains, such as two *L. reuteri* administered for 12 weeks and *L. rhamnosus* LB21 for 2 weeks, showed little or no effect on the oral environmental, albeit they did not assess inflammation (Lexner et al. 2010; Sinkiewicz et al. 2010).

Gastrointestinal Effects

There are three categories of probiotic applications to the gastrointestinal tract: one that targets the stomach, one that targets the small and large intestine, and another that acts through the gut but induces distant site effects.

The Nobel Prize discovery that *Helicobacter pylori* colonize the stomach and cause ulcers and cancer opened up a whole new area for microbiological research. It is now known that *H. pylori* is highly prevalent especially in Asian subjects, yet it remains unclear in whom the bacterium is pathogenic and in whom it is commensal. Thus, treatment is not universally administered and recurrences and reinfections are common. The necessity of using triple and quadruple antibiotic and acid-modulating therapy for *H. pylori*, leads to side effects. Lactic acid bacteria and their organic acids have been considered for use in treatment of *H. pylori*, but in vivo success has been limited. Rather, probiotics have been most used to reduce the drug side effects (Song et al. 2010), although *Bifidobacterium bifidum* 1BF-1 can affect the regulatory mechanism of the NF-κB signaling pathways, suppressing induction of IL-8 by the pathogen (Shirasawa et al. 2010).

Infectious diarrhea is a major cause of morbidity and mortality in the developing world, while in recent times *Clostridium difficile* has become a concern in the developed world. A number of probiotics have been tested to prevent and treat infectious diarrhea. Prevention of naturally acquired as distinct from antibiotic-associated diarrhea is difficult to assess and requires assumptions about equal exposure to pathogens in placebo and active groups. In one study of 204 undernourished children from an indigent peri-urban Peruvian town, *L. rhamnosus* GG in a flavored gelatin once daily, 6 days a week, for 15 months was found to prevent diarrhea, especially in non-breastfed infants (Oberhelman et al. 1999). Interestingly, in another Peruvian study, treatment with *L. rhamnosus* GG did not reduce the duration of acute watery diarrhea in infant males, although lactose intolerance may have complicated the design (Salazar-Lindo et al. 2004).

For prevention of antibiotic-associated diarrhea, successful studies have been reported for *Saccharomyces boulardii* lyo, *L. rhamnosus* GG, *Lactobacillus casei* DN 114 001, and *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R (Vanderhoof et al. 1999; Kotowska et al. 2005; Hickson et al. 2007; Gao et al. 2010). The diversity of the strains indicates a commonality of clinical effect which hypothetically could involve gut barrier enhancement that shut down fluid loss, antipathogen effects which clear the infecting strain faster, immunological modulation, or a generalized alteration of the intestinal microbiota that more quickly resolidifies the digesting foods.

Diarrhea and constipation can be problematic without an infectious cause. Many people suffer from irritable bowel syndrome, which is a group of gut ailments associated with abdominal pain, discomfort, and bloating. A number of products containing a range of lactobacilli, bifidobacteria, yeast, bacilli, *E. coli*, and a novel prebiotic trans-galactooligosaccharide have been assessed for improvements in IBS, with different levels of success (Whorwell et al. 2006; Guyonnet et al. 2007; Enck et al. 2009; Hun 2009; Silk et al. 2009; Guandalini et al. 2010). As the condition can arise with a diverse array of symptoms and signs, it is difficult to pinpoint the mechanisms whereby strains can benefit the host. When pain is the primary symptom, the ability of strains such as *L. acidophilus* NCFM to potentially induce opioid and cannabinoid receptors could be one mechanism (Rousseaux et al. 2007). Alterations in the microbiota that lead to reduced gas production could be another.

As intestinal discomfort has so many compounding factors, including differences between individuals, it seems hard to imagine that a single product could have universal benefits. Yet, probiotic foods are available to the general population. In an effort to assess effects in somewhat of a general population

(otherwise healthy but with some digestive discomfort), Guyonnet et al. (2009b) performed an open-label, controlled trial of 371 adults randomized into daily consumption of either one or two pots of probiotic yogurt over 2 weeks, or their usual diet. The test product improved digestive comfort (1-pot group 82.5 %; 2-pot group 84.3 %), compared to controls (2.9 %). The findings were confirmed in healthy women who had no digestive discomfort upon enrolment, but who reported improvement in their gut well-being (Guyonnet et al. 2009a). This same product is promoted for regularity. In simplistic terms, the product containing *B. animalis* DN 173 010 causes the motility of the gut to more effectively and consistently move the food. In scientific terms, fecal weight, sulfate-reducing bacteria counts, the concentrations of total short chain fatty acids, propionic and butyric acids, fecal pH, and methanogen counts can all influence transit time, and it remains to be determined which of these is the key to achieving regularity (El Oufir et al. 1996). The finding from animal studies that the DN 173 010 strain induces anti-inflammatory effects adds another possible mechanisms of action, or is coincidental and not related to IBS (Veiga et al. 2010).

The more serious long-term problems in the intestine are inflammatory bowel disease (IBD) which comes in the form of Crohn's Disease (CD) and ulcerative colitis (UC). While the definitive cause of IBD is not believed to be a single pathogenic species, an increase in invasive *E. coli* in the ileum has been associated with CD, and a less diverse microbiota among the phyla *Firmicutes* and *Bacteroidetes*, perhaps due to dietary changes or use of antibiotics, has been associated with IBD (Abraham and Medzhitov 2011). Treatment of CD and UC with probiotics has not been successful to date, perhaps because the inflammatory condition is too severe to be reversed by microbes. Having said that, a case of spontaneous remission has been reported following an infection, suggesting that modulation of innate immunity by bacteria may provide an effective treatment (Hoption Cann and van Netten 2011). This is contrary to the concept that treatment should focus on downregulating inflammation, rather than stimulating innate immunity. A probiotic *E. coli* Nissle 1917 has been shown to help maintain remission in UC (Kruis et al. 2004), but rather than through stimulation of an innate response, it is also believed to induce an anti-inflammatory effect (Güttsches et al. 2011). A much more realistic scenario is that the Nissle strain produces an immuno-stimulatory effect through its lipopolysaccharide (Zídek et al. 2010). Clearly, this is an area that requires more investigation in order to better select a probiotic for UC and CD.

For patients with pouchitis, an 8 strain probiotic VSL#3 has been used with pharmaceutical therapy to maintain remission, possibly by a significant increase in the percentage of mucosal CD4 + CD25(high) and CD4+ LAP-positive cells compared with baseline values (Pronio et al. 2008), and by decreasing microbial diversity (Uronis et al. 2011). The last point was proposed following animal studies, but it seems strange that adding a product rich in lactobacilli, bifidobacteria, and streptococcus would reduce diversity, unless these organisms displaced or killed other species, but the study did not explore this.

As is evident from the cacophony of applications of probiotics to the intestine, strains have different modes of action. This is beautifully depicted in the review by Bron et al. (2011) in which *L. plantarum* WCFS1 induces modulation of nuclear factor-κB signaling through Toll-like receptor 2 in intestinal epithelial cells, while *L. rhamnosus* GG induces epithelial barrier fortification and resistance to apoptosis, and *L. acidophilus* NCFM induces cytokine release and Th1 cell development.

Distant Site Effects

A number of effects likely occurring via the gut but registering at distant sites occur following probiotic use. For example, reduced duration of respiratory illness has been reported in a number of studies, presumably through a generalized priming of the mucosal immune system (Guillemard et al. 2010; Hojsak et al. 2010; Gleeson et al. 2011). In an effort to reduce the severity of seasonal allergies, two strains of lactobacilli and bifidobacteria were selected based upon in vitro antiallergy effects, then delivered in a yogurt formulation prior to grass and ragweed allergy seasons. The results of the small human study did not reach clinical significance, but several patients had marked improvements that could not be explained by placebo effects, and significant changes in immunological parameters were noted (Koyama et al. 2010). Studies aimed at treating seasonal allergies have had mixed results, with administration of *L. plantarum* No.14 in randomized, placebo-controlled, double-blind studies showing a significant improvement in ocular symptom-medication score, and Th1 cells (Nagata et al. 2010). A correlative change in the intestinal microbiota with improvements in seasonal allergy has been reported following intake of *L. rhamnosus* GG and *L. gasseri* TMC0356. The authors suggested that the intestinal microbiota might be more sensitive to exposure to environmental allergens, and a degree of stabilization achieved by probiotics could be useful in the management of seasonal allergy (Kubota et al. 2009).

As bacteria in the digestive tract can metabolize lipids, it is no surprise that probiotics have been tested for their ability to improve cholesterol levels, especially in patients at higher risk of cardiovascular disease. However, these studies have mostly resulted in failure to lower total cholesterol and low-density lipoprotein cholesterol, despite promising in vitro and animal findings. In a small study, *Enterococcus faecium* M-74, a probiotic strain used in Slovakia, was reported to lower serum cholesterol by 12 % after 56 weeks (Hlivak et al. 2005). This study needs to be repeated, but to its credit it followed subjects for a year. The standard therapy for high cholesterol is the use of statins, which block the enzyme hydroxy-methylglutaryl-coenzyme A reductase in the liver that is responsible for making cholesterol. In general, these drugs reduce cholesterol by 25 %, but they can be toxic and in some cases lethal, so alternative approaches would be welcomed. The low density lipoprotein (LDL) particles carry cholesterol and triglycerides from the liver to peripheral tissues, but their retention in the arteries leads to the formation of plaques and increased risk of

cardiovascular disease. High-density lipoprotein (HDL), or the 'good' cholesterol, is the smallest lipoprotein particle that transports cholesterol from the arteries to the liver for excretion. Cholesteryl ester (CE) in the HDL particles was increased after *Enterococcus faecium* M-74 treatment, indicating a different mechanism than the statins. This probiotic and bile salt hydrolase (BSH)-active *L. reuteri* NCIMB 30242 microencapsulated in yoghurt, taken twice per day for 6 weeks in 114 hypercholesterolemic adults and shown to be efficacious and safe for lowering LDL-C, TC, apoB-100, and non-HDL-C (Jones et al. 2011), indicates potential alternatives to the often toxic statin drugs.

One of the most exciting applications of probiotics and manipulation of the gut microbiota is their ability to influence the brain, from development to subsequent adult behavior (Heijtz et al. 2011). It is quite well known that infection and stress can adversely affect memory and cognitive function (Gareau et al. 2011). But recent studies have shown that nonpathogens can reduce stress through gut-brain axis effects. One study showed that *Bifidobacterium longum* NCC3001 normalized behavior and brain-derived neurotrophic factor (Bercik et al. 2010), while other strains reduced stress in animals through gut-brain signaling (Forsythe and Bienenstock 2010). The restoration of noradrenaline in the brainstem following *Bifidobacterium infantis* 35624 treatment further shows important events linking the gut and brain (Desbonnet et al. 2010). Pettersson's group using germ-free mice has shown that the microbial colonization process initiates signaling mechanisms that affect neuronal circuits involved in motor control and anxiety behavior (Pettersson et al. 2011). The ability of certain probiotic strains to produce neurochemicals further suggests that in the future, probiotic applications could play a profound role in mental well-being (Reid 2011). Already, there is evidence that emotional behavior can be modified via the vagus nerve and central GABA receptor (Bravo et al. 2011). Furthermore, the suggestion that *H. pylori* may be associated with Parkinson's disease (Lyte 2010), through making less dopamine in parts of the brain that control movement (Testerman et al. unpublished), has in part led to studies that assess whether eradication of certain pathogens by antibiotics, or complete regeneration of the gut microbiota using fecal transplants can alleviate or delay progression of Parkinson's disease.

What is in Store in the Future?

Predicting the applications of probiotics in the future, in addition to areas already discussed, is easy to propose but is dependent upon multiple factors. Studies on the microbiome of humans have already started to reveal some surprises in the number of species identified at various body sites. By comparing well-defined cohorts, it will be possible to identify some microbial profiles associated with health. From that will come bacterial strains that are hypothesized to effectively displace the aberrant microbiota. But what if such a probiotic contains an organism like *Bacteroides fragilis* which is pathogenic at other sites? Safety assessments in humans would be more rigorous for potential pathogens, thus perhaps limiting the types of companies who could take such strains to market. In addition, current manufacturers of nonpathogenic bacteria might be reticent to cultivate and prepare strains such as *B. fragilis*. This would be especially true if the market was small for the strain's application. Another example is *Helicobacter pylori*, a pathogen associated with stomach ulcers and cancer but which produces factors such as a neutrophil-activating factor (HP-NAP) that drives the Th-1 polarization and to display a powerful inhibition of allergic Th-2 response (Amedei et al. 2010). It seems hard to imagine using *H. pylori* to manage asthma when so much effort has been made to eradicate the organism from the stomach, but the possibility exists.

Scaling-up problems would also be encountered for novel candidate probiotic strains that are strictly anaerobic or fastidious in their growth. Thus, for example, *Bacteroides thetaiotamicron*, *Faecalibacterium prausnitzii*, and *Lactobacillus iners* might have potential as probiotics for the gut or vagina (*L. iners*), but prove difficult to produce en masse. Even more so, will be strains that only survive in conjunction with other strains because of the symbiotic association they have evolved. Indeed, currently, such coaggregates cannot even be registered at culture collection sites, where only single strains are stored as per FDA regulations. Since strains must be deposited for market approval, such mixtures could not come to market. This presents a problem for fecal transplant microbiotas and artificially derived copies of multiple fecal organisms (Allen-Vercoe et al. 2012). Given the impressive success rate for fecal transplant to cure *C. difficile* infections (Glauser 2011), and the mounting appreciation that probiotics function better if they are already growing in media mimicking their natural environment, a major challenge will occur if patients die because regulators are limited by the current setup and cannot find a way to approve such therapies.

Currently, a significant stumbling block is that many probiotics have the ability to prevent, treat, or cure disease, and by FDA and other regulatory agency policies, these products are defined as drugs. Not only does that significantly increase paperwork and amount of data required for approval, but it rules out use of the strains even in clinical trials, unless drug registration is undertaken. Thus, major advances such as use of probiotics, including recombinant strains, for management of gastroenteritis and colitis, bacterial and vaginal infections, HIV and tuberculosis, certain mental illnesses, elevated cholesterol, obesity and diabetes, may prove difficult to develop from experimental findings to human trials and consumer use. But, the burden of disease and failures of traditional chemical pharmaceutical agents might well force society to find ways to make such probiotic therapies available for testing and then general use.

In Conclusion

Probiotic research is a serious scientific discipline and the application of probiotics for humans and animals represents a widely

expanding area of commerce. This will not be a passing fad: with more bacteria and genetic information in us than human cells, the ability to modulate the microbiome will become a major method of maintaining health for decades to come. The applications will go far beyond influencing digestion and intestinal health, and will represent one of the most exciting new developments in therapeutic medicine. The next 10 years will see studies on the mechanisms of action of probiotic strains, and on what controls the microbiome within given niches, how these interlink with organisms at other body sites, and how these in turn influence sites bereft of living organisms. The beneficial microbes are coming; long live the microbes.

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4 Identification of Pathogens by Classical Clinical Tests

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Abstract
Since the recognition of bacteria as agents of human and animal disease, phenotypic methods have been used to identify them. From the time Roux first recognized curved gram negative rods in the stools of patients with cholera, microscopic examination of stained clinical specimens and cultures have been proven to be a reliable and rapid means of preliminarily identifying organisms into specific groups such as gram positive cocci or acid fast bacilli. The ability to grow under different environmental conditions such as only in the presence (aerobic), absence (anaerobic), or either in the presence or absence (facultative) of oxygen is yet another way of differentiating organisms. Simple phenotypic tests that detect a variety of enzymatic activities can be further used to identify bacteria. Detection of specific virulence factors such as protein exotoxins is another method used to detect bacterial pathogens. Some organisms are difficult or cannot be grown on artificial media. One method used in these instances is serology which tests the immune response to the organism.

Once identified, the management of infection with these organisms remains. One of the keys to managing bacterial infections in patients and animals is to determine to what antimicrobials the causative agents are resistant. This is important because patients treated with antimicrobial agents to which their causative agents are resistant will likely not have a clinical response. These methods and more are the focus of this chapter on the conventional identification of clinically relevant bacteria.

The identification of clinically relevant bacteria begins in the age of Koch, Pasteur, and Gram. For much of the microbial era, these fathers of medical microbiology would have been quite comfortable with the methods used in clinical microbiology laboratories to identify human pathogens: growth characteristics, colonial morphology, staining and metabolic characteristics, and serologic techniques. Recently, a molecular revolution in bacterial identification has occurred. It has relied upon the advent of relatively simple and cost-effective methods to determine bacterial genomic sequences and their proteomic content. Key molecular methods currently used in clinical microbiology settings included molecular amplification techniques such as PCR and LAMP; sequencing of portions of or entire conserved genes such as 16 S rRNA, *rpoB*, or *hsp*65; and proteomic analysis using MALDI-TOF mass spectrometry (Nolte and Caliendo 2011; Petti et al. 2011). Microarray-based techniques under development show great promise but for now are far too expensive for routine use in a diagnostic microbiology setting. Despite these exciting developments in molecular diagnostics, simple, reliable, conventional methods continue to be the foundation of bacterial identification in the clinical settings. A well-performed Gram stain on a clinical specimen remains a reliable, rapid, inexpensive means of diagnosing an infection. Furthermore, culture and a few simple tests to determine phenotypic characteristics remain an accurate, inexpensive means for identifying bacterial pathogens. In this chapter, we will review conventional methods that continue to be widely used in the clinical settings and explain how they are used to determine the identification of human pathogens. This chapter will concentrate on the

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organisms most frequently detected in clinical specimens or organisms which are infrequently detected but are important threats to the public health such as *Corynebacterium diphtheriae* and *Clostridium botulinum* or are biothreat agents such as *Bacillus anthracis* or *Francisella tularensis*. This chapter is not meant to be an exhaustive recitation of all the phenotypic tests that are used to identify bacteria. The interested reader is referred to either the Manual of Clinical Microbiology or Bailey and Scott's Diagnostic Microbiology for further information on the speciation of bacteria (Forbes et al. 2007b; Nolte and Caliendo 2011). Rather this chapter will expose the reader to the most common strategies for identification of bacteria and where possible give a relatively complete explanation of how many clinically important organisms are identified.

Bacterial Growth and Colony Morphology

At the most basic level, bacteria can be classified according to the in vitro conditions under which they grow. The focus of this section will be to differentiate common groups of bacteria based on their ability to grow or not under differing environmental conditions and what they look like when growth is achieved. It is important to remember that certain pathogenic organisms of humans, *Haemophilus influenzae, Bordetella pertussis*, or *Treponema pallidum*, are highly evolved such that they only associate with human or primate hosts having growth requirements which reflect their limited metabolic repertoire. Other organisms such as *Acinetobacter, Pseudomonas aeruginosa*, or *Burkholderia pseudomallei* are found in the environment whether it be a hospital air-conditioning unit or rice paddy (American Society for Microbiology 2008; Peleg et al. 2008). They have much more complex genomes resulting in much greater metabolic diversity. As a result, these genetically complex organisms are able to survive in comparatively hostile environments including those rich with antimicrobial agents such as the sink in an intensive care unit (Peleg et al. 2008).

The first differentiating factor is under what atmospheric conditions does a microorganism grow? Aerobic, anaerobic, facultative, microaerophilic, or capnophilic are terms that are used to describe the atmospheric environment tolerated by a bacterium. Aerobic organisms include those that are typically well dispersed in the environment such as *Bacillus* spp., and these organisms require oxygen for growth. Conversely, anaerobes such as *Bacteroides* spp. cannot grow in the presence of oxygen. Most clinically relevant bacteria, such as staphylococci or *Escherichia coli*, fall into the class of facultative anaerobes or those organisms that can grow either with or without oxygen, though typically there is some tropism that varies by species. Microaerophiles such as *Campylobacter* spp. require reduced levels (~5 %) of oxygen to grow. Finally, capnophiles are organisms such as *Capnocytophaga* spp. which require increased CO_2 levels (5–10 %). Although not strict capnophiles, a number of clinically important pathogens including *Haemophilus influenzae, Streptococcus pneumoniae*, and the pathogenic *Neisseria* all show enhanced growth under capnophilic conditions.

The other major environmental condition which affects the growth of bacteria is the temperature. For obvious reasons, most clinically relevant bacteria are considered mesophiles, which means that their optimal growth temperature is between 20 °C and 40 °C. Some bacteria, such as *Yersinia enterocolitica* and *Listeria monocytogenes*, can be considered psychrophilic because they are capable of growth at low temperatures. No human pathogens are true thermophiles (grow at ≥42 °C) although certain pathogenic *Clostridium* may grow at temperatures as high as 48 °C.

Because most clinically relevant bacteria utilize the same environmental conditions to grow, additional information is needed to differentiate them. A simple way of differentiating bacteria is by looking at the bacterial colony on an agar plate. Most bacteria can be grouped into categories by how they look on an agar plate, with some bacteria being able to be identified on the basis of their colony morphology alone. This is especially important considering that clinical specimens may have multiple species of bacteria present. The different colonial morphologies seen on culture plates may give an important indication about the variety of bacteria present and whether they are "friends" or "foes" in that setting.

The most widely used agar medium in the diagnostic laboratory is 5 % sheep blood agar (SBA). SBA is a nutritious medium on which most bacteria will grow. In fact, the absence of growth on SBA but permissive growth on more nutritious media such as chocolate agar provides important information in the preliminary identification of a bacterium. Due to its wide use, the colony morphologies described will be in reference to growth on SBA unless otherwise noted. An added benefit to using SBA in the diagnostic microbiology laboratory is that the presence of blood cells allows for the determination of hemolysis. Hemolysis is a reaction in which the red blood cells of the medium are destroyed due to the presence of enzymes produced by the bacteria called hemolysins. The type and degree of hemolysis are important diagnostic tools for the clinical microbiologist. Hemolytic reactions are typically divided into one of three categories: alpha or partial hemolysis, beta or complete hemolysis, and gamma or no hemolysis. Alpha hemolysis is a characteristic of organisms such as *Streptococcus pneumoniae* and *Lactobacillus* spp. and results in the agar having a green discoloration in the area around the colonies (Fig. 4.1). Beta hemolysis is often seen in organisms such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa*. It is characterized by the distinct zone of clearing around the colonies such that the surrounding medium becomes transparent. The degree of hemolysis can often give an indication of the identity of organism, as some organisms such as *Streptococcus pyogenes* give large distinct zones of clearing, whereas *Streptococcus agalactiae* often gives narrower, more diffuse zones of hemolysis. Finally, growth of some bacteria such as *Acinetobacter* spp. and *Enterococcus* spp. on SBA does not result in any hemolysis. In addition to the hemolytic reaction, the size and color of the bacterial colony on SBA can provide important clues to help direct the identification of the organism. For example, typically, the colonies of Gram-positive organisms

(see below for a description of the Gram stain and how it is used in the clinical microbiology laboratory) are smaller than those of Gram-negative organisms. Furthermore, colonies of the Gram-positive staphylococci are generally larger than those of the also Gram-positive streptococci. Additionally, some bacteria produce pigments that provide clues as to their identity. Bacteria such as *Pseudomonas aeruginosa* and *Serratia marcescens* are known for their metallic green and red pigments, respectively (Fig. 4.2). Finally, how a colony looks is often an important factor in determining what further tests to utilize to identify the bacteria or confirming the results of other tests such as a Gram stain.

Fig. 4.1
Three Bacteria demonstrating alpha, beta, and gamma hemolysis on sheep blood agar

The Gram Stain and Microscopic Morphology

The Gram stain may be the single most informative test utilized in the clinical microbiology laboratory. Its low-cost, rapid turnaround, and quality and quantity of information provided are reasons this test is so valuable. The test relies on two main features. The first is that bacterial cell walls are composed of different proteins and carbohydrates that will react differently to exposure to alcohol and that this reaction is similar for similar organisms. The second is that bacterial cells come in all shapes and sizes but in most cases are consistent within genera (e.g., all *Staphylococcus* species are round or cocci, whereas all *Pseudomonas* are rod shaped or bacilli). The Gram stain itself consists of four main reagents: the primary dye crystal violet, the iodine fixative, the alcohol decolorizer, and the safranin counterstain. In the Gram stain procedure, bacteria cells are heat fixed to a slide where they are then subjected to staining via crystal violet. The crystal violet enters the cells and is fixed to the cell walls via the addition of the iodine. The decolorizer is then applied, and some cells lose the crystal violet iodine complex. The counterstain safranin is then applied to stain these cells pink or red. The bacteria that are able to retain the crystal violet complex and remain purple after decolorization are considered Gram positive, whereas those that lose the crystal violet and stain with the safranin are considered Gram negative. It is thought that the major difference between Gram-positive and Gram-negative bacteria that leads to the differential staining is the composition of the bacterial cell wall (Forbes et al. 2007g). Gram-positive organisms have a cell wall that is much higher in peptidoglycan with many more teichoic acid cross-links. This combination of features allows the bacteria to better retain the crystal violet complex during the decolorization step, and the dominance of the crystal violet allows the cells to appear purple even if they contain safranin from the counterstain. Conversely, Gram-negative bacteria have cell walls with less peptidoglycan and fewer teichoic acid cross-links, leading to easy loss of the crystal violet complex and only safranin staining which makes the cells

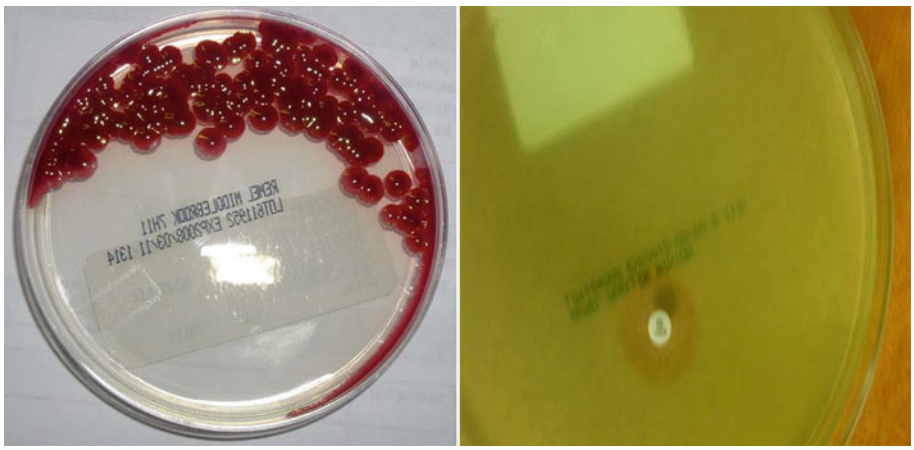


Fig. 4.2
Images of pigmented *Serratia* (*left*) and *Pseudomonas aeruginosa* (*right*)

appear red or pink. As mentioned previously, the Gram stain reaction is a characteristic that is shared by all members of a genus, allowing for easy classification of groups of bacteria by a common Gram stain characteristic.

In addition to providing information as to whether a bacterium is Gram positive or negative, the Gram stain also provides a look at the morphology of the cell. Bacteria come in many shapes and sizes making morphology a useful tool for classifying organisms. The most basic differentiation is based on whether the cells are round, referred to as cocci, or elongated, referred to as rods or bacilli. Thus, Gram-stained bacteria can be described as Gram-positive cocci, Gram-positive bacilli/rods, Gram-negative cocci, or Gram-negative bacilli/rods (Fig. 4.3). Furthermore, multiple additional morphologies can be described beyond these basic four, each of which provides information as to the identity of the bacterium. For example, Gram-positive cocci can appear in clusters (typically *Staphylococcus*), chains (*Streptococcus*) (Fig. 4.4), or as diplococci (*Streptococcus pneumoniae*). Gram-positive bacilli can be additionally described as being diphtheroid (*Corynebacterium*, branching *Nocardia*, or boxcar spaced *Clostridium* or *Bacillus*) (Fig. 4.5). Gram-negative cocci are sometimes seen in pairs

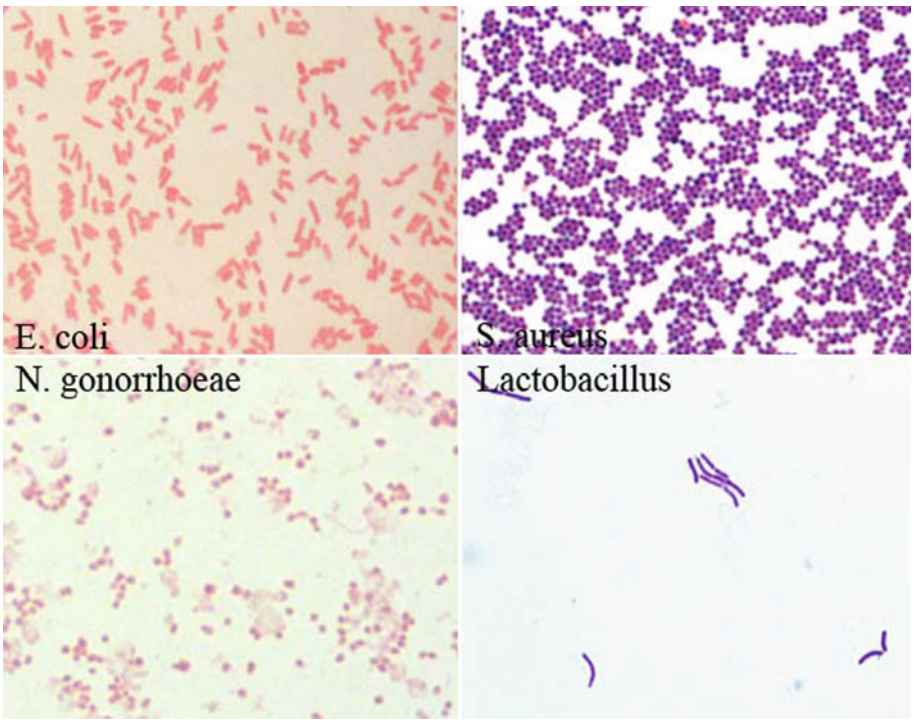


Fig. 4.3
Gram stains clockwise from *top left*: Gram-negative rods, Gram-positive cocci, Gram-positive rods, and Gram-negative cocci (some images courtesy of CDC)

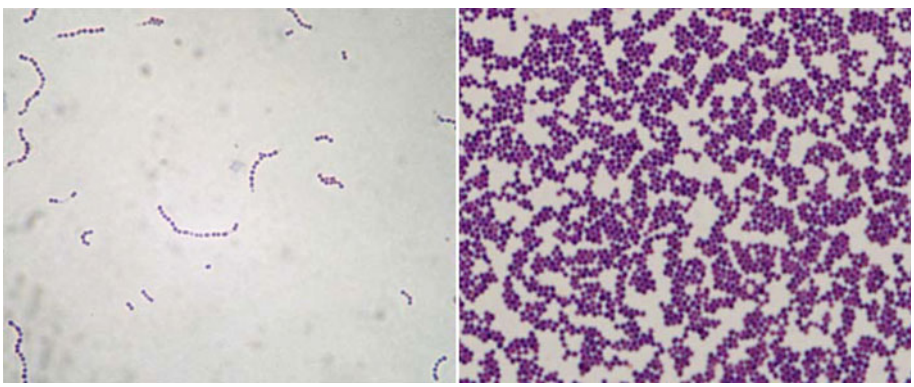


Fig. 4.4
Image differentiating Gram-positive cocci in chains (*left*) versus Gram-positive cocci in clusters

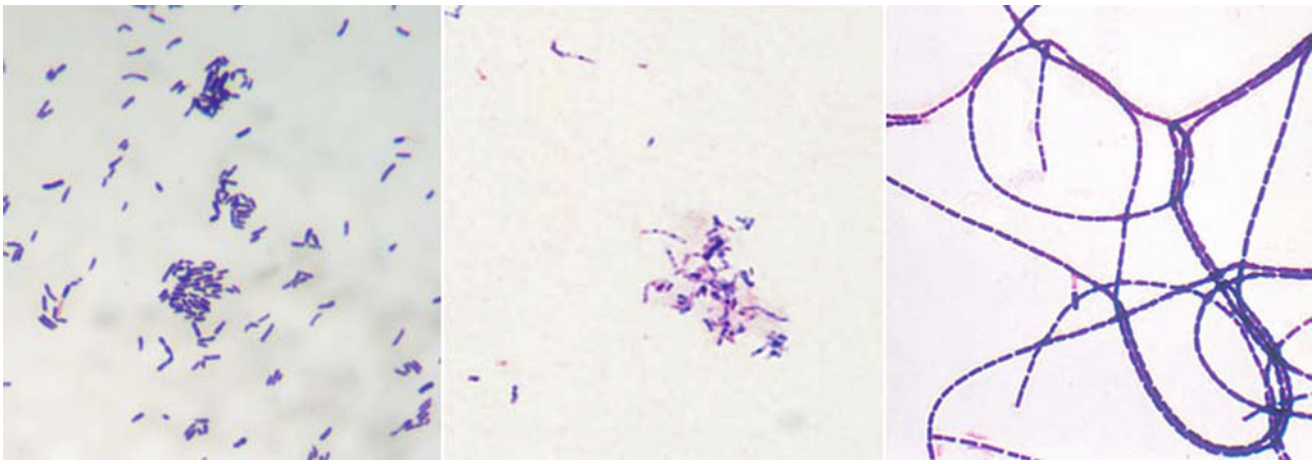


Fig. 4.5
Variations of Gram-positive rods. *From left*: diphtheroids, *Actinomyces*, and *Bacillus* (some images courtesy of CDC)

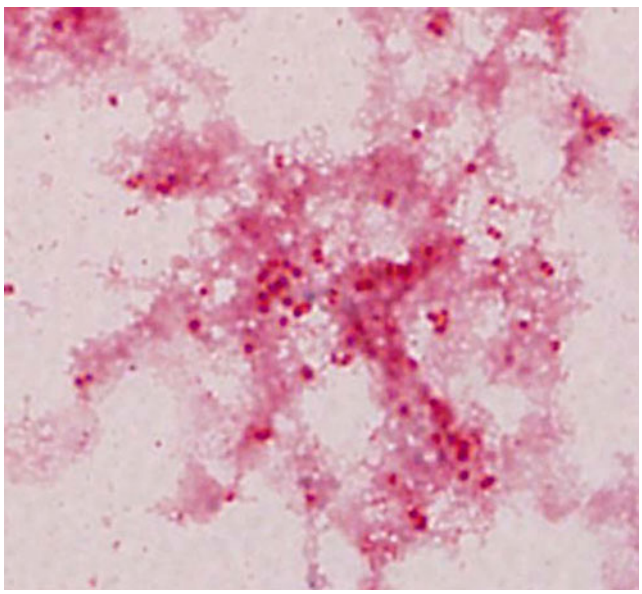


Fig. 4.6
Gram-negative diplococci

(*Neisseria*) (Fig. 4.6) or as an intermediate between cocci and bacilli called coccobacilli (*Prevotella* and *Haemophilus*). The size of Gram-negative bacilli is also informative. Small Gram-negative bacilli are often associated with bacteria such as *Brucella* or *Francisella*, while larger bacilli are associated with the enteric bacteria such as *Escherichia* or *Shigella* (Fig. 4.7). Some Gram-negative bacilli appear curved (*Vibrio*) or fusiform (*Fusobacterium*) (Fig. 4.7). Some important human bacterial pathogens do not Gram stain at all. These include *Mycobacterium* (because of a thick waxy cell envelope into which the stain cannot penetrate), *Mycoplasma, Treponema pallidum, Rickettsiae*, and *Chlamydia* species which either lack typical cell walls or are intracellular. With the amount of information that can be gathered by looking at a Gram stain under the light microscope, it is no wonder that it is considered one of the most valuable tests of the microbiology laboratory.

Simple Phenotypic Tests

Although the Gram stain provides a lot of valuable information, which is useful to rapidly narrow down suspected identities of an unknown bacterium, the Gram stain and growth characteristics alone are usually insufficient for a determinant identification. There are a number of simple phenotypic tests, however, that when combined with the Gram stain and growth characteristics provides unique insight into a bacteria's identity. Many of these tests will be discussed later as they are used to differentiate specific bacteria or groups of bacteria. The use of these simple tests have been codified into a document published by the Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2008) which describes very simple identification algorithms for many commonly encountered human pathogens including *S. aureus, Enterococcus* spp., *E. coli, Proteus mirabilis, P. vulgaris, P. aeruginosa, H. influenzae, Moraxella catarrhalis, S. pyogenes, S. agalactiae, and S. pneumoniae.*

There are a few tests, however, that are performed on a majority of either Gram-positive or Gram-negative organisms. Oftentimes, the first test done on a Gram-positive organism is the catalase test. This determines the presence of the enzyme, catalase, which is present in only some bacteria. The catalase test is performed by adding a 3 % hydrogen peroxide solution to a smear of the organism and by looking for the production of bubbles that is indicative of the reaction of H_2O_2 being broken down into O_2 and H_2O. This test is especially useful in differentiating Staphylococci (catalase +) from Streptococci (catalase −). For Gram-negative bacteria, the first test performed is frequently the oxidase test which tests for the presence of cytochrome c oxidase. In the presence of this enzyme, a colorless reagent, tetramethyl-para-phenylenediamine, turns purplish blue (Fig. 4.8). This test

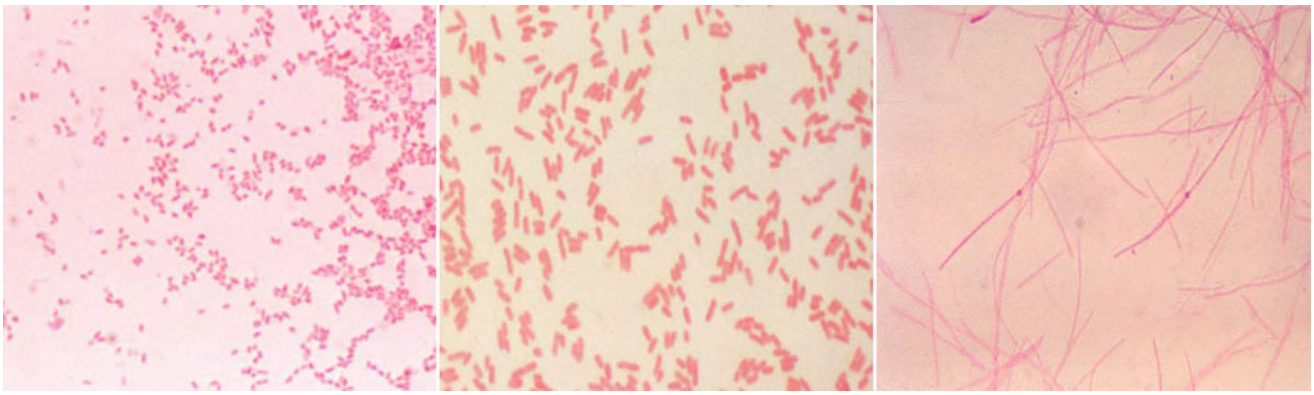


Fig. 4.7
Variations of Gram-negative rods. *From left*: *Brucella*, *Escherichia coli*, and *Fusobacterium* (images courtesy of CDC)

Fig. 4.8
Positive oxidase reaction

is frequently used to differentiate Gram-negative bacilli, especially clinically important non-fermenting Gram-negative bacilli such as *P. aeruginosa* and *Burkholderia cepacia* complex from the glucose-fermenting *Enterobacteriaceae*. *Neisseria* species are also oxidase positive, and this is a useful preliminary step in the identification of the pathogenic species, *N. gonorrhoeae* and *N. meningitidis*.

Even though they are not specifically "tests," a frequent source of information for the microbiologist is the ability of a bacteria to grow on specialized media referred to as "selective media" and "differential media." Selective media select the growth of one type of bacteria over another. Typically, they enrich for either Gram-positive or Gram-negative organisms. Colistin and nalidixic acid agar (CNA) is a medium that enriches the growth of Gram-positive organisms over Gram-negative organisms. The antimicrobials, colistin and nalidixic acid, inhibit the growth of Gram-negative but not Gram-positive ones. On the other hand, MacConkey agar (MAC), due to the presence of the Gram-positive inhibitors, bile salts, and crystal violet, enriches the growth of Gram-negative bacilli at the expense of Gram-positive organisms and Gram-negative cocci (*Fig. 4.9*). Differential media provide additional information about the microorganism other than ability to grow or not grow. Sheep blood agar is a differential medium because it allows for the determination of hemolysis. In addition to being a selective medium, MAC is also a differential medium as it contains high levels of lactose and a pH indicator that differentiates colonies that ferment lactose from those that do not (*Fig. 4.10*). Because MAC is both a selective medium as well as a differential one, it is typically referred to as a "selective-differential" medium. The determination of the colonial morphology and ability to ferment lactose is widely used to differentiate Gram-negative bacilli in clinical specimens. For example, *E. coli* is a lactose-fermenting organism with a distinctive colonial morphology (see *Fig. 4.10*) which can be easily differentiated from the non-lactose, pigment-producing *P. aeruginosa* on MAC. Commonly encountered lactose-fermenting Gram-negative bacilli include ***K**lebsiella* spp., ***E**. coli*, and ***E**nterobacter* spp. or KEE (pronounced "key"), while common non-lactose fermenters are "PS" organisms: ***P**roteus*, ***P**rovidencia*, ***P**seudomonas*, ***S**almonella*, ***S**higella*, and ***S**erratia*. Perhaps as many as 90 % of clinically encountered Gram negative will belong to the KEE or PS classification, though discrepancies do exist (e.g., some *E. coli* do not ferment lactose).

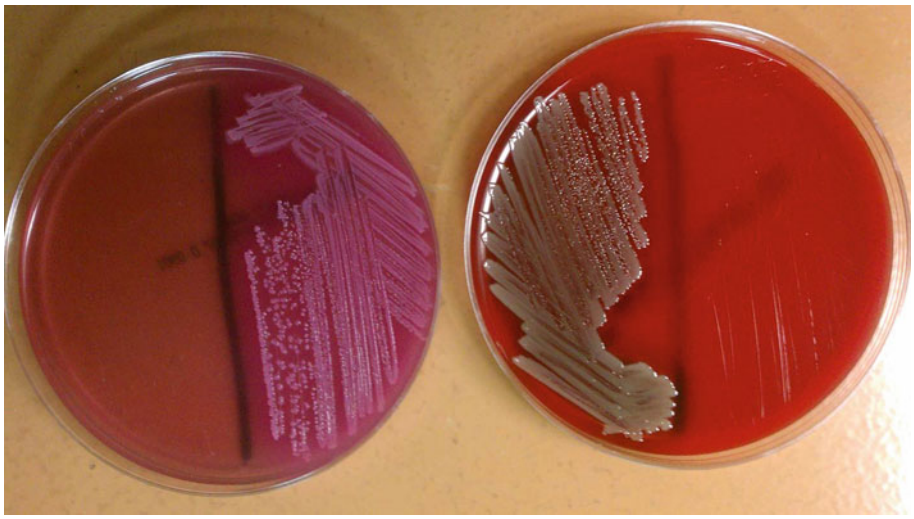


Fig. 4.9
Demonstration of selective media. The MacConkey plate does not permit growth of *Staphylococcus aureus* (*left side of plate*), though it does permit growth of *Escherichia coli* (*right side of plate*). The reverse is shown on CNA media

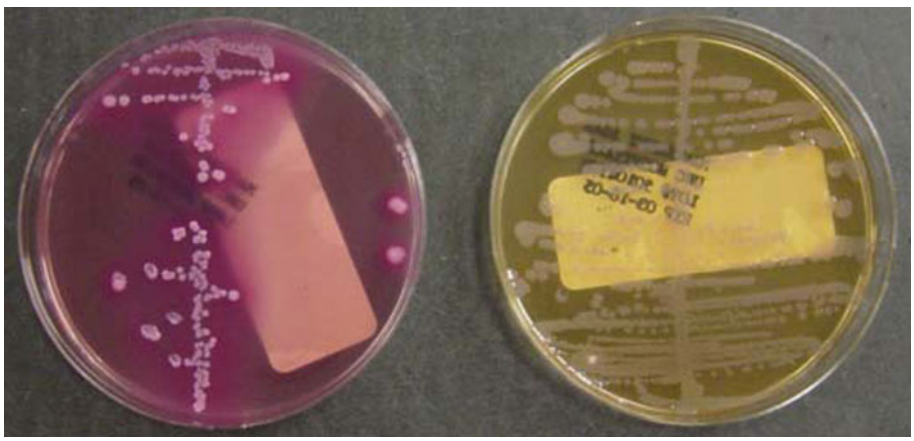


Fig. 4.10
Depiction of growth of organisms that ferment lactose (*left*) versus ones that do not on MacConkey media

Useful Tests for the Identification of Aerobic Gram-Positive Cocci

After obtaining general information regarding the bacteria's identity, typically a more algorithmic approach to narrowing down the precise species that is causing the infection is utilized. There are three important genera of clinically relevant Gram-positive cocci: *Staphylococcus, Streptococcus*, and *Enterococcus*. Typically, *Staphylococcus* spp. are normal inhabitants of human skin, though some species are very capable of causing infection. *Staphylococcus aureus* is the most widely encountered human staphylococcal pathogen. It causes a variety of skin and soft tissue infections that may progress to more serious deep infections. The progression and severity of infections caused by *S. aureus* is dependent upon the variety of virulence factors that may be expressed by individual strains. Other species known to be associated with specific infections include *S. epidermidis*, a cause of infections of foreign bodies including intravascular catheter and prosthetic devices; *Staphylococcus lugdunensis*, an emerging cause of skin and soft tissue infections; and *S. saprophyticus*, which is seen in urinary tract infections (Becker and von Eiff 2011; Frank et al. 2008). *Streptococcus* spp. can be commonly found in small numbers as members of the respiratory tract; however, large numbers of streptococci in the respiratory tract can be indicative of disease. The most common streptococci isolated as pathogens from clinical specimens include group A streptococci (*S. pyogenes*), group B streptococci (*S. agalactiae*), and *Streptococcus pneumoniae* (Spellerberg and Brandt 2011). Formerly considered a group of streptococci, *Enterococcus* is a related genus that is typically found in the gastrointestinal tract but is a cause of bloodstream, wound, and urinary tract infections (Teixeira et al. 2011).

Enterococci are particularly worrisome because of their high natural resistance to many commonly used antibiotics.

As noted above, the Gram stain morphology can provide some clues as to the identity, but the catalase test can accurately differentiate staphylococci from streptococci and enterococci in a matter of seconds. Staphylococci are catalase positive; streptococci and enterococci are catalase negative. For suspected staphylococci, the next test performed is often the coagulase test. The coagulase test checks for the ability of the bacterium to clot plasma (usually rabbit). The coagulase test occurs in two parts. If either of the tests is positive, the bacterium is considered coagulase positive. The first part of the coagulase test is to check for bound or cell-associated coagulase. In this test, a small amount of the organism is mixed on a slide with plasma. If clumping begins to occur, the test is considered positive; if no clumping occurs, a second test is typically performed. The second test checks for free or extracellular coagulase by incubating bacteria in a tube of plasma for 4 h at 37 °C. After 4 h, the tube is checked for the formation of a clot. If no clot is present, the tube is incubated an additional 12–24 h at room temperature before calling negative (Forbes et al. 2007g). Although there are a few staphylococci that are coagulase positive (Boucher et al. 2009), the only one that frequently causes human infections is *Staphylococcus aureus*. Therefore, in lieu of the coagulase test, some laboratories look specifically for the presence of *S. aureus* using a commercially available latex agglutination kit. In this kit, latex beads are coated with plasma as well as antibodies to proteins in the cell wall of *S. aureus*. Bacteria are mixed with the reagent beads, and agglutination occurs in the presence of *S. aureus*, allowing the test to be considered positive (Forbes et al. 2007g). Bacteria that are not coagulase positive by the latex tests are typically grouped together as "coagulase-negative staphylococci" or CNS. Speciation of CNS only occurs for a select group of organisms that are of particular clinical interest. Usually, this identification is the result of a battery of different biochemical reactions and carbohydrate assimilations. One species, *S. saprophyticus*, is able to be presumptively identified on the ability to grow in the presence of novobiocin (Becker and von Eiff 2011). Most CNS are susceptible to novobiocin, so a strain recovered from urine that is capable of growing in the presence of the drug is most likely *S. saprophyticus*. Another species with colony morphology and hemolysis similar to *S. aureus* is *S. lugdunensis* (Fig. 4.11). It is important to differentiate *S. lugdunensis* from other CNS in the clinical microbiology laboratory because *S. aureus* susceptibility breakpoints are used for this organism (Clinical and Laboratory Standards Institute 2011; Frank et al. 2008). Fortunately, this can be achieved via a couple of simple phenotypic tests. Two unique characteristics of *S. lugdunensis* are that it is both PYR as well as ornithine decarboxylase positive (Becker and von Eiff 2011; Forbes et al. 2007g).

If the catalase test on the Gram-positive coccus is negative, the suspected culprit becomes *Streptococcus* or *Enterococcus* spp. Whether a presumptive *Streptococcus* spp. is alpha or beta hemolytic determines the next step in the identification algorithm (Fig. 4.12). The two main groups of beta-hemolytic

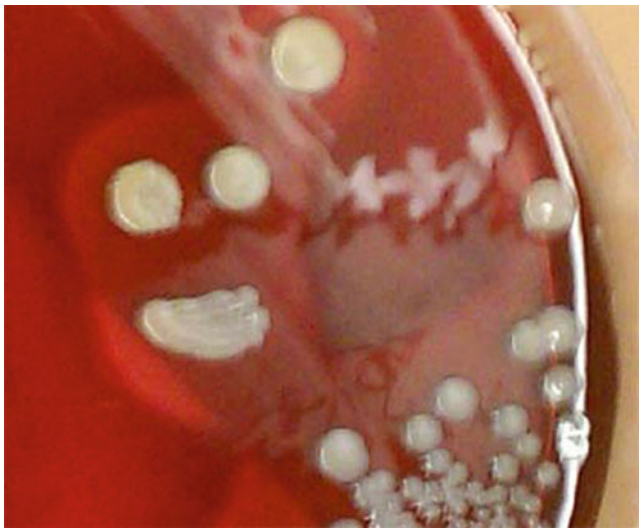


Fig. 4.11
Staphylococcus lugdunensis

streptococci are group A, *S. pyogenes*, and group B, *S. agalactiae*. As discussed previously, the intensity of the beta-hemolytic reaction, weak for group B streptococci and strong for group A streptococci, determines the next step in identification. Two of the tests commonly used to differentiate group A streptococci from other groups are the bacitracin test and the PYR test, though neither test is 100 % specific (Forbes et al. 2007g). Group A streptococci are bacitracin susceptible, and *S. pyogenes* is the only species of beta-hemolytic streptococci that is PYR positive. The bacitracin test is performed by placing a 0.04U bacitracin (A) disk onto a lawn of bacteria on a blood plate. If there is a zone of growth inhibition around the A disk, then the isolate is identified as a presumptive group A streptococcus (GAS) (Fig. 4.13). The isolate is given a presumptive identification because a small percentage (<5 %) of group C and G beta-hemolytic streptococci are also bacitracin susceptible. These groups are encountered less frequently as causes of infection, though they may cause tonsillitis (Llor 2011; Wessels 2011). A rapid spot test called PYR looks for the ability of an organism to produce beta-naphthylamine from L-pyrrolidonyl-beta-naphthylamide. This reaction can be detected by spotting the unidentified organism onto L-pyrrolidonyl-beta-naphthylamide soaked filter paper. A drop of N,N-methyl-aminocinnamaldehyde is added to the organism, and if beta-naphthylamine is present, a reaction occurs which produces a red color. Even though they are infrequently beta hemolytic, enterococci are PYR positive, which needs to be considered in the context of a PYR-positive beta-hemolytic colony. These organisms are difficult to confuse, however, as the intensity of the hemolytic reaction is much less for enterococci compared to GAS.

Group B streptococci are typically identified using the CAMP test. The test looks for the presence of a secreted factor (CAMP) that can augment the hemolysis of staphylococcal beta-lysin. To perform the test, a test organism is streaked perpendicular to a streak of a beta-lysin producing *Staphylococcus aureus* strain on a sheep

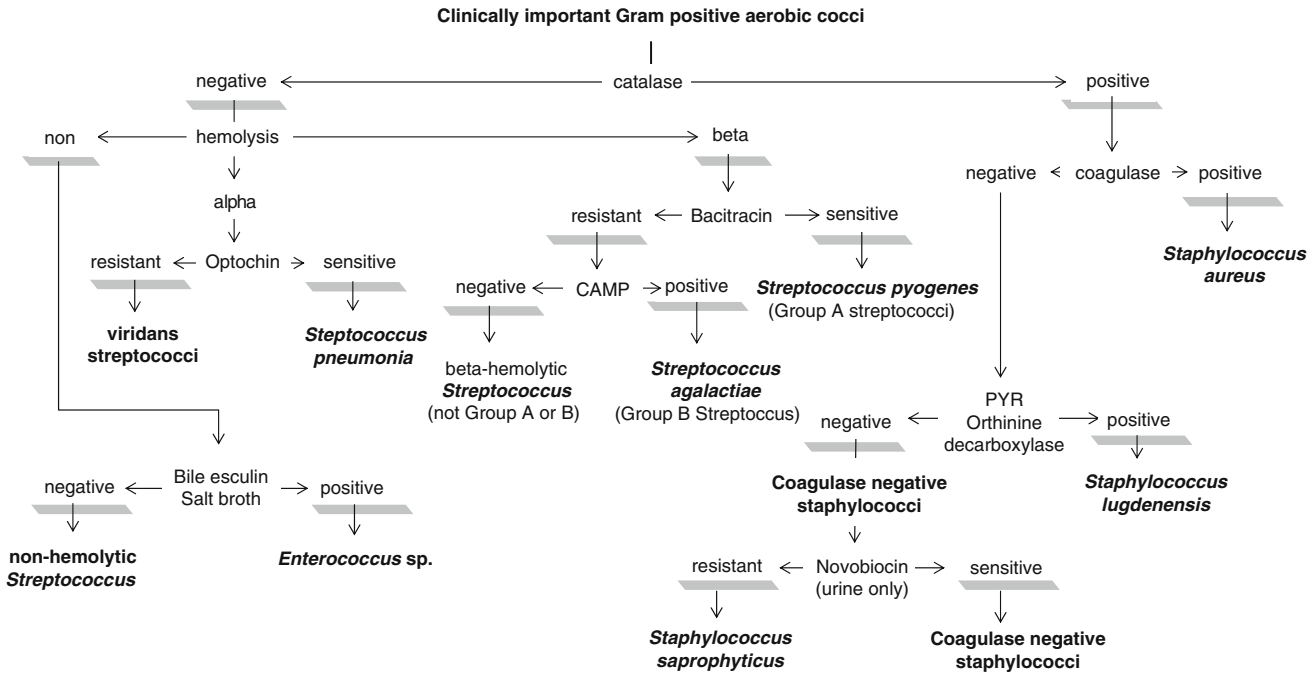


Fig. 4.12
Identification algorithm for clinically important Gram positive cocci

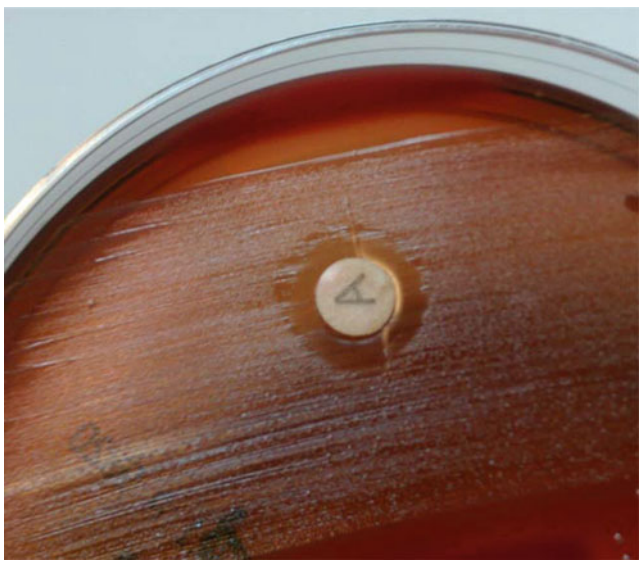


Fig. 4.13
Inhibition of growth of *Streptococcus pyogenes* by bacitracin

Fig. 4.14
Positive CAMP reaction

blood plate and incubated overnight. The plate is then analyzed for the presence of CAMP factor by looking for enhanced hemolysis in the area near where the test organism intersects with the *S. aureus*. The appearance of an arrowhead-shaped area of enhanced hemolysis can presumptively identify the organism as a group B streptococcus (Forbes et al. 2007j) (Fig. 4.14).

The identification scheme for alpha-hemolytic strep is based on the optochin test. The optochin test is similar to the bacitracin as it looks for the ability of an organism to grow in the presence of the compound ethylhydrocupreine hydrochloride (optochin). While most alpha-hemolytic streptococci can grow in the presence of optochin, *S. pneumoniae* cannot (Forbes et al. 2007j). Therefore, similar to a group A streptococcus in a bacitracin test, the organism of interest, *S. pneumoniae*, is identified by an inhibition of growth in the presence of the test compound (Fig. 4.15). Optochin-resistant organisms are classified as viridians streptococci group. These organisms are typically found as members of the oropharyngeal flora, though some members, such as the *S. anginosus* group of viridians streptococci, have been associated with liver, lung, and brain abscesses (Karchmer et al. 2011). The *S. anginosus* group can be differentiated from the other viridians streptococci on the basis

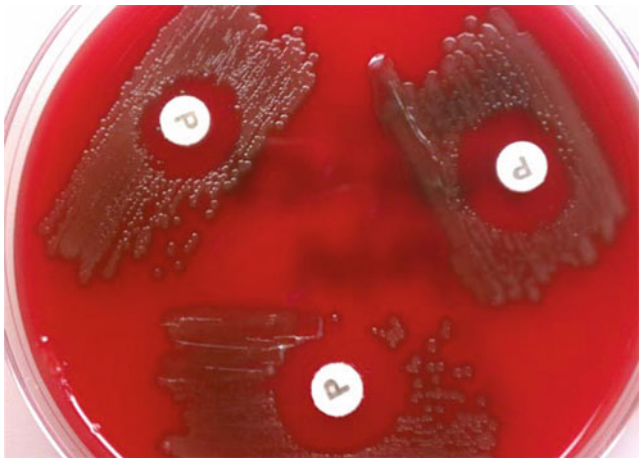


Fig. 4.15
Inhibition of growth of *Streptococcus pneumoniae* by optochin

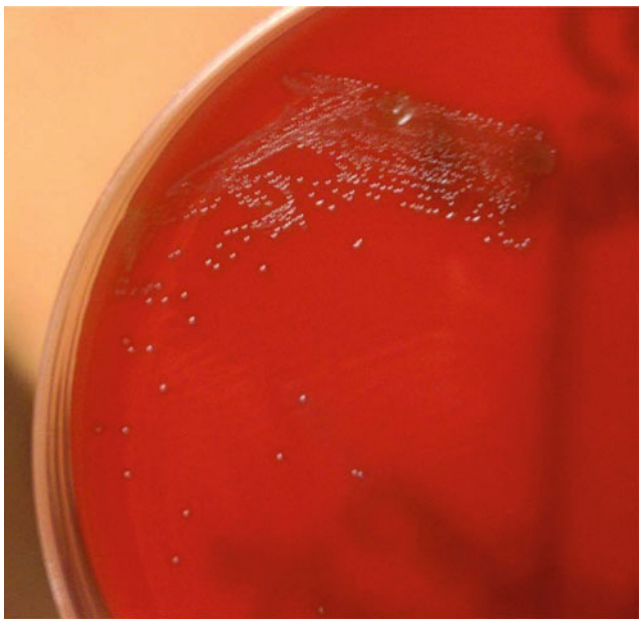


Fig. 4.16
Enterococcus on sheep blood agar

of a distinctive caramel or vanilla odor when the plate is opened and characteristic small, often molar toothlike colonies (Clinical and Laboratory Standards Institute 2008).

Enterococci are typically alpha or gamma hemolytic with reasonably distinctive colonial morphology (*Fig. 4.16*). They are differentiated from other nonhemolytic streptococci by performing a PYR test or a bile esculin and 6.5 % NaCl test (Clinical and Laboratory Standards Institute 2008; Teixeira et al. 2011). Given the appropriate colonial morphology, PYR is a reliable test for identifying enterococci. Alternatively, the combination of bile esculin and 6.5 % NaCl test may be used to identify enterococci to the genus level. The bile esculin test checks for two characteristics, the ability to grow in the presence of high bile concentrations and the capability of hydrolyzing esculin. It is performed by inoculating the bacteria of interest to media containing high concentrations of bile, esculin, and ferric citrate (Teixeira et al. 2011). Enterococci are able to grow on the media as well as hydrolyze the esculin into esculetin which reacts with the ferric citrate to produce a black color and are therefore considered bile esculin positive; group D streptococci are also bile esculin positive; therefore, additional tests are needed to distinguish enterococci. Enterococci will grow in broth with a high salt content (6.5 % NaCl), while group D streptococci will not. Enterococcal speciation requires additional phenotypic characterization. The interested reader is referred to the Manual of Clinical Microbiology or Bailey and Scott's Diagnostic Microbiology for further details (Forbes et al. 2007g, j; Teixeira et al. 2011).

Identification of Aerobic Gram-Positive Bacilli

Unlike the aerobic Gram-positive cocci, most clinically relevant aerobic Gram-positive bacilli can only be presumptively identified to the genus level in the absence of extensive biochemical testing (*Fig. 4.17*). Fortunately, however, this can usually be rapidly achieved via growth characteristics in lieu of phenotypic tests. The more clinically relevant aerobic Gram-positive bacilli include members of the *Bacillus*, *Corynebacterium*, *Listeria*, *Lactobacillus*, and *Nocardia* genera. Although many *Bacillus* spp. rarely cause infection and can be considered environmental contaminants, two species, *B. cereus* and *B. anthracis*, are seen as pathogens. Of these two species, *B. anthracis*, the etiologic agent of anthrax, is a much more serious threat, as it is highly pathogenic in humans. Anthrax tends to be more widely known for its association with bioterrorism (American Society for Microbiology 2010); however, natural reservoirs and infections do exist. Natural infections occur in cases of contact with infected animals, especially animal hides, lending to the name "wool-sorters disease." Anthrax causes a myriad of diseases depending on the route of inoculation; cutaneous, pulmonary, and gastrointestinal are all different forms of disease caused by *B. anthracis*. *B. cereus* is less frequently a cause of severe infection but can be associated with food poisoning. Importantly, *B. cereus* is recognized as an important agent of bacteremia in neutropenic patients especially those with indwelling intravascular devices (Inoue et al. 2010).

Similar to *Bacillus* spp., most *Corynebacterium* spp. are saprophytic. However, some species are human pathogens, including *C. diphtheriae*, the etiologic agent of the respiratory disease diphtheria; *C. ureolyticum*, which is associated with difficult to treat urinary tract infections; and *C. jeikeium*, a lipophilic, multidrug-resistant species associated with intravascular device-related bacteremia (Forbes et al. 2007a).

One of the most clinically important aerobic Gram-positive bacilli to cause disease in the developed world is *Listeria monocytogenes*. Because this organism can replicate at 4 °C, it is usually associated with outbreaks due to contaminated food. Disease which occurs during these outbreaks is typically most

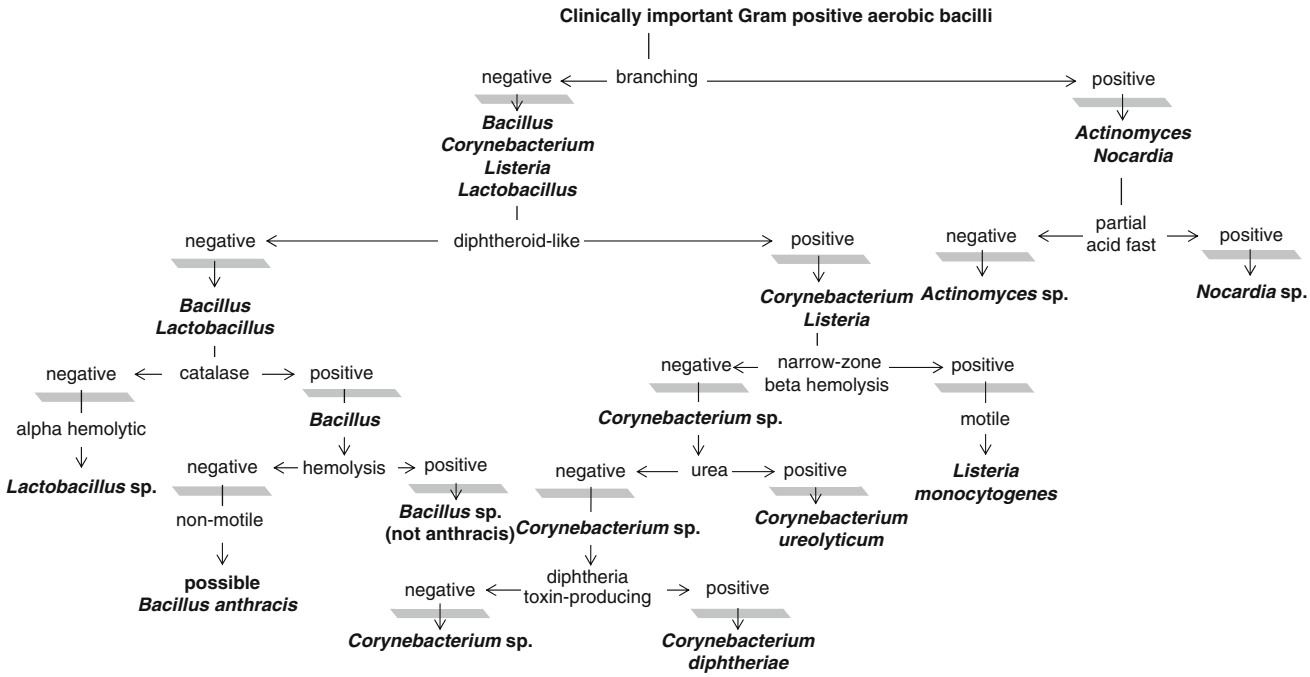


Fig. 4.17
Identification algorithm for clinically important Gram positive aerobic bacilli

severe in immunosuppressed populations including newborns and pregnant women (Centers for Disease Control and Prevention 2011a; Forbes et al. 2007a).

Lactobacillus spp. are facultative, frequently curved Gram-positive rods. These organisms will most frequently be seen in poorly collected urine specimens from females. Lactobacilli make up an important part of the protective microbiome in the lower female genital tract, and imbalance in the amounts of lactobacilli there can result in bacterial vaginosis (Lamont et al. 2011). It is also often part of over-the-counter probiotic mixtures or can be found in large quantities in yogurt.

Finally, *Nocardia* spp. are part of a group of aerobic Gram-positive bacilli known as the aerobic actinomycetes. These bacteria share the common characteristic of forming elongated, branching structures that resemble fungal hyphae. *Nocardia* spp. are usually seen in skin infections of the immunocompetent host and can be causative agents of mycetomas, skin abscesses, and cellulitis. *N. brasiliensis* is the most likely culprit behind these infections. *N. asteroides* complex organisms such as *N. nova, N. asteroides, N. cyriacigeorgica*, and the drug-resistant *N. farcinica* are frequently associated with invasive pulmonary and central nervous system infections of immunocompromised individuals (Forbes et al. 2007i; Sorrell et al. 2010).

As mentioned previously, absolute identification of the aerobic Gram-positive bacilli usually requires extensive biochemical identification. However, due to the low number of organisms that cause diseases, presumptive identifications can be made on the basis of simple growth characteristics. For example, *Bacillus* spp. can be identified by the presence of spores within the Gram-positive bacilli when visualized via Gram stain. Then,

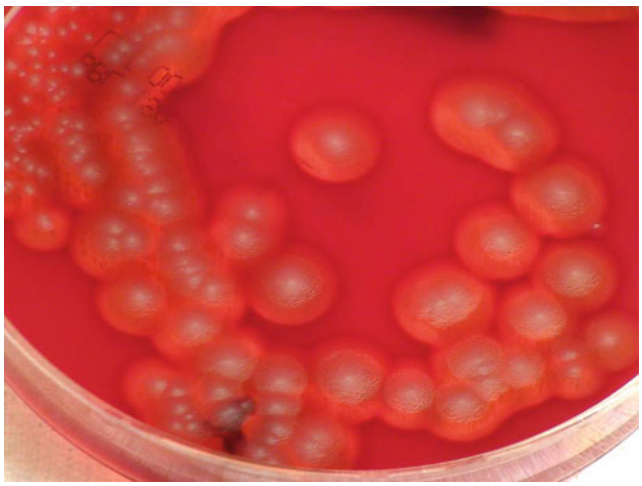


Fig. 4.18
***Bacillus cereus* on sheep blood agar (image courtesy of CDC)**

B. anthracis can be differentiated from other *Bacillus* spp. by a characteristic lack of hemolysis on sheep blood as well as being nonmotile (American Society for Microbiology 2010). *B. cereus* can be presumptively identified on the basis of a strong hemolytic reaction and characteristic colonial morphology (*Fig. 4.18*). *C. diphtheriae* is usually differentiated from other respiratory flora via its ability to grow on media containing tellurite (e.g., cystine-tellurite blood agar and/or Tinsdale's medium) (Meyer and Reboli 2010). *C. ureolyticum* is recovered in high numbers in a urine culture, has a typical diphtheroid-like

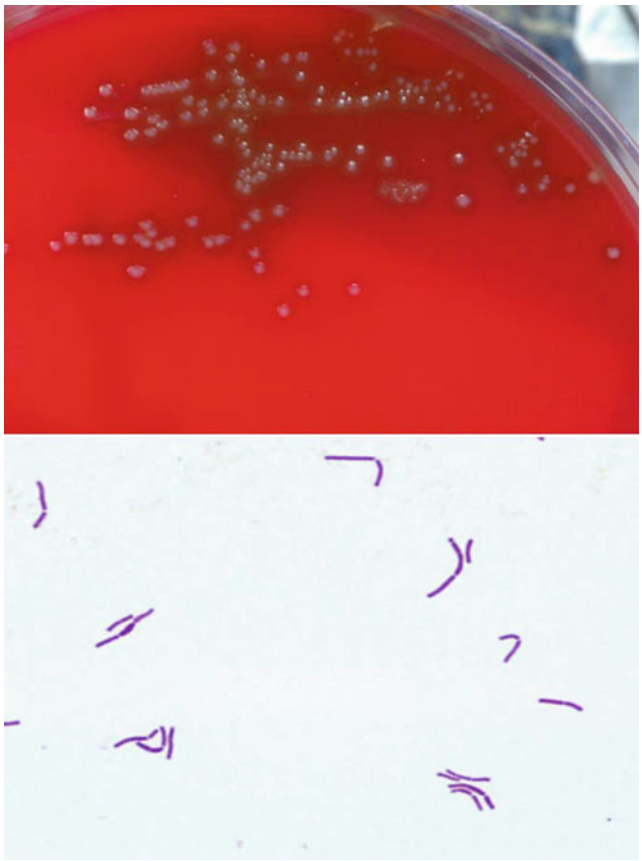


Fig. 4.19
(*Top*) Lactobacillus on sheep blood agar and Gram stain (*bottom*)

Gram stain morphology, and is a potent urea splitter. *C. jeikeium* is a lipophilic organism that produces small colonies on most commonly used media and is highly drug resistant only being reliably susceptible to vancomycin (Johnson et al. 2003; Meyer and Reboli 2010). *Listeria monocytogenes* has similar morphology to group B streptococci both on Gram stain, Gram-positive coccobacilli that can easily be confused with the diplococci of group B streptococci and a grayish white, entire colony which is weakly beta hemolytic, which again is also characteristic of Group B streptococci. These two organisms can be differentiated on the basis of a catalase test (*L. monocytogenes* is positive; group B streptococci, negative), and *L. monocytogenes* has a characteristic tumbling motility that can be visualized on a wet mount or, when inoculated to motility medium, demonstrates umbrella motility (Forbes et al. 2007a; Meyer and Reboli 2010).

Three phenotypic characteristics are all that is needed to identify isolates as belonging to the genus *Lactobacillus*. Isolates that are Gram-positive rods and that may be curved, alpha hemolytic on blood agar, and catalase negative are reliably identified as *Lactobacillus* spp. (*Fig. 4.19*). Speciation is rarely needed as lactobacilli are not a common human pathogen, but when required it will be accomplished by molecular means.

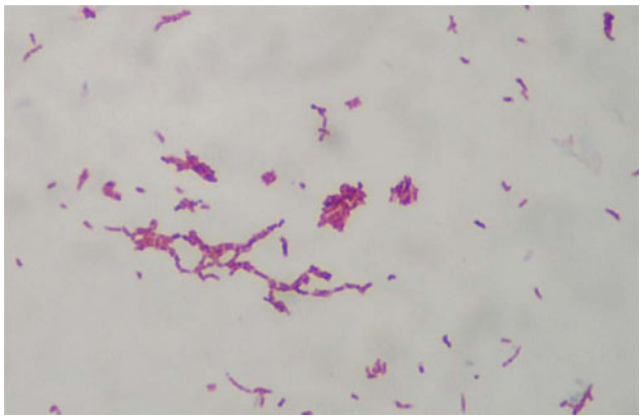


Fig. 4.20
Acid-fast stain of *Nocardia* showing partial acid-fastness

Finally, *Nocardia* spp. have two major microscopic characteristics that differentiate them from the other common aerobic Gram-positive bacilli. The first is their ability to form hyphal-like branching structures. The second feature is related to the composition of their cell wall which allows them to stain positive using acid-fast staining techniques. Acid-fast staining refers to the ability of a bacterium to retain stain in the presence of an acidified alcohol decolorizer. *Nocardia* are considered partially acid fast (Forbes et al. 2007i; Sorrell et al. 2010). The decolorizing agent in an acid-fast stain contains 3 % hydrochloric acid dissolved in ethanol. *Nocardia* are actually capable of retaining stain in the presence of acid, but not in the presence of alcohol. To prove an organism is *Nocardia*, a modified acid-fast stain is performed where the alcohol-acid decolorizer is replaced by a dilute acid solution (*Fig. 4.20*).

Identification of Gram-Negative Cocci

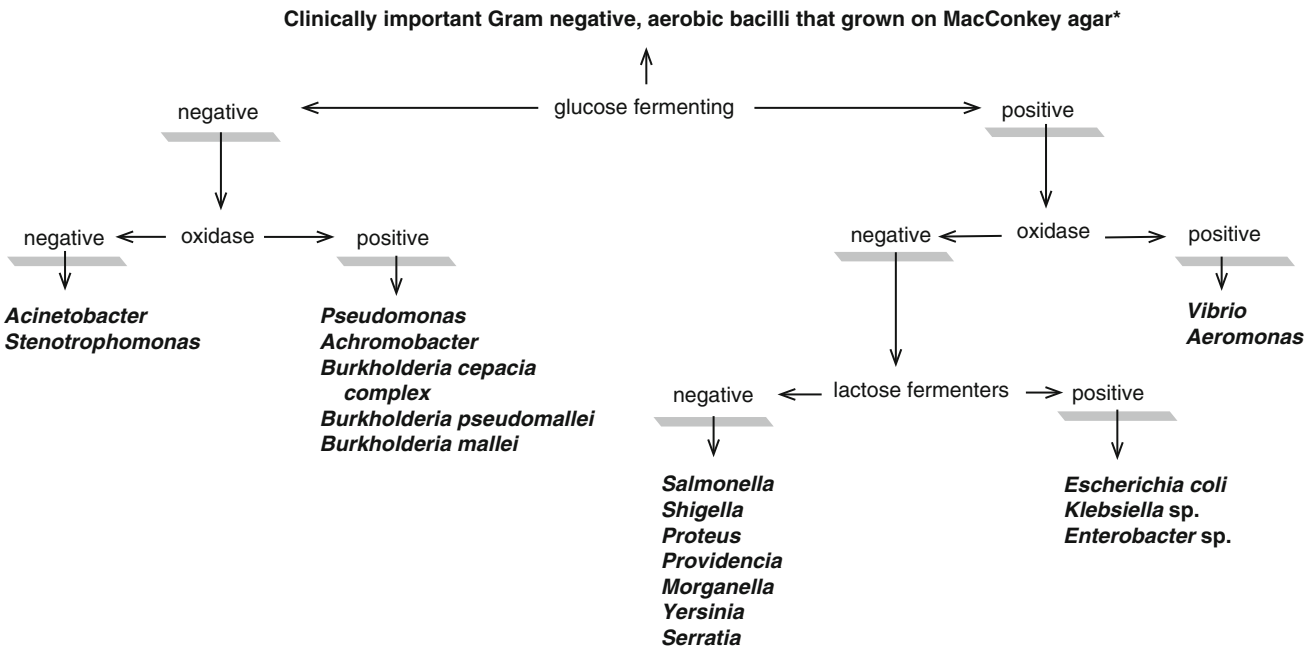
The most frequently encountered clinically relevant Gram-negative cocci are *Neisseria gonorrhoeae*, the causative agent of gonorrhea; *N. meningitidis*, a leading cause of bacterial meningitis; and *Moraxella catarrhalis*, an important cause of upper respiratory infections including otitis media and conjunctivitis. When it comes to Gram-negative cocci, the identification of the organism as a true Gram-negative coccus is the most important step. Following Gram stain, an oxidase test is performed since all clinically relevant Gram-negative cocci are oxidase positive. A negative oxidase test on Gram-negative cocci may indicate the organism is *Acinetobacter*, an important cause of a variety of nosocomial infections including bacteremia. Acinetobacter is typically thought of as a short Gram-negative bacillus but may appear as a Gram-negative diplococcus (Peleg et al. 2008). *Moraxella* is easily differentiated from the pathogenic *Neisseria* on the basis of a positive "hockey puck" test. In this test, a wooden stick, the "hockey stick," can be used to move an isolated colony along the surface of the agar as if it were a "hockey puck". Pathogenic *Neisseria* colonies will not move

along the surface of the plate. Additionally, *Moraxella catarrhalis* fails to oxidize glucose while both pathogenic *Neisseria* will. Differentiation of the pathogenic *Neisseria* is based on carbohydrate utilization or more modern nucleic acid techniques. *N. meningitidis* oxidizes both glucose and maltose, while *N. gonorrhoeae* oxidizes only glucose (Elias and Vogel 2011).

Identification of Aerobic Gram-Negative Bacilli

Perhaps the most extensive group of organisms to identify using simple tests is the Gram-negative bacilli. This is mainly due to the large number of organisms that are clinically relevant that need to be differentiated from one another. These organisms can be separated into groups based on similar characteristics (Fig. 4.21). For example, there is a large family of organisms called the *Enterobacteriaceae* that share common characteristics such as facultative growth, a negative oxidase reaction, and the ability to ferment glucose (Forbes et al. 2007e). Many of the species within this family are members of the indigenous intestinal microflora including *Citrobacter, Enterobacter, Klebsiella, Morganella, Proteus, Providencia*, and *Serratia* species, as well as many strains of *Escherichia coli*. *Salmonella, Yersinia*, and *Shigella* as well as selected strains of *Escherichia coli* are pathogens rather than commensals in the gastrointestinal tract causing diarrheal disease. It is important to note that many of the "normal" flora *Enterobacteriaceae* can also be considered pathogens in sites other than the gastrointestinal tract (e.g., the urinary tract).

Another large group of clinically important Gram-negative bacilli are commonly referred to as the glucose non-fermenters. The glucose non-fermenters are aerobic environmental Gram-negative bacilli which oxidize rather than ferment glucose or produce energy by other means. These organisms tend to have very large genomes as compared to more highly evolved human pathogens and commensals. This allows them to readily adapt to a variety of hostile environments making them ideal health-care-associated pathogens. Two glucose non-fermenters which are viewed as community acquired rather than health care associated are *Burkholderia pseudomallei* and *Burkholderia mallei* (American Society for Microbiology 2008). On the other hand, clinically relevant glucose-fermenting health-care-associated pathogens include *Pseudomonas aeruginosa, Acinetobacter baumannii, Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* (Boucher et al. 2009; Jones 2010; LiPuma 2010; Peleg et al. 2008). These organisms are most frequently recovered from patients with ventilator-associated pneumonia, surgical site infections especially in burn patients, catheter-related urinary tract infections, and intravascular catheter-related bacteremia. Occasionally, these organisms can be obtained outside the health-care setting such as in cystic fibrosis patients developing pulmonary infections due to *P. aeruginosa* or members of the *B. cepacia* complex, but the vast majority of infections due to these organisms are obtained in



*Extensive phenotypic characterization is needed to separate to genus, complex or species level.

Fig. 4.21
Identification algorithm for clinically important Gram negative, aerobic bacilli that grow on MacConkey agar

health-care settings (Boucher et al. 2009; Jones 2010; LiPuma 2010).

A third group of clinically relevant aerobic Gram-negative organisms are the oxidase-positive glucose-fermenting organisms. This includes the genera *Vibrio, Aeromonas, and Pasteurella*. Clinically relevant species include *V. cholerae* (etiologic agent of cholera), *V. parahaemolyticus* (bloody diarrhea associated with ingestion of contaminated shellfish), *Vibrio vulnificus* (wound infections/septicemia especially in patients with liver disease), *Aeromonas hydrophila* (diarrheal disease and wound infections), and *Pasteurella multocida* (bite wounds following cat (common) or dog (less common) bites) (Forbes et al. 2007l; Horneman and Ali 2011).

Identification of these three groups of Gram-negative bacilli has traditionally been done by using a variety of biochemical and carbohydrate reactions (Forbes et al. 2007c; Forbes et al. 2007e). These organisms all grow on MacConkey agar (Fig. 4.21). A useful starting point in the identification of these three groups is to determine an isolate's reaction on a triple sugar iron or TSI slant (Forbes et al. 2007j). This medium slant contains one part glucose and 10 parts of sucrose and lactose as carbohydrate sources with a phenol red indicator which can detect fermentation of the sugars. In order for the indicator to work, the medium is initially buffered at an alkaline pH around 7.4. If an organism is capable of fermenting the sugars, the medium becomes more acidic and changes from red to yellow at pH 6.8. The medium also contains the chemicals ferrous sulfate and sodium thiosulfate allowing for the detection of hydrogen sulfide. Taken together, reactions that are determined are the color of the slant over the color of the butt as well as hydrogen sulfide production. For example, if the bacteria cannot ferment any of the sugars in the medium, the result would be alkaline/no change (K/N) because the medium would not change from its original pH of 7.4 (Forbes et al. 2007j). This result is observed with glucose non-fermenters, and the organism is not a member of the *Enterobacteriaceae*, since all members of the family have the ability to at least ferment glucose as do the oxidase-positive genera *Vibrio, Aeromonas*, and *Pasteurella*. If bacteria that can only ferment glucose are inoculated, the result is alkaline/acid (K/A) with or without H_2S production. It is important to note that this is the reaction result after 18–24 h incubation. Earlier time points may provide false acid/acid results as the glucose is quickly fermented. At later time points, the bacteria have to resort to oxidizing the peptones in the medium, creating alkaline by-products and raising the pH. This turns the slant portion of the medium back to alkaline; however, the butt portion of the medium remains acidic because oxidation does not occur under the anaerobic conditions seen in the butt. The final fermentation reaction is acid/acid (A/A), with or without H_2S, which is the result of bacteria that are capable of fermenting either the lactose or the sucrose in the medium after utilizing all of the glucose. This causes the medium to remain acidic throughout since the amount of acid produced when the large quantities of sucrose or lactose are fermented far exceeds the ability to neutralize as a result of later oxidation of peptones. The other key indicator in the TSI slant is the H_2S indicator. When bacteria that can generate H_2S are placed in an acidic environment with sodium thiosulfate, H_2S gas is released as a by-product. This gas then reacts with the ferric ions from the ferrous sulfate to form ferrous sulfide which is a black precipitate. The different TSI reactions begin to separate different bacteria from one another and usually serve as the first level of an algorithmic approach to identification (Fig. 4.22).

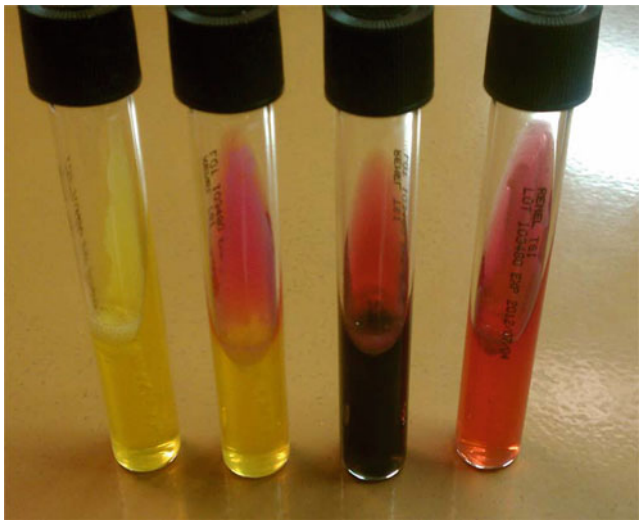


Fig. 4.22
TSI reactions. *From left*: acid/acid, alkaline/acid, alkaline/acid with hydrogen sulfide, and alkaline/no change

Although the TSI reactions are a good starting point for the identification of these three groups of organisms, a number of other tests are useful for the identification of *Enterobacteriaceae*. Following are a description of a limited number of tests useful in abbreviated schemes for the identification of these three groups of organisms. For more extensive discussion of phenotypic identification of these three groups of organisms, the reader is referred to either the Manual of Clinical Microbiology or Bailey and Scott's Diagnostic Microbiology (Forbes et al. 2007b, e, g, j; Horneman and Ali 2011; Nataro et al. 2011).

A particularly useful test in the CLSI abbreviated identification scheme (Clinical and Laboratory Standards Institute 2008) is the spot indole test. In this test, the ability of organism to deaminate tryptophan to the intermediate indole is determined by smearing a colony from a blood-containing or chocolate agar plate on the surface of a piece of filter paper that has been soaked with para-dimethylaminobenzaldehyde which gives a purple-red color when positive or para-dimethylaminocinnamaldehyde which gives a blue color when positive (Forbes et al. 2007j). Oxidase negative, lactose fermenters with appropriate colony morphology on MacConkey agar and a positive spot indole test can be identified as *E. coli* (Clinical and Laboratory Standards Institute 2008). Additionally, spot indole can be used to differentiate swarming *Proteus. P. mirabilis* is spot indole negative, while *P. vulgaris* is spot indole positive (Clinical and Laboratory Standards Institute 2008). *P. multocida* can also be identified with great accuracy based in part on a positive spot indole test (Forbes et al. 2007k). Isolates from animal bite

wounds, typically wounds on the hands, arms, legs, and feet which are oxidase positive, fail to grow on MacConkey agar, are K/K on TSI, and are spot indole positive, have a high likelihood of being *P. multocida* and can be identified as such (Zurlo 2010).

Motility can be a useful test when screening fecal specimens for potential pathogens as *Shigella* and *Yersinia enterocolitica* will be both nonmotile when incubated at 35–37 °C (Forbes et al. 2007f, g). A variety of semisolid media exist to detect motility (Nataro et al. 2011; Schriefer and Petersen 2011). Motile strains are capable of diffusing through the media, whereas nonmotile strains are restricted to where they are inoculated. *Shigella* is a non-lactose fermenter on MacConkey agar and will give a K/N reaction with no H_2S production on TSI slant. An isolate from a fecal specimen which is a nonmotile, non-lactose fermenter that is spot indole negative has a high likelihood of being a *Shigella*. These simple tests are useful in developing algorithms for detecting these pathogens in fecal specimens. This approach is commonly used to determine the identification of many isolates encountered in the clinical laboratory. Many of the biochemical tests have been miniaturized into commercial automated testing systems which utilize complex algorithms based on the biochemical reactions on different substrates that different species produce. These commercial systems such as VITEK (bioMerieux, Durham, NC), MicroScan (Siemens, Deerfield, IL), and Phoenix (BD Micro Systems, Cockeysville, MD) are widely used in clinical settings and give highly accurate identifications for commonly recovered Gram-negative bacilli (Forbes et al. 2007e).

Vibrio and *Aeromonas* are both oxidase positive, glucose fermenters that will grow on MacConkey agar. Additionally, *Aeromonas hydrophila*, the most commonly encountered *Aeromonas* species, is beta hemolytic on sheep blood plates and ampicillin resistant. Placing a 20 ug ampicillin disk on a sheep blood agar plated is a useful isolation strategy when attempting to recover this species from stool specimens or wound specimens likely to have multiple organisms present (Forbes et al. 2007l). Detection of resistance to the vibriostatic agent O/129 will differentiate *A. hydrophila* from the *Vibrio* species. Another useful strategy for differentiating *Aeromonas* and *Vibrio* spp. is the string test. In this test, organisms are suspended in 0.5 % sodium deoxycholate. If the organism is a *Vibrio*, it will be lysed and DNA will be released from the organism. If a loop is placed in the surface of the suspension and slowly raised, a "string" will form indicating a positive test (Forbes et al. 2007l). All *Vibrio* spp. are string test positive, while *Aeromonas*, the genus most closely related to the *Vibrio* genus, is string test negative (Fig. 4.23).

Vibrio species are typically recovered from stool specimens using a specific isolation medium, thiosulfate citrate bile salts sucrose or TCBS agar designed specifically for this purpose. *Vibrio cholerae* will form yellow to orange colonies on this medium; *V. parahaemolyticus*, on the other hand, will produce blue or blue-green colonies on this medium. *V. vulnificus* is typically recovered from either wound specimens or occasionally the blood, so TCBS medium is not typically used in its isolation (Forbes et al. 2007l).

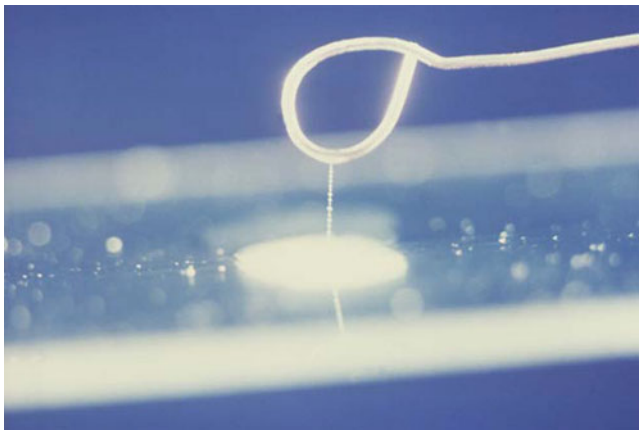


Fig. 4.23
String test (image courtesy of CDC)

There is a large group of organisms including *Pseudomonas* and *Burkholderia* spp. that do not ferment glucose; they either oxidize the sugar or do not use it at all. TSI reactions for these organisms are K/N. Whether these organisms can oxidize glucose or other sugars can be determined by performing oxidation-fermentation (OF) carbohydrate tests. These tests are performed by inoculating two identical tubes with peptones and glucose as the sole carbon source. One tube is left alone, allowing it access to oxygen in the air (oxidation), while the other tube is overlaid with mineral oil to prevent access to oxygen (fermentation). A pH indicator is included in the medium to allow for easy visualization of carbohydrate utilization. When the carbohydrate is utilized, the medium acidifies changing the color of the pH indicator. If the organism is a fermenter, both tubes will change color. If the bacteria in question cannot ferment the sugar, then only the tube that is exposed to air will change color. Finally, if the organism is unable to utilize the carbohydrate, then neither tube will change color. These organisms are then differentiated from one another using a battery of biochemical reactions similar to those used to distinguish the *Enterobacteriaceae* from one another (Forbes et al. 2007j). Commercial systems used to identify *Enterobacteriaceae* have been modified so that they can identify glucose non-fermenters as well, although the accuracy of identifying glucose non-fermenters by these systems is not as robust as that for the *Enterobacteriaceae* (Kiska et al. 1996). Unlike the *Enterobacteriaceae*, the glucose non-fermenters are not typically part of the human microflora but rather are common in the environment. Therefore, it is important to identify them within a clinical context in the laboratory. The most commonly isolated, clinically important, non-fermenting Gram-negative bacillus is *Pseudomonas aeruginosa*. Rapid identification schemes are readily applicable to *P. aeruginosa*. The organism is oxidase positive, motile, and gives a TSI reaction of K/N. On sheep blood agar, *P. aeruginosa* is often strongly beta hemolytic and can produce different diffusible pigments such as pyocyanin. These diffusible pigments may give the organism a characteristic green metallic sheen when grown on blood

Fig. 4.24
Mucoid *Pseudomonas aeruginosa*

agar. A final helpful attribute is a characteristic "grapey" or "corn taco" odor produced by the culture (Clinical and Laboratory Standards Institute 2008). When all these characteristics are present, the organism can reliably be identified as *P. aeruginosa*. Some isolates of *P. aeruginosa* may be poor pigment producers; they typically form mucoid colonies where the organisms are growing within an alginate matrix. These mucoid colonies (Fig. 4.24) are characteristic of *P. aeruginosa* causing chronic lung infections in patients with cystic fibrosis.

Achromobacter xylosoxidans is another important glucose non-fermenting, oxidase positive, motile nosocomial pathogen. Importantly, the organism oxidizes xylose more strongly than glucose. These test findings are suggestive of this organism being *A. xylosoxidans*, but confirmation using commercial identification systems or 16 S rRNA sequencing is recommended.

Two oxidase-negative, glucose non-fermenters commonly encountered clinically causing health-care-associated infections are *Acinetobacter* spp. and *Stenotrophomonas maltophilia*. *Acinetobacter* has a distinctive coccobacilli Gram stain and is nonmotile which may aid in its preliminary identification. Otherwise, identification can be readily accomplished to the genus level with commercial phenotypic identification systems (Forbes et al. 2007b). Accurate means of identification to the species levels by phenotypic means is less reliable but is probably not of great clinical significance. *S. maltophilia* is reported to be oxidase negative although a recent report suggests that as many as 20 % may be oxidase positive (Carmody et al. 2010). *S. maltophilia* isolates are K/N on TSI, oxidize maltose, and glucose and produce the enzyme DNase. This simple scheme will reliably identify this important health-care-associated pathogen.

Important human pathogens within the genus *Burkholderia* include *B. pseudomallei, B. mallei*, and members of the *B. cepacia* complex. *B. pseudomallei* can cause an acute, fulminant pulmonary infection as well as chronic infections that mimic tuberculosis. It is an agent found primarily in tropical regions especially in rice growing areas (American Society for Microbiology 2008). *B. mallei* is a zoonotic agent which primarily causes infection in equine species but can be spread to humans (American Society for Microbiology 2008). Both organisms have been recognized as potential agents of bioterrorism, and algorithms for "ruling out" these organisms in clinical specimens are readily available (American Society for Microbiology 2008). Their identification is not something that is routinely attempted in the clinical laboratories in the North America or Europe although *B. pseudomallei* is common enough in Southeast Asia and Australia to warrant special attention there.

B. cepacia complex is made up of 17 different species, some of which are associated with chronic lung infection in cystic fibrosis patients. *B. cenocepacia, B. multivorans*, and *B. dolosa* are the species most commonly associated with these infections (LiPuma 2010). Special isolation media, all of which contain colistin or the closely related antimicrobial, polymixin, are needed to reliably recover this group of organisms from CF respiratory specimens. Isolates that grow on these media should be transferred to nonselective media, preferably one containing blood or hemin, so a reliable oxidase test can be performed. These organisms are oxidase-positive, motile, glucose non-fermenters with a characteristic musty odor. Commercial systems cannot differentiate these species, and so the only reliable means of speciating organisms with these characteristics—growth on *B. cepacia* selective and MacConkey agar; motile, oxidase-positive, K/N on TSI (some species have yellow pigment on this medium); and musty odor on blood agar—is to do sequence analysis. The initial sequence analysis is done using 16 S rRNA. This will accurately identify the organism to the *B. cepacia* complex. Differentiation of different species requires partial sequencing of the *recA* gene (Cesarini et al. 2009).

The fourth group of facultative, Gram-negative bacilli is not easily classified on the basis of phenotypic characteristics and is usually not identified using a battery of biochemical tests as the first three groups of organisms are. This group includes the fastidious Gram-negative rods which have special growth requirements and/or grow slowly (Fig. 4.25). Unlike the first three groups of organisms, these organisms will not grow on MacConkey agar. The more fastidious organisms in this group are not identified by commercial identification systems. Organisms represented in this group include *Haemophilus influenzae, Haemophilus ducreyi*, the HACEK organisms (***H****aemophilus parainfluenzae*, ***A****ggregatibacter actinomycetemcomitans, A. aphrophilus*, ***C****ardiobacterium spp.*, ***E****ikenella corrodens*, and ***K****ingella kingae*), *Bartonella* spp., *Brucella, Francisella*, and *Capnocytophaga*. *Haemophilus influenzae* is a common cause of upper respiratory tract infections including otitis media, sinusitis, and conjunctivitis. It used to be the most common cause of childhood bacterial meningitis in the industrialized world, but with the widespread use of conjugated *H. influenzae* type b vaccine, that infection has essentially disappeared (MacNeil et al. 2011). *H. ducreyi* is the etiologic agent of the sexually transmitted infection, chancroid, a painful ulcerative disease of the genitalia. The HACEK organisms are commensals of the oropharynx. In patients with heart valve abnormalities, transient bacteremia following tooth brushing or flossing may result in endocarditis with these organisms (Brouqui and Raoult 2001). Other prominent infections with these organisms include infections of the hands due to *Eikenella* secondary to closed fist

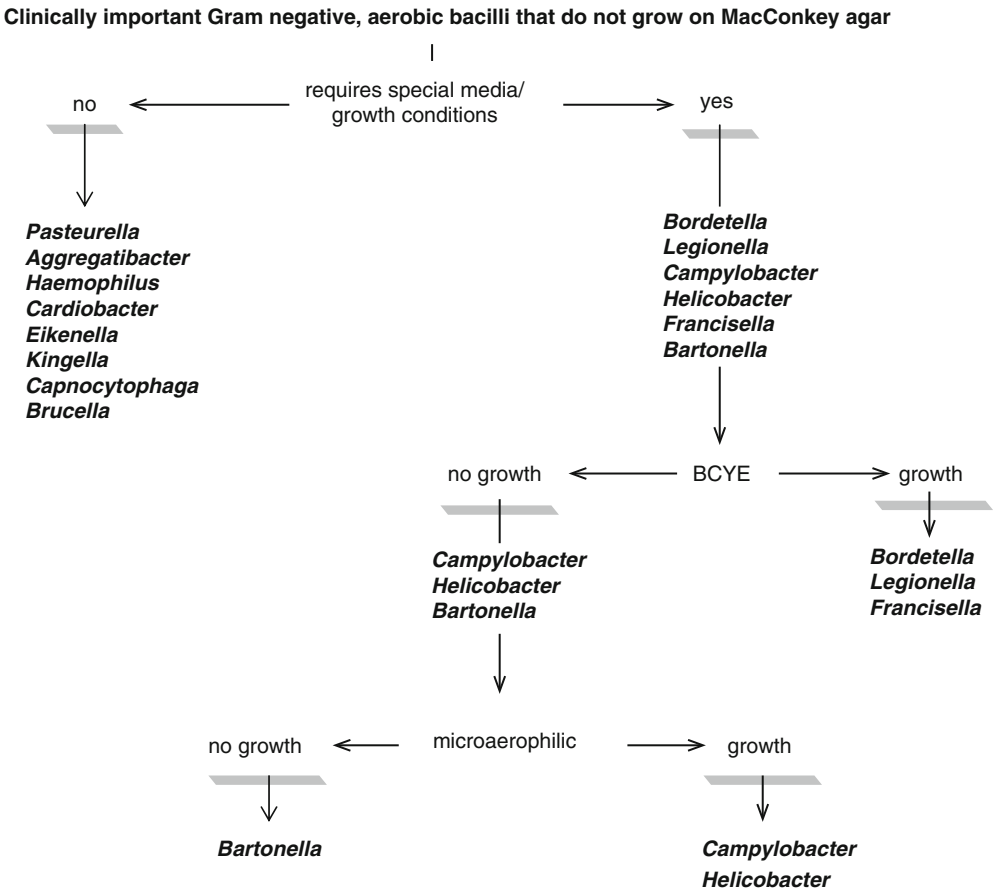


Fig. 4.25
Identification algorithm for clinically important Gram negative bacilli that do not grow on MacConkey agar

injuries and septic arthritis in children due to *Kingella* (Dubnov-Raz et al. 2008). *Bartonella* can cause bacteremia in HIV and transplant patients as well as a lymphadenitis referred to as cat scratch disease primarily in children (Eremeeva et al. 2007). Both *Brucella* and *Francisella* are zoonotic infections (American Society for Microbiology 2004; Feldman et al. 2001). *Brucella* causes a chronic indolent, febrile illness with many of the same clinical characteristics as tuberculosis including fatigue, night sweats, intermittent fevers, and weight loss (American Society for Microbiology 2004). *Francisella* causes a variety of acute and chronic infection depending upon the manner in which the organism is obtained. A recent outbreak of disseminated disease was likely due to inhalation of the organisms perhaps from the dead carcasses of animals (Feldman et al. 2001). The organism may also be spread from other mammals via tick bites. Although neither has a particularly high attributed mortality, both have been weaponized and are considered to be select agents by the United States government (Chang et al. 2003). Two species of *Capnocytophaga* are clinically important. *C. ochracea* which is part of the indigenous microflora of the human mouth has been associated with bacteremia in neutropenic host with poor dentition (Kristensen et al. 1995). *C. canimorsus* is a member of the indigenous oral microflora of canines. It can be associated with fulminant sepsis especially in those with cirrhosis of the liver, other iron overload states, and splenectomy (Janda et al. 2006; Low and Greenwood 2008).

Campylobacter and *Helicobacter* are microaerophilic organisms that infect the gastrointestineal tract causing either diarrheal disease (*Campylobacter*) or peptic ulcer disease and gastric cancers (*Helicobacter*). Finally, two respiratory tract pathogens, *Bordetella* and *Legionella*, grow best on medium that contains charcoal with Regan-Lowe agar favored for the growth of *Bordetella* and buffered charcoal yeast extract (BCYE) agar for *Legionella*. Neither of these organisms will grow on common laboratory medium such as sheep blood agar (*Fig. 4.25*). *Bordetella pertussis* is the etiologic agent of whooping cough, a disease whose incidence waxes and wanes in the industrialized world depending upon the percentage of children who are vaccinated against this organism (Rohani and Drake 2011). Two other species of *Bordetella*, *B. parapertussis* and *B. bronchiseptica*, can also cause a milder version of whooping cough. *Legionella pneumophila* is an agent referred to as an "atypical" cause of community-acquired pneumonia. It is free living in the environment and is obtained by inhalation. It is most common in immunocompromised patients (Newton et al. 2010).

Identification of these groups of organisms is driven in large part by Gram stain reaction and morphology, colony morphology, and growth characteristics. Spot test such as catalase and oxidase may be useful for selected organisms within this group. *Haemophilus* spp. commonly encountered in clinical specimens can be identified based on Gram stain reaction and morphology, growth requirement, and hemolytic reactions on horse blood agar. All haemophili are Gram-negative coccobacilli (Fig. 4.26). *Haemophilus* does not grow on SBA but will show "satellite" grow along a streak of *S. aureus*. To demonstrate satelliting, a suspension of the unknown bacterial is struck on the surface of the agar, and a staph streak is then added. If pinpoint colonies grow only along the staph streak, the organism is in all likelihood a *Haemophilus* species. The delta-aminolevulinic or ALA test is used to determine if the organism has the enzymes required to make porphyrins from the ALA reagent. *H. influenzae* and *H. haemolyticus* do not, a negative test, while *H. parainfluenzae* and *H. parahaemolyticus* can, a positive test (Forbes et al. 2007f, j). *H. influenzae* and *H. parainfluenzae* can be differentiated from their hemolytic counterpart based on hemolysis on rabbit or horse blood agar plates or if unavailable based on the hemolytic reaction around the colonies on the staph streak plate if that is discernable. If an organism is recovered from a genital tract specimen that is consistent with a *Haemophilus* species based on Gram stain and colony morphology but does not show satelliting on a staph streak, that isolate could be *H. ducreyi* (Forbes et al. 2007f). Either 16 S rRNA sequencing or preferably referral to a reference laboratory for identification is recommended.

Commonly encountered species of *Aggregatibacter* include *A. actinomycetemcomitans* and *A. aphrophilus*. *A. actinomycetemcomitans* has characteristic adherent colonies, and after several days of growth, examination of the colonies using a dissecting microscope may reveal starlike appearance in the center of the colony (Forbes et al. 2007c). This organism is also catalase positive which may also help in its identification. *A. aphrophilus* has a fairly nondescript colony morphology and does not satellite or require hemin for growth. It is ONPG positive which may assist in its identification. Accurate identification of this organism requires sequence analysis.

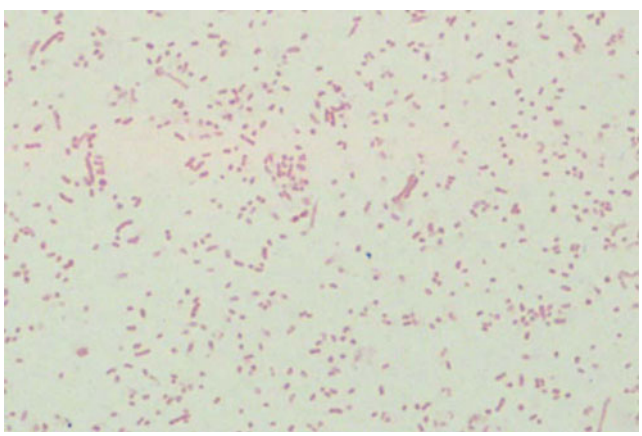


Fig. 4.26
Gram stain of *Haemophilus*

Cardiobacterium, Eikenella, and *Kingella* are reasonably easily identified by a limited number of tests. *Cardiobacterium* is a pleiomorphic Gram-negative rod which can be observed in clusters. It is also oxidase and spot indole positive. It will grow on sheep blood agar but is nonhemolytic. Organisms with these characteristics can be presumptively identified as *Cardiobacterium* (Forbes et al. 2007c), but when clinically important, confirmation should be done by 16 S rRNA sequence analysis. *Eikenella* has a characteristic colonial morphology of pitting the agar on sheep blood or chocolate agar. It also is nonhemolytic and has a distinctive bleach-like odor. These characteristics are usually sufficient to identify this organism in clinical specimens (Forbes et al. 2007d). *Kingella* is typically a coccoid, Gram-negative rod that produces large, smooth beta hemolytic on SBA. It is also oxidase positive. Its large, oxidase-positive, hemolytic colonies and failure to grow on MAC are key distinguishing features of this organism (Forbes et al. 2007c). Confirmation of this organism is best accomplished by 16 S rRNA sequencing.

Bartonella is rarely recovered from clinical specimens, and when it is recovered, it is only after prolonged incubation usually lasting for a minimum of 2 weeks on highly enriched medium such as chocolate agar which is freshly prepared. The colonies even after 2 weeks will be tiny, pinpoint colonies which can be either tan and moist in appearance or dry, raised, irregular in appearance (Maggi et al. 2011). On Gram stain, the organisms are small Gram-negative rods which frequently are clumped. The organisms are oxidase negative. These organism are phenotypically inert so molecular methods are needed to identify them. The 16 S rRNA sequences are quite similar at the species level, so other targets are needed for speciation although identification to the genus level is sufficient in most clinical situations (Kiska et al. 1995).

Special focus has been paid to the identification of *Brucella* and *Francisella*, in part, because these organisms are considered potential agents of bioterrorism (American Society for Microbiology 2004; American Society for Microbiology 2001; Chang et al. 2003). *Brucella* is one of the most commonly laboratory-acquired organisms, and so any isolate that might be *Brucella* should be handled in a biological safety cabinet (Baron and Miller 2008; Noble 2011). How do you know when you might be dealing with a *Brucella* isolate? Organisms that take 2–4 days to grow aerobically on enriched medium but fail to grow on selective media and are small Gram-negative coccobacilli should be considered to be *Brucella* until proven otherwise. When such organisms are suspected, all testing after the initial Gram stain should be done in a biological safety cabinet. *Brucella* are oxidase and catalase positive and produce a rapid urease reaction (color changes beginning within 2 h of incubation) on standard urea slants (American Society for Microbiology 2004). These isolates should be referred to a Laboratory Response Network reference laboratory for confirmatory identification. Like *Brucella*, *Francisella* grows slowly on enriched medium, does not grow on commonly used selective agars, is a small Gram-negative

coccobacilli on Gram stain, and has been laboratory acquired, and so the same safety rules apply to *Francisella* that apply to *Brucella*. *Francisella* is oxidase, catalase, and urea negative, but positive tests that may be helpful in presumptively identifying this organism are a positive beta-lactamase test and the ability to grow on the cysteine-enriched buffered-charcoal yeast extract (BCYE) agar (American Society for Microbiology 2001). As with *Brucella*, isolates should be referred to a Laboratory Response Network reference laboratory for confirmatory identification. For those who want further information on these two potential agents of bioterrorism, the reader is referred to:

http://www.asm.org/images/pdf/Clinical/Protocols/brucella10-15-04.pdf
http://www.asm.org/images/pdf/Clinical/Protocols/tularemia.pdf

Capnocytophaga ochracea and *C. canimorsus* have considerably different appearance on culture media. The organism usually requires 48–72 h or longer to grow from clinical specimens. *C. ochracea* demonstrates gliding motility with a thin film of growth spreading over the surface of the medium. These organisms may also have yellow or orange pigmentation. *C. ochracea* is oxidase and catalase negative and are thin Gram-negative rods which may have tapered ends. These characteristics are sufficient to identify this organism presumptively as *C. ochracea* although other species of *Capnocytophaga* not *ochracea* can share these characteristics. Differentiating them into species is clinically of little importance. *C. canimorsus* is reported to be catalase, oxidase, and ONPG positive. It does not have spreading colonies but does have the same type of Gram stain morphology as *C. ochracea*. As with many organisms within the group of fastidious Gram-negative rods, this organism can be best identified by 16 S rRNA sequencing (Janda et al. 2006).

Campylobacter spp. are most commonly recovered from stools cultured on selective blood containing medium grown under microaerophilic conditions. Although there are numerous *Campylobacter* species, the major diarrheal pathogens, *C. jejuni* and *C. coli*, both grow at 42 °C. Growth at this temperature is a useful tool for both their isolation and identification. After 48 h of microaerophilic incubation at 42 °C, the colonies are typically small, smooth, glistening, and may have a pinkish hue. On Gram stain, these organisms will be curved and may exhibit a "gull-wing" appearance. If these organisms are oxidase and catalase positive with the aforementioned Gram stain morphology, this is sufficient information for clinical purposes to identify them as *Campylobacter* species (Fitzgerald and Nachamkin 2011).

Isolation and identification of *Helicobacter pylori* is rarely attempted in the clinical microbiology laboratory because alternative diagnostic techniques such as rapid urease test, histology, urea breath test, and stool *H. pylori* antigen tests all have found favor with gastroenterologists (Talley and Vakil 2005). When culture is attempted, it is performed primarily to recover the organism for drug susceptibility testing in patients who are failing antimicrobial therapy. Recovery of *H. pylori* can be accomplished by culture of gastric biopsy samples on a wide variety of enriched media such as BHI-blood agar with incubation at 35 °C in microaerophilic conditions. Growth is typically observed after 3 days of incubation with colony morphology similar to that of campylobacter, small, smooth colonies, but a pinkish hue is not seen. Microscopically, the organism is curved or may be spiral shaped. Like campylobacter, these organisms are oxidase and catalase positive, but they have a potent urease that will start to change a Christensen's urea slant within 30 min. This is a highly reliable means of identifying *H. pylori* (Lawson 2011).

Direct detection of *B. pertussis* by PCR has become the clinical care standard for the diagnosis of pertussis (Wirsing von Konig et al. 2011), so culture for this organism as a diagnostic tool has been abandoned in most clinical situations. Isolation of *Bordetella* species then occurs most commonly either on BCYE plates in patients, typically immunocompromised ones, who are suspected of having legionellosis or isolated from the respiratory tract of patients with cystic fibrosis. On BCYE, *B. pertussis* and *B. parapertussis* will produce shiny, pinpoint colonies after 3–7 days of growth which on Gram stain will appear as short coccobacilli. All *Bordetella* are catalase positive, and both *B. bronchiseptica* and *B. pertussis* are oxidase positive; *B. parapertussis* is not. *B. bronchoseptica* will also grow on MAC and is urease positive (Wirsing von Konig et al. 2011). These organisms will not give a satelliting reaction, so they should not be confused with *Haemophilus* spp. Differentiating it from the other fastidious Gram-negative bacilli and some glucose non-fermenters may be difficult, so again this is a group of organisms that should be identified by 16 S rRNA sequencing. Because speciation is less certain with this gene, identification to genus level is recommended with referral to a reference laboratory with access to typing reagents for definitive identification.

In most clinical settings, *Legionella* is identified on the basis of growth characteristics and Gram stain. The organism grows on BCYE agar forming white, round, convex colonies after 3–5 days of incubation at 35 °C in ambient air. Suspicious colonies should be subcultured to SBA and BCYE agar. If the organism grows on BCYE but fails to grow on SBA, it is an indication that the organism may belong to the genus *Legionella* (Fig. 4.27). Gram stain of the organism grown on agar plates reveals small, thin Gram-negative bacilli that may form fairly long filaments. Importantly, carbolfuchsin, the stain used in acid-fast stains such as Ziehl-Neelsen, is a much more effective counterstain for *Legionella* and is recommended in lieu of safranin (Edelstein 2011). Isolates with Gram stain and growth characteristics consistent with *Legionella* are best identified by molecular means with 16 S rRNA sequencing working best for identification to genus level, while *nim* gene sequence will reliably differentiate *L. pneumophila* from other clinically relevant *Legionella* spp.

Anaerobic Bacteria

In recent years, we have begun to appreciate the complexity of the relationship of the human host and its microbiome. The microbiome consists of 10^{12} bacteria that grow on the surface of

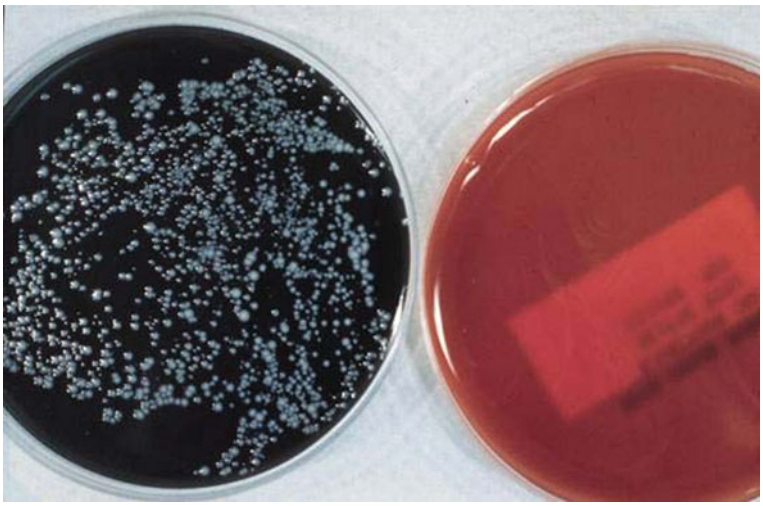


Fig. 4.27
Positive *Legionella* culture on BCYE (*left*) with no growth on sheep blood agar (*right*)

the human host primarily on mucous membrane. It is estimated that >99 % of the human microbiome is made up of anaerobes (Palmer et al. 2007). Anaerobes are the predominant flora in the oropharynx and the gastrointestinal and female genital tracts. With the advent of sophisticated molecular methods which allow us to genetically characterize the microbiome, we now recognize that only a small percentage perhaps as little as 10 % of the microbiome can be cultivated and that the agents that are uncultivable are likely to be anaerobic bacteria. We are beginning to understand the importance of these organisms in human health and their importance in such disparate clinical conditions as obesity, inflammatory bowel disease, and diabetic foot infections (Relman 2011). In this section, we will focus on those bacteria that are cultivated under anaerobic conditions and have proven roles in human infection. In general, these organisms are members of our microbiome that find themselves dislocated to tissues as a result of trauma or disease processes resulting in infection. The reader is encouraged to remember that the organisms described herein represent a small sampling of the richness of anaerobic bacteria that make up our microbiome.

Special devices are needed to generate anaerobic conditions so that we can grow these bacteria on agar media in the laboratory. All anaerobic devices are dependent upon using a catalyst to produce a reaction that removes O_2 from the culture environment. This can be accomplished in gas impermeable pouches, boxes, or chambers (*Fig. 4.28*). Furthermore, most of the medium that is utilized to recover anaerobic bacteria is itself reduced by placing it in an anaerobic environment to rid it of oxygen that may accumulate in the medium prior to its inoculation with clinical specimens.

Of the clinically relevant anaerobic bacteria, there are a limited number of species which are Gram-positive cocci. *Peptostreptococcus* and *Finegoldia magna*, previously classified as a *Peptostreptococcus*, are the most frequently anaerobic Gram-positive cocci. *F. magna* is typically associated with skin and soft

Fig. 4.28
(*Top*) Anoxomat system and glove box (*bottom*) for anaerobiasis

tissue infections, though infections involved sterile body sites have been documented as well. *P. anaerobius* is frequently found as part of a mixed infection, especially those associated with abscess formation. In terms of identification, *F. magna* is noted for its large cell size, and *P. anaerobius* can be separated from many anaerobic cocci by its inability to grow in the presence of sodium polyanethol sulfonate (Song and Finegold 2011).

A variety of Gram-positive rods are recognized as important human pathogens. *Propionibacterium acnes* is an anaerobic Gram-positive diphtheroid that is commonly found as part of normal skin flora but has become increasingly recognized as an important cause of prosthetic devices infections especially in the central nervous system (Wade and Kononen 2011). *Propionibacterium* grow as small, white pinpoint colonies that are catalase positive. This coupled with Gram stain showing characteristic Gram-positive diphtheroids is sufficient to identify the organism as *Propionibacterium* spp. *P. acnes* is easily separated from other propionibacterium on the basis of a positive spot indole reaction (Wade and Kononen 2011).

The genus *Clostridium* includes some of the most clinically important anaerobes. A distinct feature of this organism is its ability to produce spores which is important in its pathogenesis as well as diagnostics. Spores allow strict anaerobes to survive in aerobic environments until conditions become favorable (anaerobic) for germination and replication. Most clostridial pathogens are obtained as spores from the environment. There are two different major groups of clostridial pathogens, one in which pathogenesis is the result of the activity of a specific protein exotoxin or exotoxins and the other that produces myonecrosis through the activity of specific cytolysins called phospholipases (Stevens et al. 2011). The three toxin producers are C*lostridium difficile, C. botulinum*, and *C. tetani* (Stevens et al. 2011). *C. botulinum* and *C. tetani* produce neurotoxins, botulinum toxin (*C. botulinum*) which causes a flaccid paralysis (botulism) and tetanospasmin (*C. tetani*) which causes a spastic one (tetanus). *C. difficile* produces two toxins called toxins A and B that act as glucosyltransferases inactivating small GTPases. This inactivation has numerous biologic effects including disruption of the intestinal epithelium and cell death (Davies et al. 2011). Diagnosis of these intoxications is either on clinical grounds (tetanus) or by toxin detection (botulism and *C. difficile* infection). Means of toxin detection will be described in a later section in this chapter.

The other important infection due to clostridial species is myonecrosis or as it is commonly called, gas gangrene. The severity of infections in this disease is due to the rapid growth of these organisms in tissues coupled with the production of potent cytolysins such as phospholipase C. This disease is most commonly seen following trauma due to crush injuries to the extremities, but an atraumatic form is well described. The introduction of clostridial spores into devitalized tissue is the key event in the pathogenesis of this infection. Devitalized tissue is avascular and creates an ideal environment for the germination of spores and the growth of these anaerobes. Although several clostridial species can cause myonecrosis secondary to trauma, two species are found, most frequently *C. perfringens* and *C. septicum.* Both may enter wounds following trauma but may also be part of the human gastrointestinal microbiome. *C. septicum* is the main cause of atraumatic myonecrosis. In this disease process, the organism enters the bloodstream from the GI tract and causes myonecrosis typically in the arms or legs. The patient often has significant pain and may have a cellulitis (inflammation of skin characterized by redness and warmth to the touch) at the infection site. Gram stain of the tissue characteristically will have few white blood cells and the presence of Gram-positive bacilli often with subterminal spores, a finding that is characteristic of *C. septicum* myonecrosis. Interestingly, patients with *C. septicum* atraumatic myonecrosis need to be examined for the presence of a gastrointestinal malignancy because this malignancy is associated with this infection (Delbridge et al. 2005). Differentiation of *C. perfringens* from *C. septicum* is important because of the association of GI malignancy with *C. septicum* infections. Identification of *C. perfringens* can be easily made on the basis of a double zone of beta hemolysis on anaerobic blood agar. This double zone of beta hemolysis can be enhanced by placing the plate at 4 °C for an hour or longer. *C. septicum* demonstrates swarming growth on blood agar and less intense hemolysis. The finding of subterminal spores on Gram stain of clinical specimens further support the identification as *C. septicum* since spores are almost never in clinical specimens containing *C. perfringens* (Stevens et al. 2011). Identification of other *Clostridium* species is rarely important clinically, and the use of the term, *Clostridium* species not *perfringens* or *septicum*, is clearly acceptable in most clinical situations.

Actinomyces is a genus composed of both facultative and strict anaerobes. These organisms can be part of the microbiome of the oropharynx, gastrointestinal tract, and urogenital tracts of both males and females. The organism is an important cause of head and neck infection and is particularly associated with dental infections especially chronic osteomyelitis of the jaw (Robinson et al. 2005). *Actinomyces* may also be associated with lung and brain abscesses secondary to aspiration pneumonia. Serious actinomyces infection secondary to intrauterine devices leading to sterility are well described (Westhoff 2007). In most infections involving *Actinomyces*, multiple species of bacteria may be present and may obscure this rather slow growing organism on culture. Determining whether *Actinomyces* is present is important because antimicrobial treatment is prolonged, and especially in dental infections, failure to recognize this organism may result in inadequate antimicrobial treatment leading to significant bone destruction. Direct examination of clinical specimens should include looking for the presence of "sulfur granules" which should be crushed and examined for the presence of branching Gram-positive rods which is characteristic of *Actinomyces* (Wade and Kononen 2011). If branching, beaded Gram-positive bacilli are observed, *Nocardia* as well as *Actinomyces* should be considered. These organisms can be differentiated on the basis of a modified acid stain. *Nocardia* will be positive; *Actinomyces* will be negative (❯ *Fig. 4.29*). Although all species of *Actinomyces* will grow anaerobically, many of the species are aerotolerant and may grow in 5 % CO_2. As a result, Gram stain morphology, branching Gram-positive rod, and a negative catalase reaction are needed to place the organism within the genus *Actinomyces.* As with so many other organisms, accurate speciation is best accomplished by 16 S rRNA sequencing. Speciation of *Actinomyces* is limited in most clinical situation because all infections will require prolonged antimicrobial therapy.

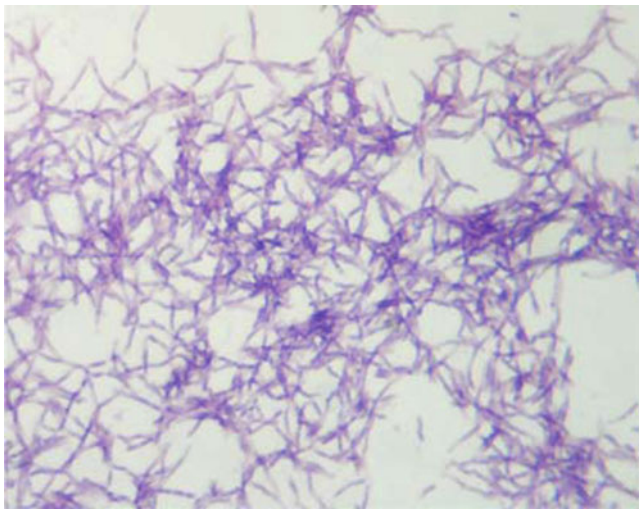


Fig. 4.29
Gram stain of *Actinomyces*

Veillonella spp. are the only anaerobic Gram-negative cocci that are commonly recovered from clinical specimens in humans. It has very limited pathogenic potential. Identification can be based on establishing it as being only able to grow anaerobically and by Gram stain morphology.

Anaerobic Gram-negative bacilli make up approximately 95–99 % of the gastrointestinal microflora (Palmer et al. 2007; Relman 2011). These organisms can be found on any of the mucous membranes in the body that provide an anaerobic niche. Key genera of anaerobic Gram-negative bacilli in human infections include the *Bacteroides fragilis* group, *Prevotella, Porphyromonas*, and *Fusobacterium.*

B. fragilis group is a major inhabitant of the gastrointestinal tract and causes problems when the GI barrier is breached either due to disease or surgical procedures. When it escapes from its normal environmental niche, the organism in concert with other members of the GI microbiome can cause intra-abdominal abscesses and bacteremia, two of the most common infections associated with this organism (Garrett and Onderdonk 2010). On Gram stain, the organism is short, plump Gram-negative bacilli whose appearance is similar to *E. coli.* The organism grows well anaerobically on anaerobic selective medium such as kanamycin-vancomycin laked sheep blood. This organism group is easily identified based on a positive 15 % catalase test and by their ability to grow in 20 % bile (Kononen et al. 2011).

Prevotella and *Porphyromonas* spp. are small Gram-negative coccobacilli that are prominent microflora in the oropharynx cavity. They play a prominent role in periodontal disease, other dental infections, and may also be associated with aspiration pneumonia and its complications such as lung and brain abscess (Garrett and Onderdonk 2010). These organisms can also be associated with intra-abdominal and pelvic infections. Some of these species are readily identified as members of the *Prevotella* or *Porphyromonas* genera based on their ability to produce pigments, some of which are visible under ambient light while others are visible using ultraviolet light, typically producing a brick red color. Differentiating them into specific species is a limited importance since antimicrobial treatment and pathogenic potential are similar.

Like *Prevotella* and *Porphyromonas, Fusobacteria* are part of the oropharyngeal flora. They tend to be associated with more invasive infections of the head and neck such as Ludwig's angina and Lemierre's syndrome (Garrett and Onderdonk 2010). These infections can be life-threatening in part because swelling in the neck and submandibular space may lead to airway obstruction (Garrett and Onderdonk 2010). These organisms may also play a role in the same types of infections as *Prevotella* and *Porphyromonas.* Differentiation of *Fusobacterium* from the other anaerobic Gram-negative bacilli can be done on the basis of a negative 15 % catalase test, susceptibility to kanamycin, and characteristic Gram stain morphology. On Gram stain, *Fusobacterium* are thin, fusiform Gram-negative rods. *F. nucleatum* can be reliably identified by virtue of having tapered ends and a positive spot indole test (Forbes et al. 2007g). *Fusobacterium* species are susceptible to 1 mg kanamycin disk, while *Prevotella, Porphyromonas*, and *Bacteroides fragilis* groups are all resistant (Kononen et al. 2011).

Organisms That Do Not Gram Stain

There are several organisms in this group. Several of them are best detected by serologic means which will be described in the next section of this chapter.

Mycobacterium

The key genus of pathogenic bacteria that is refractory to Gram staining is *Mycobacterium.* The refractory nature of this genus is due to the presence of thick, waxy cell envelope which is rich in mycolic acids. *Mycobacterium* spp. are relatively slow growing obligate aerobic bacilli. Most take at least 2 weeks to grow in broth medium and longer on solid medium. Infections caused by one species, *Mycobacterium leprae*, are diagnosed by histologic and molecular diagnostic means as the organism cannot be grown on artificial media (Forbes et al. 2007h; Richter et al. 2011). There are five groups of *Mycobacterium* that can be grown on artificial medium: the *Mycobacterium tuberculosis* complex which consists of *M. tuberculosis, M. bovis*, and *M. africanum* and four groups of environmental mycobacteria, the photochromogens which are pigmented only when exposed to light; the scotochromogens which produce pigment in the absence of light; the nonchromogens which, as the name implies, are not pigmented; and the rapid growers (Forbes et al. 2007h; Richter et al. 2011). The term "rapid grower" is a bit of a misnomer since the definition of a rapid grower is that it will grow on solid medium within 7 days of subculture from an initial culture of a clinical specimen (Forbes et al. 2007h). The initial isolation of rapid grower from a clinical specimen may

take up to 2 weeks. "Environmental mycobacteria," unlike *M. tuberculosis* and *M. leprae* which are only obtained via person to person spread, are obtained by exposure to contaminated environmental sources such as water and soil. Because they are organisms that are best adapted to grow in the natural environment rather than a human host, many grow better at 30 °C than at 37 °C. Key human pathogens within these four groups are as follows (Forbes et al. 2007h; Pfyffer and Palicova 2011; Richter et al. 2011):

Photochromogens: *M. marinum, M. kansasii, M. asiaticum*, and *M. simiae*
Scotochromogens: *M. scrofulaceum* and *M. gordonae* (which is actually the most common contaminant of mycobacterial cultures rather than a pathogen)
Nonchromogens: *M. avium* complex, *M. genavense, M. ulcerans*, and *M. haemophilum* which requires hemin for growth
Rapid growers: *M. fortuitum* group and *M. chelonae/abscessus* group

Because of the slow growth of many of these organisms, molecular identification such as 16 S rRNA, *rpoB*, and *hsp65* sequencing in large part has replaced phenotypic identification methods. However, certain phenotypic tests are still used to supplement and support molecular identification. The most important of these remains the acid-fast stain (Fig. 4.30). Other organisms such as *Nocardia* and *Rhodococcus* can be recovered from mycobacterial cultures and may have similar colonial and microscopic morphology but will not be truly acid fast (Nataro et al. 2011). One microscopic morphologic characteristic that may be helpful in differentiating *M. tuberculosis* complex from the other mycobacterial species, if present, is cording (Fig. 4.30). However, cording is present in only approximately 25 % of *M. tuberculosis* cultures, and additionally environmental mycobacterium may infrequently demonstrate it so its value is limited (Morris and Reller 1993). Once it is established that the organism is acid fast, growth rates, pigmentation, and colony morphology will next be considered. Since liquid medium such as that used in the MGIT system (BD Microsystems, Cockeysville, MD) is how mycobacterium is first recovered from clinical specimens, subculture to solid medium such as 7H11 will be necessary for determination of colony morphology and pigmentation (Richter et al. 2011). If the organism does not grow on 7H11, it may be because the organism requires either hemin (*M. haemophilum*) or mycobactin (*M.* genavense) for growth (Richter et al. 2011). Fortunately, these organisms are only rarely encountered in clinical specimens. The rate at which the colonies appear on this medium will allow the determination of whether the organ is a rapid ($\leq$7 days) or slow (>7 days) grower. Rapidly growing mycobacterium is best identified by sequence analysis of *hsp65* or *rpoB* (Adekambi et al. 2006).

Colonial morphology and pigmentation are useful tools for the presumptive identification of slow growing mycobacterium. For nonpigmented organisms, the two most commonly encountered organisms, *M. tuberculosis* and *M. avium* complex, have quite distinctive and different colonial morphologies. On 7H11

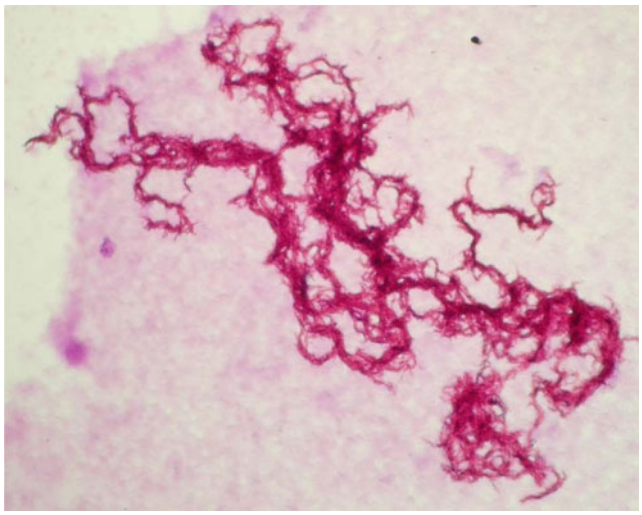


Fig. 4.30
Positive acid-fast stain of *Mycobacterium tuberculosis* showing cording

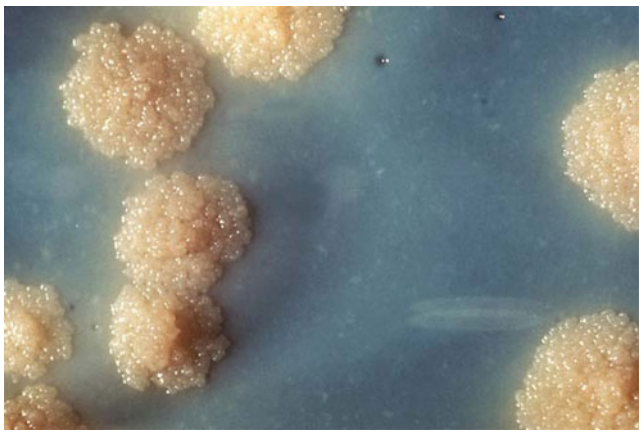


Fig. 4.31
Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen media (Image courtesy of CDC)

or Lowenstein-Jensen slants, *M. tuberculosis* has a dry, rough, wrinkled appearance with a buff or off-white color (Fig. 4.31), while *M. avium* complex are smooth and often clear on 7H11 making them difficult to appreciate during the early stages of growth (Fig. 4.32). In the molecular era, identification of these organisms can be rapidly and reliably done by 16 S rRNA sequencing making phenotypic characterization superfluous.

For pigmented organisms, determination of whether the organism is a photochromogen or scotochromogen is useful in a presumptive identification scheme. This is done by placing equal portions of inoculum typically onto two Lowenstein-Jensen slants and incubating one tube in the dark and the other in the light. If the organism produces pigment both in the light and dark, it is considered a scotochromogen (Fig. 4.33). The most commonly encountered scotochromogen in clinical specimens is *M. gordonae* which is typically considered

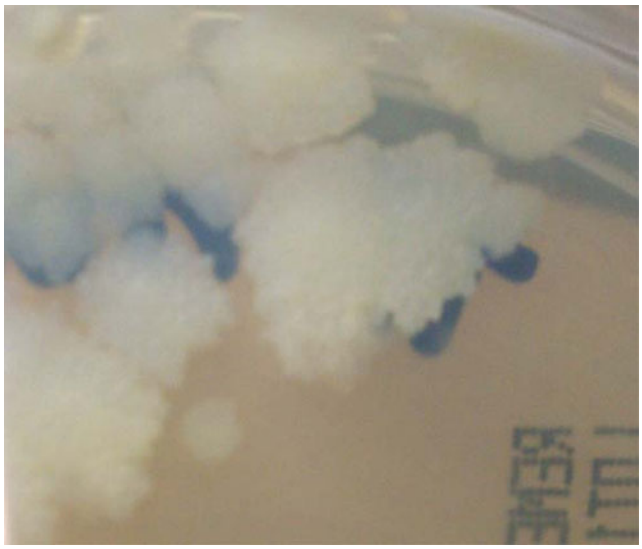


Fig. 4.32
Colonies of *Mycobacterium avium* on 7 H11 media

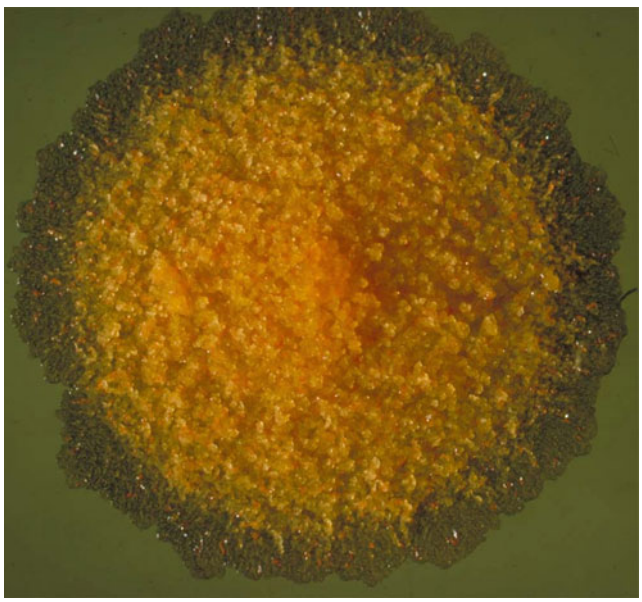


Fig. 4.33
Scotochromogen (Image courtesy of CDC)

a contaminant. Photochromogens are organisms that only produce pigment when exposed to light, so the tubes incubated in the dark will be nonpigmented, while those incubated in the light will produce pigments. *M. kansasii* and *M. marinum* are the most commonly clinically encountered photochromogens. *M. marinum* is typically associated with skin and soft tissue infections and will grow best at 30 °C and may grow poorly or not at all at 37 °C, while *M. kansasii* will be recovered most frequently from the respiratory tract and will grow at 37 ° C. Laboratories may opt to culture skin and soft tissue specimens at 30 °C or even at ambient temperature to assure the recovery of *M. marinum* (Richter et al. 2011). As with other groups of mycobacterium, a definitive identification is preferably done by 16 S rRNA sequencing.

Chlamydia trachomatis

The genus *Chlamydia* represents a group of obligate intracellular pathogens, the most important of which is *C. trachomatis*. In the industrialized world, *C. trachomatis* is a leading cause of sexually transmitted infections. Because initial stages of infections may be asymptomatic, pelvic inflammatory disease (PID), a late stage complication, may result. PID is a leading cause of female infertility globally (Haggerty et al. 2010). It is also an important agent of ocular infection and is a leading cause of blindness in the developing world (Burton and Mabey 2009). This organism has an unusual bacterial life cycle. It can be cultured from infected individuals using an immortalized cell line most commonly McCoy cells. In this method, the elementary (infective) body of *C. trachomatis* found in a clinical specimen adheres and is taken up by the cells. The organism then differentiates into the reticulate (reproductive) form and divides by binary fission. The organism then differentiates back to elementary bodies and is released from the by cell lysis (Abdelrahman and Belland 2005). Fluorescent antibody-based methods can be used to detect the elementary bodies directly in clinical specimens or as inclusion bodies in cell cultures. Fluorescent antibody-based methods typically use either monoclonal or polyclonal antibodies against species-specific antigens. They may target a single or a mixture of species-specific antigen(s) (Gaydos and Essig 2011). Diagnosis of *C. trachomatis* infection is primarily done by molecular techniques.

Mycoplasma and *Ureaplasma*

Mycoplasma and *Ureaplasma* have the smallest genome of any commonly encountered human pathogen. Although there are several species of *Mycoplasma*, three species are encountered clinically with some degree of frequency. *M. pneumoniae* causes "walking pneumonia," meaning patients have pneumonia symptoms but are not especially ill. Their disease is self-limited typically. *M. hominis* can be indigenous flora in the genitourinary and gastrointestinal tracts. It can cause postpartum bacteremia and wound infections. Finally, *Ureaplasma ureolyticum* can be found in the genitourinary tract and has been implicated both as a cause of nongonococcal urethritis and may be associated with male infertility.

Most clinical laboratories do not attempt to isolate these organisms. *M. pneumoniae* requires both highly specialized growth medium and prolonged incubation of 21 days or longer. *Ureaplasma ureolyticum* requires both special medium and an inverted microscope to view the microscopic colonies produced by this organism. Only *M. hominis* is likely to be encountered in the clinical laboratory. It can grow in blood cultures and also on commonly used nonselective agar such as sheep blood agar.

The organism is identified based on colony morphology. Colonies are clear, have a "fried egg" appearance, and fail to Gram stain. This is sufficient information to presumptively identify the organism as *M. hominis* (Dumler 2011)

Organisms That Are Detected Primarily by Serologic Methods

Infections due to organisms that are not easily grown in the clinical laboratories are often made by detecting antibodies produced in response to infection caused by these organisms. Because detection of the organism is dependent upon the generation of an immune response and it may be days to weeks before that immune response can be measured, serologic diagnosis is considered a "retrospective" diagnosis. Importantly, serologic tests are often negative in the acute phase of the disease, so they are not as sensitive as culture. Additionally, organisms may share similar antigens resulting in a "false-positive" test. A false-positive serologic test occurs when a positive test result is found, but the patient is not infected with the target organism (Jackson 2008). An important reason for a false-positive test is an antigen of another organism, frequently an organism that is part of the patient's resident microflora that is not causing any pathology, rather than the target organism that is responsible for the positive result. In spite of these shortcomings, serologic testing is widely used for the detection of several organisms. In this section, we will discuss some of the most widely used serologic tests for bacterial agents. The interested reader who wants to learn more about the use of serologic tests in the diagnosis of bacterial infections is referred to the Manual of Clinical Immunology (Rose et al. 2002).

There are two major approaches in looking for antibodies in bacterial pathogens. One is a fairly simple approach where enzyme immunoassays or indirect immunofluorescent antibody test are performed to look for antigen-specific antibodies (Carpenter 2011). Two tick-borne diseases caused by obligately intracellular pathogens, *Ehrlichia chaffeensis* and *Rickettsia rickettsii*, are diagnosed serologically. These organisms cause severe and sometimes fatal disseminated infections. Both diseases can be characterized by fever, headache, and petechial skin rashes although skin rashes may be absent (Raoult 2010). The disease caused by the rickettsial agent is called Rocky Mountain spotted fever or RMSF. This name is a bit of a misnomer as the disease is found most commonly in Oklahoma, Arkansas, and the Southeastern United States (Centers for Disease Control and Prevention 2011b). Ehrlichiosis has a similar geographic distribution (Centers for Disease Control and Prevention 2011b). The most widely used test in the USA for diagnosis of these two organisms is the indirect immunofluorescent antibody (IFA) assay. In this test, multiple spots of cells infected by the bacteria for which the patient is being tested are placed on a glass slide. Different dilutions of the patient's serum are applied to a spot. The mixture is incubated and gently washed with a buffered solution. If antibodies specific to the organism's antigen are present, they will remain bound to the spot even after washing. The antibodies are detected by applying an antihuman antibody labeled with a fluorescent dye to the spots, incubating and washing, and then observing the slide under a fluorescent microscope. If fluorescence is observed in the infected cells, then the reaction is positive (Carpenter 2011; Dumler 2011). For the patient to be considered infected, a specific minimal dilution of serum must give a positive reaction. To do serology most accurately, paired sera obtained two or more weeks apart are needed to demonstrate a current immune response. Paired sera are tested and the results of the "acute" (the initial specimen obtained) and the "convalescent" (the second specimen obtained at least 2 weeks before the first) sera compared. If the patient had an acute infection, there should be a fourfold change in titer (titer being the reciprocal of the dilution tested). If the titer is negative or remains the same, the patient either is not infected or had an infection in the past, respectively. However, the way serology is usually done is by performing the test in a patient with appropriate signs and symptoms of the disease, and if the patient has a high enough titer, the patient is considered to have an infection with that organism.

In Europe, serologic testing is widely used to make the diagnosis of *B. pertussis* infections through detection of antibodies using solid-phase enzyme immunoabsorbent assays (EIA) (Guiso et al. 2011). The principle is the same as that used for IFA. Organism-specific antigens are bound on a solid phase in this assay—plastic wells. Patient antibodies are added, incubation and washing follow, and then are added antihuman antibodies to which an enzyme has been linked. Again, there is incubation and washing. The substrate that will give a color change is added to the well, and if enzyme is present, a color reaction will occur. The intensity of that color change is directly proportional to the amount of antibody present in the specimen. That color reaction is compared to a positive control and a titer can be determined.

Another strategy that is used to detect bacterial infections serologically is the "screening" and the "confirmatory" test. This approach has long been used to make the diagnosis of syphilis which is caused by the spirochete, *Treponema pallidum*, which cannot be grown on an artificial medium (Loeffelholz and Binnicker 2012; Radolf et al. 2011). A screening test has certain characteristics which are important:

1. It must be highly sensitive.
2. It must be easily performed.
3. It must be inexpensive.
4. Preferably it is rapid.

A widely used test for syphilis screening is the rapid plasma reagin test or RPR. The RPR test detects antibodies which target antigens released from *T. pallidum* damaged tissues. The reagent contains cholesterol and cardiolipin and charcoal particles which add in the detection of the formation of antibody-antigen complexes. When these complexes are formed, they are visualized due to the clumping of the charcoal particles which are trapped in the complexes. There are three stages of syphilis: primary, secondary, and late syphilis. RPR has a sensitivity of ~85 % in primary disease, 100 % in secondary syphilis, and ~75 % in late syphilis (Larsen et al. 1995). The major difficulty

with this test is that false-positives are not uncommon and may lead to a poor positive predictive value of the screening test in low incident populations such as pregnant women or those with rheumatologic disorders (Larsen et al. 1995). Because any diagnosis of a sexually transmitted disease can have consequences beyond infectious ones, accurate diagnosis is paramount. As a result, positive RPR tests need to be confirmed by the use of a "confirmatory test."

Characteristics of a confirmatory test include the following:

1. It must be highly specific, meaning it must have few false-positives.
2. It often is more complex and expensive than a screening test.

A widely used confirmatory test for syphilis is the *Treponema pallidum* particle agglutination (TP-PA) test. In this test, *T. pallidum*-specific antigens are absorbed onto the surface of gelatin particles. Serum or plasma from RPR-positive individuals is reacted with the antigen-coated particles as well as non-antigen-coated ones. A patient is diagnosed with syphilis if there is agglutination of the antigen-coated particles and no agglutination of the non-antigen-coated ones. This test has high specificity, approaching 100 % when properly performed (Loeffelholz and Binnicker 2012).

Another spirochetal disease which uses a screening and confirmatory testing approach is for the diagnosis of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. When diagnosing this infection, an EIA test for antibodies is performed, and if positive a confirmatory Western blot test is performed. Patients with negative EIA results are considered negative. In this Western blot assay, lysates of *B. burgdorferi* are separated by SDS polyacrylamide electrophoresis and transferred to a nitrocellulose strip. These strips are incubated with EIA-positive sera, washed with buffer and then reacted with antihuman antibodies that are coupled with an enzyme, and washed again and reacted with enzyme substrate that will give a chromogenic reaction. Specific banding patterns are observed when patients are infected with *B. burgdorferi*. As with syphilis, the two-step approach is done to improve the specificity of diagnosis, especially for populations who live in a geographic locale where this organism is endemic (Schriefer 2011).

Detection of Microorganism Using Antigen Tests

With the advent of molecular diagnostics and the widespread use of vaccines for bacterial meningitis, the use of antigen detection to diagnose infections has declined significantly. However, antigen detection tests are still widely used to diagnose group A streptococcal pharyngitis, *Legionella pneumophila* pneumonia, and as a screening test for *C. difficile* infection.

Group A streptococcal (GAS) pharyngitis is one of the most common reasons why children consult a physician. This is not an infection that is easily distinguished from viral pharyngitis clinically resulting in a need for diagnostic GAS testing since antimicrobial therapy is recommended for children with GAS pharyngitis to prevent post-streptococcal sequelae as well as prevent spread in school settings (Song and Finegold 2011). The development of a rapid test which could detect GAS on swabs taken from the pharynx of infected children was most welcome. In this assay, group A streptococcus-specific carbohydrate antigen can be extracted from a throat swab using mild acid solution. The antigen can be detected using variety of immunoassay approaches. These assays have a reported sensitivity when compared to a culture between 70 % and 95 % with the conventional wisdom putting that sensitivity at around 80 % (Wessels 2011). That means that 20 % of infected children will have a negative rapid GAS test. As a result, it is recommended that children, because they are at greatest risk of developing post-streptococcal sequelae and therefore benefit most from antimicrobial therapy, should have "backup" cultures if negative. Most agree that these tests, if properly performed, are very simple and have a specificity of >98 %, meaning that positives can be considered true positives (Wessels 2011).

When an individual is infected with certain bacterial as well selected fungal pathogens, they often excrete these organisms' antigens in urine. Because urine is easily and reliably obtained from most patients, urinary antigen tests for difficult to detect infections have been developed. One of the most widely used bacterial urinary antigen tests detects *Legionella pneumophila* serogroup 1 which is a notoriously difficult organism to culture from patients with legionellosis. This antigen can be detected using immunochromatographic devices which work on the same principle as a urine pregnancy test and are as easy to perform. The limitation of this assay is that it only sensitively detects *L. pneumophila* serogroup 1. Fortunately, *L. pneumophila* serogroup 1 is the predominant type causing this disease. The test is highly specific, but a shortcoming is that antigen excretion can last for months, so at least in theory, false-positive tests in previously infected individuals may occur (Mandell et al. 2007).

The final antigen test that has found wide usage in the clinical microbiology laboratory is the detection of the *C. difficile* glutamate dehydrogenase (GDH) enzyme (Gilligan 2008). Large quantities of GDH are produced by *C. difficile*, and it can be detected in the feces of infected patients by either EIA or immunochromatographic assays (ICA) using antibodies specific for this *C. difficile* enzyme (Fig. 4.34). The test is reported to have a sensitivity of between 90 % and 100 % (Wilcox et al. 2010), but it is produced by all *C. difficile* strains including those that do not produce toxin. Non-toxigenic strains are avirulent

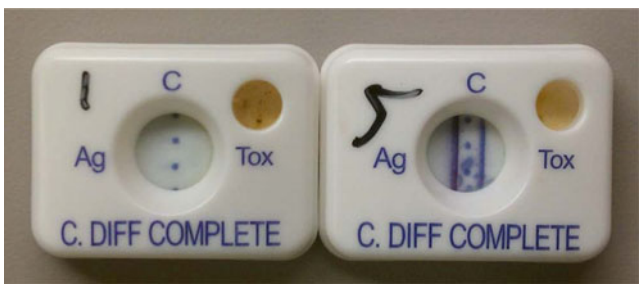


Fig. 4.34
Negative and positive (*right*) *Clostridium difficile* EIA tests

but can be found in the gut. Thus, GDH assays are highly sensitive but are not highly specific. This combination of characteristics means that this test has a poor positive predictive value for toxigenic organisms, as low as 50 % (Stevens et al. 2011). Therefore, positive *C. difficile* GDH assays must be confirmed by a more specific assay such as *C. difficile* cytotoxin neutralization assays (see below) or polymerase chain reaction (PCR) assays for *C. difficile* toxin genes (Wilcox et al. 2010).

Detection of Clinically Important Bacterial Virulence Factors

Although we depend on the detection of bacterial primarily by culture, the diagnosis of some infectious diseases is dependent on the demonstration of that organism's ability to produce a specific toxin either in vitro or in vivo. The major type of virulence factor that is searched for in the clinical laboratory is toxins. Although toxin genes can be detected by PCR, we still rely on toxin detection to determine the presence of certain infectious agents.

Diphtheria is a rare disease in the industrialized world because of effective toxoid vaccination, but pockets of infection may occur especially in individuals who either choose not to be vaccinated or have not received appropriate booster vaccination (Eskola et al. 1998). The pathogenesis of disease occurs when the organism locally infects the pharynx and produces the virulence factor diphtheria toxin that causes the formation of a localized lesion called a "pseudomembrane." Diphtheria toxin can enter the bloodstream and can damage a variety of tissues most importantly the heart (MacGregor 2010). Toxin is encoded in a lysogenic phage, and if that phage is not present, the organism cannot produce toxin or cause disease. *C. diphtheriae* can be found in the throat of individuals with the infection and perhaps as importantly in individuals who do not have diphtheria (MacGregor 2010). Since non-toxigenic strains of *C. diphtheriae* can be recovered from healthy individuals, the recovery of the organism alone is not sufficient to make the diagnosis of diphtheria. Rather the ability to produce diphtheria toxin by the isolate recovered from an ill patient must be demonstrated in order to make this diagnosis. To demonstrate toxin production, the organism must be grown under iron-limiting conditions. A strip of filter paper into which antitoxin for diphtheria toxin is absorbed is placed on a medium that will support the growth of a toxin-producing control strain and the isolates in which toxin production is being determined. The toxin-producing strain and the unknown strain are struck in parallel approximately 10–15 mm apart at right angle from the antitoxin impregnated strip. After 1–2 days incubation at 35 °C, the plates are examined for lines of precipitation due to the formation of antibody-antigen complexes. Importantly if the unknown strain is producing toxin, it should produce an "arc of identity" with the positive toxin-producing control strain (Forbes et al. 2007a). Unknown strains showing this reaction are toxin producers.

Escherichia coli can be a commensal part of the intestinal microflora but can also be a diarrheal pathogen if it has obtained specific toxin genes. These genes include those that allow it to cause attaching and effacing lesions (enteroadherent *E. coli* or EAEC), invade the epithelium (enteroinvasive *E. coli* or EIEC), produce a cholera-like toxin or a heat stable toxin which can induce diarrhea (enterotoxigenic *E. coli* or ETEC), or produce shiga toxin (shiga toxin producing *E. coli* or STEC) (Gilligan 1999). In the industrialized world, STEC which can be spread through contaminated food and water has become a cause of public health concern. Large-scale disease outbreaks have been due to ingestion of a variety of contaminated food substances including undercooked hamburgers, spinach, and bean sprouts (Frank et al. 2011). STEC infections are problematic because a subset of infected individuals develop a complication of this infection called hemolytic uremic syndrome due to shiga toxin's ability to damage the kidney. The morbidity and mortality due to hemolytic uremic syndrome is significant (Frank et al. 2011). The gene for shiga toxin is located on a lysogenic phage and can be found in a wide variety of *E. coli* strains. In order to differentiate STEC from commensal *E. coli* strains which are found in the gut of almost all humans, it is necessary to demonstrate that the stool contains organisms that can produce shiga toxin. One of the simplest ways to do this is to inoculate broth with feces, incubate it overnight, and then use either an EIA or ICA to detect toxin in feces. This approach is the standard method recommended by the Centers for Disease Control to diagnose this infection (Marcon et al. 2011).

The diagnosis of botulism is dependent upon the demonstration of botulinum toxin either in food or in a variety of clinical specimens. There are four major syndromes associated with botulism: infant (most common), food-borne, wound, and child and adult of unknown origin (Lindstrom and Korkeala 2006). Diagnostic specimens for infant botulism are stool, frequently requiring an enema because of constipation, and serum; for food-borne, the potential tainted food in original containers, stool, serum, and gastric aspirates/vomitus (if available); for wound botulism, serum and tissue exudates/biopsies; and for child and adult of unknown origin, stool and serum. The testing is done at public health laboratories, so appropriate handling (specimens should be refrigerated but not frozen), packaging, and shipping is important. The test that is performed is a mouse lethality test where filtrates of the various substances other than serum (which can be tested directly) are injected intraperitoneally into a mouse and the mouse is observed for symptoms consistent with botulism. To show that the symptoms that are occurring are due to botulinum toxin, specimens that have been preincubated with botulinum antitoxin are also injected; these animals should remain symptom free. Finally, specimens which have been heat treated to inactivate the toxin are used as negative controls in this assay. The specimen contains botulinum toxin if the untreated specimen causes botulinum-like symptoms in the mouse (slowly ascending paralysis starting in the hind legs and tail), while mice who receive antitoxin and heat treated specimens remain well (Lindstrom and Korkeala 2006).

The final toxins that are widely sought diagnostically are *C. difficile* toxins. *C. difficile* produces two toxins, A and B, which are responsible for the pathogenicity of this organism.

As with diphtheria and STEC, non-toxigenic strains of *C. difficile* which do not cause disease can be found in feces (Wilcox et al. 2010). Therefore, demonstration of toxin production rather than recovery of the organism is needed for the diagnosis of *C. difficile* infection. Two major approaches have been used for detection of these toxins: tissue culture cytotoxicity neutralization assay or CTN and EIA/ICA for *C. difficile* toxins.

C. difficile as a cause of diarrheal disease was first discovered because the toxins it produced in vivo were present in fecal filtrates of *C. difficile*-infected patients and caused morphologic changes in a variety of tissue culture cell lines. With the recognition that this "cytotoxic" effect could be "neutralized" by antitoxin made against these two toxins, the cytotoxin neutralization or CTN assay was born (Wilcox et al. 2010). This assay has been found to be reasonably sensitive and highly specific and for many years has been the gold standard for the diagnosis of this infection (Stevens et al. 2011). Recently, it has been superseded by PCR for toxin genes that appear to be more sensitive for disease detection although likely not to be as specific. EIA/ICA for detection of *C. difficile* toxins remains in use in many laboratories for the diagnosis of *C. difficile* infection. However, there is a growing body of literature that suggests that this testing has an adjunct rather than primary role in the diagnosis of this infection (Wilcox et al. 2010).

Susceptibility Testing

Identification of microorganism alone is often not sufficient for the management of patients. Additional information that is frequently necessary for optimal patient care includes determining to what antimicrobials an organism may be resistant and what virulence factors the organism might produce.

The Gram stain reaction provides valuable information about the antimicrobial susceptibility of an organism. As a general rule of thumb, Gram-positive organisms are susceptible to certain antimicrobials to which Gram-negative organisms are typically resistant. The reverse is also true. For example, Gram-positive organisms are generally susceptible to vancomycin and macrolide antimicrobials, while the most commonly encountered aerobic Gram-negative rods are typically resistant to them. Conversely, aerobic Gram-negative rods are usually but not always susceptible to polymixin antimicrobials, while Gram positives are resistant. This resistance is referred to as intrinsic resistance and is due to general structure of the cell envelope of Gram-positive and Gram-negative organisms. Bacteria can also acquire resistance either through mutation or recombination events where organisms obtain genes that encode for any number of resistance mechanisms (Miller and Gilligan 2008).

Acquired antimicrobial resistance can fall into five broad categories (Miller and Gilligan 2008):

1. *Enzymatic inactivation*: Beta-lactamases which are enzymes that can degrade the beta-lactam ring in beta-lactam antimicrobials inactivating the antimicrobial.
2. *Modification of the antimicrobial binding site*: Penicillin binding site protein 2a in *S. aureus* which confers resistance by modifying the antimicrobial target site preventing the binding of the penicillinase stable penicillins (oxacillin and methicillin); antimicrobials that cannot bind are not active.
3. *Active efflux*: Efflux pumps remove antimicrobials such as macrolides and tetracyclines from the bacterial cells.
4. *Alterations in outer membrane permeability*: Alterations in porin proteins in the outer membrane of Gram-negative bacteria prevent the entry of beta-lactam drugs such as carbapenem into *Pseudomonas aeruginosa.*
5. *Alteration in enzymatic pathways*: Occurs when organisms can take up thymidine from their environment rather than using a synthetic pathway to make it which is blocked by an antimicrobial, trimethoprim/sulfamethoxazole.

Antimicrobial resistance can be detected by examining the interaction of a bacteria and the antimicrobial agent which is being considered for use in treatment of an infection due to that organism. This testing is called antimicrobial susceptibility testing. It is dependent upon the pharmacokinetics of a particular antimicrobial and the activity of the antimicrobial against a particular organism. One caveat that should always be remembered is that all drugs including antibacterial agents have toxicity associated with them, so unlimited amounts of antimicrobials cannot be administered (Miller and Gilligan 2008).

Each antimicrobial used to treat bacterial infections has antimicrobial susceptibility breakpoints that have been established during the development of the particular agent (Miller and Gilligan 2008). These breakpoints establish whether an antibacterial is likely to be active clinically (the organism is susceptible) or not (resistant) against the infecting organism being tested. The simplest approach to determining whether an organism is susceptible or resistant to a particular antimicrobial is to perform a minimum inhibitory concentration determination or MIC. MICs are done by making twofold serial dilutions over a range of drug concentration from quite susceptible to resistant. These dilutions can be done in either broth or agar. For example, penicillin G MIC for *Streptococcus pneumoniae* will be tested in a range of 0.03–16 ug/ml with the "breakpoint" for a susceptible isolate being $\leq$2 ug/ml and resistant being $\geq$8 ug/ml. An MIC = 4 ug/ml would be considered "intermediate." Intermediate means that the drug may or may not work; it is dependent on the pharmacokinetics of the drug in the body site that is infected. In difficult to treat infections, physicians may increase the dose of antimicrobials which have intermediate activity if this can be safely done to enhance the likelihood that such an infection can be treated. Because *S. pneumoniae* is a common cause of bacterial meningitis and penicillin G does not penetrate well into the meninges, there are separate, much lower penicillin G "meningitis" breakpoints for *S. pneumoniae* of $\leq$0.06 (susceptible) and $\geq$0.12 ug/ml (resistant). MIC methods have been automated, and automated MIC detection systems are widely used in clinical laboratories (Kiska et al. 1995).

There are two alternative susceptibility techniques that are widely used. One is the disk diffusion method and the other is

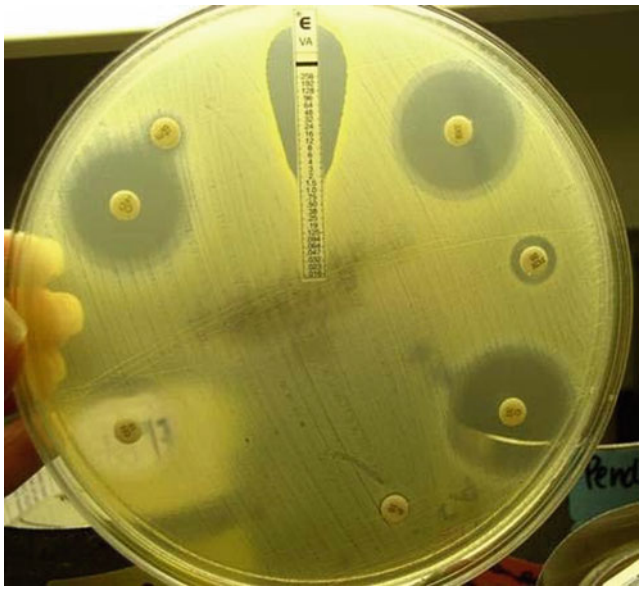


Fig. 4.35
Antibiotic susceptibility testing of *Staphylococcus aureus*

Fig. 4.36
Penicillin E-test of *Streptococcus pneumoniae*

the E-test (Fig. 4.35). In the disk diffusion method, disks are impregnated with specific concentration of antimicrobials, placed on a lawn of bacteria that has been spread on the surface of a Mueller-Hinton agar (MHA) plate, incubated overnight, and then the zone of inhibition are measured. As with MIC determination, the "zone sizes" determine the relative activity of the drug against the test organism, and the size of the zone size will determine if the organism is susceptible (larger zone of inhibition) or resistant (smaller zone of inhibition). The zones of inhibition that denote whether an organism is susceptible or resistant to a particular antimicrobial is based in part on how well the antimicrobial diffuses in agar. Some large, highly charged molecules such as aminoglycosides and polymixin diffuse poorly and thus may have small zones of inhibition denoting the organism being susceptible, while other antimicrobials such as the beta-lactams readily diffuse in agar and may have comparatively large zones of inhibition denoting susceptibility.

The E-test is a combination of disk diffusion and MIC. The approach to the E-test is similar to disk diffusion. However, instead of a disk being placed on a plate, a strip impregnated with different concentrations of antimicrobials along its length is placed on the lawn of bacteria. The place where the elliptical zone of inhibition intersects with the strip indicates the organism's MIC to the antimicrobial in the E-test strip (Fig. 4.36). This test has been particularly useful in determining *S. pneumoniae* MICs, something that cannot be accurately done with some automated MIC detection systems (Kiska et al. 1995).

With the recognition of "new" antibacterial resistance mechanisms, alternative susceptibility testing methods were required. Emergence of resistance due to "new" beta-lactamases which are

Fig. 4.37
Depiction of a positive ESBL test. The organism shows greater growth inhibition in the presence of ceftazidime plus clavulanic acid (*left*) than with ceftazidime alone

not easily detected by standard MIC or disk diffusion methods lead to new methods for detecting these mechanisms. Two that are particularly problematic are extended spectrum beta-lactamases or ESBLs and the *Klebsiella pneumoniae* carbapenemases or KPC (Bush 2010). ESBL confers resistant to all classes of beta-lactams except carbapenems and can be detected by placing a lawn of the bacterium in question on Mueller-Hinton agar and placing cefotaxime, cefotaxime-clavulanic acid, ceftazidime, cetazidime,

Fig. 4.38
Positive modified Hodge test

and ceftazidime-clavulanic acid on the plate. Clavulanic acid is a beta-lactamase inhibitor active against ESBLs, so an ESBL positive organism has a zone of inhibition at least 5 mm greater with the clavulanic acid combinations compared to the cefotaxime or ceftazidime alone (*Fig. 4.37*) (Clinical and Laboratory Standards Institute 2011).

Carbapenemases can be detected with a modified Hodge test. This test is done by placing a lawn of susceptible *E. coli* on MHA and placing a meropenem and ertapenem disk at least 30 mm apart on the plate. The test organism is then inoculated on the plate in a straight line starting from the edge of the disk and moving outward to the periphery. If there is an invagination in the zone of inhibition along the test organism streak, the organism is a carbapenemase producer (*Fig. 4.38*) (Clinical and Laboratory Standards Institute 2011). Carbapenemase-producing organisms are resistant to all beta-lactamase producers and may also be resistant to all other antimicrobials except colistin/polymixin b and tigecycline (Bush 2010).

Conclusions

Although the molecular revolution will continue to advance the identification of clinically relevant microbes, there will always be a place for conventional methods. Gram stains, colony morphology, and simple phenotypic tests can still provide a vast amount of information in a short amount of time. Most of all, these techniques are accessible to anyone, not just those trained in, or capable of, performing molecular biology. Even as we identify more bacteria via 16 S sequencing, the questions of "What does it look like on Gram stain?" or "Does it grow on sheep blood agar?" still remain. For these reasons and more, conventional methods will always remain conventional, not outdated, not outdone.

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5 Identification of Pathogens by Nonculturing Molecular Techniques

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Abstract
This chapter will focus on the use of non-culture-based methods to identify prokaryotic human pathogens. The methods discussed rely primarily on nucleic acid detection and analysis for pathogens commonly encountered in diagnostic and clinical research settings. Diagnostic capabilities in clinical microbiology have exponentially grown due to the impact of molecular methods and tools, like (but not limited to) polymerase chain reaction (PCR) and DNA sequencing, for the detection of human pathogens. Increasingly, technologies such as mass spectrometry and next-generation sequencing that have been incorporated into routine research laboratory use for sometime will be implemented for routine use in a clinical setting along with PCR-based assays. In addition, the pace and continued development of molecular methods for clinical applications of pathogen detection may ultimately transform diagnostic microbiology into a "culture less" diagnostic science, in the future.

Introduction

This chapter will focus on non-culture-based methods to identify prokaryotic human pathogens, commonly encountered in diagnostic and clinical research settings. Diagnostic capabilities in clinical microbiology have exponentially grown due to the impact of molecular tools to detect human pathogens. New technology will continue to rapidly influence the diagnostic sciences in the future.

The current technologies have already improved diagnostic performance and stand ready to be used as tools to improve patient care and disease management. The benefits of these technologies are their speed, increased sensitivity and specificity, greater versatility, and more accurate results that are not dependent on isolation of pathogens in culture. Their limitations can include higher costs, inability to detect emerging genetic sequences that may not be targeted by primer or probe regions, and the inability to access cultivated bacteria for susceptibility testing or genotyping.

We will begin by reviewing real-time polymerase chain reaction (PCR), arguably the molecular method with most impact to the clinical laboratory in the last two decades. The focus of this chapter will be on the applied use of this technology and the circumstances and challenges faced by clinical and diagnostic laboratories as we strive to impact health care and serve as

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prudent stewards of resources. We will finish with a review of emerging technology and DNA sequencing methods that may impact clinical microbiology over the next two decades.

Real-Time PCR

Real-time PCR has quickly integrated into diagnostic microbiology practice as a rapid method to identify or quantify many common pathogens (Espy et al. 2006; Bravo and Procop 2009; Wittwer and Kusukawa 2011; Wolk and Hayden 2011). Related methods continue to evolve and redefine its role in clinical diagnostics. With the development of simpler and even more rapid methods, multi-target chemistries, and smaller instruments that integrate extraction and amplification, require minimal manipulation, and impose less-stringent biosafety requirements (Espy et al. 2002, 2006), real-time PCR methods are steadily impacting the laboratory's ability to improve patient care. Real-time PCR methods for pathogen identification are becoming commonplace, and there is an emerging trend to explore the use of this technology for determining antimicrobial resistance of organisms by analyzing the genes that confer resistance opposed to merely discerning the phenotypic determinants (Espy et al. 2006; Marlowe and Wolk 2006; Wolk et al. 2009c, d; Marner et al. 2011). The development of bacterial panels for direct detection of pathogens and their resistance mechanisms from blood cultures is likely to impact antimicrobial stewardship practices (Tenover and Rasheed 2004).

Such benefits currently decrease the time to result, the hands-on time demand, and the requirements for qualified molecular technologists to perform the testing, and although the evidence-based laboratory intervention literature is currently sparse, there is hope that these improvements will increase the cost-benefit of the test. There are now several assays and instruments available, which are classified as "moderate complexity" by the Clinical Laboratory Improvement Act, making them amenable to testing by most laboratory staff, 24/7 testing, and use in small community hospital laboratories and large reference laboratories alike (Espy et al. 2006; Bravo and Procop 2009). With the advent of completely integrated systems, the ability to apply such a technology to direct point-of-care testing and bedside identification is promising.

Currently, two main types of diagnostic real-time PCR assays are used for the detection of pathogens: (1) multi-step real-time PCR assays that require off-platform specimen processing, extraction of nucleic acid from the specimen, PCR assay setup and amplification/detection and (2) integrated assays that incorporate the three steps into the subsequent amplification/detection step. Examples of the latter type, in the order of appearance to the US market, include Cepheid's GeneXpert® system, Becton Dickinson's BD Max™ system, BioFire FilmArray®, and Focus Diagnostic's Simplexa Assays on the Integrated Cycler. The benefits of the integrated real-time PCR assays are decreased hands-on time, no manipulation of the extracted nucleic acids, which decreases the risk of contaminating the work area, and in most cases decreased reaction time and overall turnaround time.

Since there are hundreds of real-time PCR examples, all cannot be reviewed here and we refer readers to recent extensive review of methods (Wittwer and Kusukawa 2011; Wolk and Hayden 2011). Likewise, it is not possible to review the entire breadth and depth of laboratory developed tests (LDTs). We will review examples of common or emerging technology with impact to the clinical microbiology laboratories.

Multi-step Real-Time PCR Assays: Requirements for Upstream Nucleic Acid Extraction

Although, some of the newly developed PCR instruments are becoming fully integrated assays, there remains a large demand for multi-step PCR assays, which require upstream nucleic acid (NA) extraction; therefore, assays of this type continue to be created for Unites States and international patient care purposes. A summary of some common extraction methods used in diagnostic laboratories is presented in *Table 5.1*.

Nucleic extraction from clinical samples is accomplished by either manual or automated methods (see *Table 5.1* for commonly available methods). Determining which method to use is a critical step in successful nucleic acid purification and subsequent amplification assays. The extraction methods that may work for one pathogen and specimen type may not necessarily be effective for other specimen types and pathogens. In addition, structural characteristics of microorganisms and specimen-inhibiting substances can affect nucleic acid extraction.

Automated extraction systems tend to be more common and practical for clinical laboratories and are thought to provide several benefits over manual extraction methods. Automated NA extraction systems yield more reproducible NA recovery, while reducing the risk for contamination of other samples and the work environment (Hill 2011). Since they perform the NA extraction steps robotically, automated extraction instruments ensure greater workload efficiency; once the instrument is set up it requires very minimal manipulation or attention (Wolk et al. 2001; Knepp et al. 2003). An example of an automated extraction instrument, the Qiagen QIAsymphony SP system, is shown in *Fig. 5.1* and is designed to couple with the Qiagen Rotor-Gene Q real-time PCR instrument, pictured in front. Integrated extraction systems, such as the QIAsymphony, typically provide prefilled reagent cartridges, bar coding of samples and reagents, and touch-screen operation. Many systems allow continuous loading of 1–96 samples per run and can perform different purification procedures within the same run of 96 samples. In clinical laboratories, technology is expected to offer inventory control and full process documentation.

Despite the advantages of automated systems, there are circumstances when manual methods may be preferred or used in combination with automated methods. For example, manual methods may offer higher DNA yield or purity, or may be useful when microbial cell walls or inhibitors in specimen matrices become problematic. Manual extraction kits are usually less

Table 5.1
Manual and automated nucleic acid extraction methods for real-time PCR assays

	Kit/Platform	Manufacturer	Technology	Throughput samples/time	Specimen type
Manual	Highly pure	Roche Applied Science	Glass fiber fleece immobilized in a plastic filter	24/h	Serum, whole blood, plasma, urine, stool, sterile body fluids, respiratory tract specimens, genital and dermal swabs
	QIAamp	Qiagen	Silica gel membrane in column	DNA-24/h RNA-24/1.5 h	Serum, whole blood, plasma, urine, stool, sterile body fluids, respiratory tract specimens, nasal and fecal swabs
	IsoQuick	Orca Research	Ethanol precipitation	DNA-24/h RNA-24/2 h	Whole blood, plasma, stool, respiratory tract specimens, sterile body fluids, dermal, fecal, and genital swabs
	NucliSENS® miniMAG®	bioMeriéux	Proprietary Boom® technology with magnetic silica	DNA 12/batch RNA 12/batch	Most sample types (plasma, serum, whole blood, stool, respiratory samples, etc.) 50–1,000 μL
Automated	MagNA Pure LC	Roche Applied	Magnetic silica particles	32/90 min 8/30 min 96/2 h	Serum, whole blood, plasma, stool, sterile body fluids, respiratory tract specimens, dermal and genital swabs
	MagNA Pure Compact	Science			
	Cobas Ampliprep				
	BioRobot EZ1	Qiagen	Magnetic silica particles	6/20 min 24/1.5 h 96/1.5 h 96/2 h	Dermal, genital, and nasal swabs
	QIAcube				
	QIAagility				
	QIAsymphony				
	Need to add bioMerieux Easy Mag as they are common in clinical labs				
	Also M2000 from Abbott				
	NucliSENS® EasyMAG®	bioMeriéux	Proprietary Boom® technology with magnetic silica	DNA 24/batch RNA 24/batch	Most sample types (plasma, serum, whole blood, stool, respiratory samples, etc.) 50–1,000 μL
	ABI Prism 6100	Applied Biosciences	Silica fiber membrane	96 per 30 min	Serum, plasma, swabs, cell culture media, blood, semen
	ABI Prism 6700		Magnetic particles	96 per 74 min	
	MagMax Express				
	MagMax Express-96				
	Maxwell 16	Promega	Paramagnetic particles	16 per 30–45 min	Blood, serum, plasma, cells, fresh/frozen or FFPE tissue

expensive, but do require several manipulations, offsetting the savings in reagent costs. Manual methods are time-consuming and are generally less reproducible than automated methods.

Trending Real-Time PCR Assays

Simplexa Assays

Simplexa (Focus Diagnostics; Cypress, CA) PCR assays on the 4-channel 3M Integrated Cycler (*Fig. 5.2*) allow the processing of multiple pathogen target genes simultaneously. The Simplexa PCR assays are available in two formats, depending on the target pathogen(s): chemistries that require nucleic acid extraction prior to sample loading and direct specimen and reagent loading. PCR amplification and detection of up to 96 samples is rapid with results available in about an hour. The assays and instruments are extremely versatile with software programs for DNA and RNA, qualitative and quantitative PCR reactions, and a small instrument footprint. Their growing menu of molecular chemistries allow detection of *Clostridium difficile*, *Bordetella pertussis/parapertussis*, *Chlamydophila pneumoniae*, Group A streptococcus, *Mycoplasma pneumonia*, and *Borrelia* spp. from a patient specimen without requiring preliminary growth of the pathogen on agar media (Emmadi et al. 2011; Lanotte et al. 2011; Touati et al. 2009). The Simplexa assays that

Fig. 5.1
QIAsymphony SP instrument, an integrated extraction system from Qiagen, Valencia, CA, is designed to work in conjunction with the Qiagen Rotor-Gene Q, real-time PCR Instrument shown in front

Fig. 5.2
The 3M Integrated Cycler, sold by Focus Diagnostics

are fully integrated and do not require the upstream nucleic acid extraction steps allow for direct specimen testing, decreased turnaround time, and more widespread use by smaller laboratories where engineered molecular laboratory space is typically not available.

IntelligentMDx Assays

IntelligentMDx (Cambridge, MA) uses a unique proprietary bioinformatics process to provide analysis and verification of the genetic sequences, thus producing assays with the potential to quickly adapt to genetic change. The Intelligent MDx bioinformatics process PriMD has been specifically developed to address complex and evolving infectious targets. The PriMD system and IMDx processes enable a better understanding of nucleic acid interactions and thus can enhance the development of tests of any molecular application. Accelerated production of assays occurs by preselection of metrics, algorithms, and rules necessary to build robust, reproducible molecular assays for detection of pathogens. Theirs is the first process to use a systems approach and multifactorial computational capability that solves complex problems in assay commercialization. The *in silico* design and analysis process avoids the long trial-and-error approach and requires less wet lab experimentation prior to assay production. Built into this process is the integration of inputs from expert end-users to preset the clinical indications and specifications for an assay. In theory, the IMDx assay should be robust and easily adapted to emerging genetic sequences as pathogens evolve. Time will tell if the IMDx system will enhance clinical practice; yet, the strategy to prepare for genetic change, as pathogens evolve and mutate, and the ability to pretest assay design and check the cross-reactivity *in silico* prior to wet laboratory testing appear to add value to the existing commercial processes for diagnostic assay development and upkeep. Real-time PCR assays are designed with similar reaction conditions so that they can be multiplexed, run in parallel, or run separately, allowing clinical laboratories to optimize throughput and workflow.

Currently, there are two IMDx assays commercially available in Europe. The IMDx *C. difficile* assay for the Abbott *m*2000 is a PCR assay for the qualitative detection of conserved regions within toxin A and toxin B genes of the *C. difficile* genome in human symptomatic patients. The assay is intended for use on the Abbott *m*2000 RealTime system—a high-throughput system ranging from 24 to 96 samples/batch. The assay detects toxigenic *C. difficile*, including NAP1/027 hypervirulent strain and toxin B-variant type strains. In addition, the IMDx VanR assay detects *vanA* and *vanB* vancomycin resistance genes directly from human peri-rectal or rectal swabs and stool samples from patients at risk for Vancomycin Resistant Enterococcus (VRE) colonization.

Integrated PCR Assays

GeneXpert Assays

The Cepheid GeneXpert (Cepheid, Sunnyvale, CA) is a completely integrated single-use cartridge real-time PCR instrument for detection of pathogens directly from specimens. Specimen processing and decontamination occurs along with DNA extraction, amplification, and detection of the genetic target all

Fig. 5.3
Cepheid GeneXpert systems range in throughput from 1 cartridge to 96 cartridges

in the same cartridge. The result is rapid, that is, within 2 h, for all assays and requires minimal hands-on time. The system is composed of the GeneXpert instrument, with throughput designed to range from 1 to 96 samples simultaneously (*Fig. 5.3*) and a computer containing the required assay software to interpret the results. The various cartridges all incorporate *Bacillus globigii* spores, which serve as internal controls for extraction and amplification making the test technically effortless with little manipulation by the operator. Currently, Cepheid offers assay cartridges for several important pathogens (listed with reference to first published method comparisons), such as *Staphylococcus aureus* (Parta et al. 2009), *methicillin-resistant S. aureus* (Rossney et al. 2008), *C. difficile* (Novak-Weekley et al. 2010), vancomycin-resistant *Entercoccus* (Bourdon et al. 2010), Group B streptococcus (Gavino and Wang 2007), and *Mycobacterium tuberculosis* (Moure et al. 2011a, b), the latter assay being associated with a recent eruption of publications (Evans 2011; Hillemann et al. 2011; Ioannidis et al. 2011; Laudat et al. 2011; Lawn 2011; Marlowe et al. 2011; Miller et al. 2011; Spencer et al. 2011; Zeka et al. 2011).

The GeneXpert instrument is a random access instrument with STAT testing capabilities. Each individual reaction module can detect up to six genetic targets in a single cycler. Another advantage for pathogen detection is its multiplex capabilities, detecting the pathogen as well as some antibiotic-resistance genes. Strain typing capabilities are also available, as in the case of presumptive identification of *C. difficile 027/NAP1/B1 strains* (Babady et al. 2010). Methicillin resistance of *S. aureus*, vancomycin resistance of enterococci, and rifampin resistance of *M. tuberculosis* are discriminated by the system (Rossney et al. 2008; Shore et al. 2008; Wolk et al. 2009b, c, d; Bourdon et al. 2010; Dekeyser et al. 2011; Gazin et al. 2011; Hanif et al. 2011; Hillemann et al. 2011; Ioannidis et al. 2011; Marlowe et al. 2011; Marner et al. 2011; Miller et al. 2011; Moure et al. 2011a, b; Scanvic et al. 2011; Spencer et al. 2011; Zabicka et al. 2011; Zeka et al. 2011), but as with any laboratory method, vigilance is required, because antibiotic resistance genes can mutate quickly and exceptions can occur (Brenwald et al. 2010; Ciardo et al. 2010; Blanc et al. 2011; Shore et al. 2011).

Fig. 5.4
Idaho Technology's FilmArray

BioFire Film Array System: "The FilmArray"

The FilmArray system (BioFire Inc, Salt Lake City, UT) is a novel and completely integrated real-time multiplex PCR instrument (*Fig. 5.4*) that incorporates succeeding single-plex PCR reactions (Endimiani et al. 2011; Poritz et al. 2011). Originally launched for the detection of respiratory viruses (Loeffelholz et al. 2011; Pierce et al. 2011; Poritz et al. 2011; Rand et al. 2011), it does have the capability to identify bacterial pathogens, but

Fig. 5.5
The Luminex 100/200 system (*left*) and the Luminex MAGPIX instrument (*right*) provide detection of targets downstream to multiplex PCR

those targets have not yet been cleared by the Food and Drug Administration (FDA) for use in clinical laboratories. All nucleic acids are extracted and purified directly from the unprocessed sample. Then, the FilmArray performs a nested multiplex PCR assay. During the nested PCR, the FilmArray performs a single, large volume, multiplexed reaction with primers specific to several targets. Following the multiplex PCR reaction an individual single-plex second-stage PCR reaction detects the products that are amplified during the first round of PCR. Endpoint melting curve analysis of the amplicons and the FilmArray software generate a target result from the data. Targets in development include a 23 target gastrointestinal panel, 8 target sexually transmitted infection panel, and a 27 target Biothreat Panel.

Multiplex PCR

Bead-Based Assays

While none of the bacterial multiplex assays are currently cleared by the FDA, their widespread use and FDA product lines for Clinical Virology make it likely that panels of bacteriology targets will become the next stage of development. Luminex xTAG Technology (Luminex Corp, Austin TX) assays, performed on Luminex 100/200 instruments (*Fig. 5.5*), and Qiagen Liquichip (Qiagen Inc, Valencia, CA) assays are the two most common instrument platforms.

The Luminex assays are composed of a multiplexed PCR reaction that amplifies the regions of interest in the target pathogen genes followed by treatment with exonucleases to remove excess nucleotides and primers. Subsequently, a primer extension step that is specific for the pathogen target being analyzed is included in the PCR reaction. The 5' end of the primers is attached to a universal tag sequence. The 5' universal tag sequence is hybridized to the complementary anti-tag sequence coupled to a particular xMAP bead set. After performing a wash step, detection is initiated. The hybridized beads are read by aspirating the assay samples, one after another, into the reader. The hybridized beads are carried by a microfluidic system in a stream of fluid through the measurement cuvette where they are individually irradiated by dual reporter lasers. A red laser identifies each bead (or pathogen) by its color-coding, while a green laser detects the hybridization signal associated with each bead (indicating the presence or absence of a particular pathogen). Instrumentation reads the color-coded beads that attach to specific nucleic acid sequences, and results are analyzed by specific data analysis software (Dunbar 2006; Dunbar and Jacobson 2007a, b).

Luminex xMAP technology is supported by the principles of flow cytometry, and the major advantage of the technology is centered on the bead color-coding, which enables up to 100 possible separate interactions that can be analyzed simultaneously (Dunbar and Jacobson 2007a, b). The entire procedure can be completed in approximately 5 h making it a quick method to identify multiple genetic targets. Other significant advantages over traditional methods include: the speed and accuracy, the versatility of one system to analyze several target types, and the standardization that is observed because of the reproducibility of high-volume production of xMAP microspheres within a single lot. The new Luminex instrument, the MAGPIX (*Fig. 5.5*), is a compact instrument launched to support bacterial panel testing.

Luminex xTAG panels are available outside the USA as xTAG® Gastrointestinal Pathogen Panel (xTAG GPP) to detect *Salmonella, Shigella, Campylobacter, Yersinia enterocolitica*, Enterotoxigenic *E. coli* (ETEC), *E. coli* O157, Shiga-like Toxin-producing *E. coli* (STEC), *Clostridium difficile* Toxin A/B, and *Vibrio cholera* in addition to viruses and parasites associated with gastrointestinal disease (Battaglia et al. 2011; Dunbar and Jacobson 2007a, b). The multiplex aspect of the xMAP panels enables the identification of infections due to multiple pathogens with rapid diagnostic results for diagnosis and patient management.

Qiagen QIAplex Panels are commercially available for the detection of gram-positive cocci in clusters (StaphPlex) and pathogens associated with community-acquired pneumonia (Resplex I: *Mycoplasma pneumonia, Chlamydophila pneumonia, Legionella pneumophila, Streptococcus pneumonia, Neisseria*

meningitidis, and *Haemophilus influenza*) (Tang et al. 2007; Benson et al. 2008; Brunstein et al. 2008). The Staphplex differentiates between 18 different targets and provides species-level identification of *Staphylococci*, as well as the identification of antimicrobial resistance determinants and genes encoding Panton-Valentine leukocidin (PVL) (Tang et al. 2007). The QIAplex assays are performed on the Qiagen LiquiChip 200 Workstation, a branded form of the Luminex 100/200 instrument.

Transcription-Mediated Amplification (TMA)

Transcription-mediated amplification (TMA) is the synthesis of a DNA strand complementary to a target nucleic acid, usually RNA (Kwoh et al. 1989). The newly synthesized cDNA becomes the template for the subsequent in vitro transcription reactions. An excess of RNA is created and is used as the substrate for another round of transcription. Much like PCR this process continues at an exponential rate and several million copies of the target can be created. The amplified target is then detected using various methods. Gen-Probe (Gen-Probe Incorporated, San Diego, CA) has launched the PANTHER, the smaller version of the TIGRIS, a fully automated random-access TMA instrument for high-volume testing. Like the TIGRIS, the PANTHER also utilizes Gen-Probe's target capture technology for sample processing, TMA amplification, and HPA detection and decontamination into one instrument (Graber et al. 1998). The PANTHER boasts the ability to test for four targets from one specimen using Gen-Probe's APTIMA assays for *N. gonorrhoeae*/*C. trachomatis*, human papilloma virus, and *Trichomonas vaginalis*, providing a multi-targeted STD testing option. Currently, the assays are limited to endpoint assays; however, the PANTHER boasts of assays currently in development with real-time TMA capabilities to compete with quantitative PCR applications (Templeton et al. 2001).

Loop-Mediated Isothermal Amplification of DNA (LAMP)

Loop-mediated isothermal amplification of DNA (LAMP) is a novel method that is quickly impacting molecular detection of pathogens (Notomi et al. 2000). This method rapidly amplifies DNA under isothermal conditions with high specificity and efficiency. The *illumigene* (Meridian Bioscience Inc, Cincinnati OH) DNA amplification assays use specific primers that continuously facilitate isothermal amplification producing the by-product, magnesium pyrophosphate, in the reaction. The accumulation of magnesium pyrophosphate results in a precipitated solution. The resulting turbidity from the precipitate can be detected by the instrument, which functions as both an incubator and turbidometer (Mori et al. 2001). When the pathogen, specifically the gene target of interest, is not present, precipitate does not form and the amplification reaction is negative. When the gene target is present, the resulting amplification produces a turbid solution and a positive result. The *illumigene C. difficile* assay detects the pathogenicity locus (PaLoc) of toxigenic *C. difficile*, by targeting a region of *tcdA*, and the illumigene Group A Streptococcus assay targets the pyrogenic exotoxin B (speB) gene, with results available in about an hour (Kato and Arakawa 2011; Lalande et al. 2011; Noren et al. 2011). LAMP assays have also been published for *Legionella* spp., Group B strep, *Vibrio* spp., *Salmonella, Shigella, Listeria monocytogenes, Staphylococcus, Mycobacterium, Neisseria*, and *Brucella*.

During the LAMP process, six different primers, specifically designed to recognize six or eight distinct regions on the target sequence, participate in amplification and detection of the target sequence, which can be completed in a single step (Nagamine et al. 2001; Tomita et al. 2008). A DNA Polymerase with strand displacement activity forms a dumbbell-like DNA structure, which is the starting structure for LAMP cycling. A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. A primer anneals to the 5' end of the single-stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary regions. Next, starting from the 3' end, DNA synthesis starts using the DNA itself as a template and releases the complementary strand. The released single strand then forms a dumbbell-like structure at both ends. The steps are repeated through self-primed DNA synthesis starting from the 3' end. An additional primer anneals to the 3' end and primes strand displacement DNA synthesis, releasing the primed DNA strand with a stem-loop structure. Accordingly, similar stem-loop structures as well as the dumb-bell structure are produced. Another primer anneals to the single-stranded region of the produced stem-loop, and DNA synthesis continues by displacing double-stranded DNA sequence. Various-sized structures made up of alternately inverted repeats of the target sequence on the same strand are created. This cycling results in the amplification of the target.

Direct Probe Technology

FISH

Fluorescence in situ hybridization (FISH) utilizes fluorescent dyes linked to nucleic acid probes that target conserved molecules found abundantly in microorganisms (Muresu et al. 1994; Hayden, Kolbert et al. 2001a, b). Detection and identification can be accomplished without cultivation, and this method could potentially be applied directly to almost any sample. Following a simple hybridization procedure, fluorescence of probe-target hybrid molecules is visualized with a fluorescence microscope. Identification of samples containing mixed organisms can be achieved, although individual probes for each pathogen are required. If commercial or published probe

sequences are not available, they can be obtained by designing probes specific to the target sequence of interest (i.e., rRNA or DNA). Optimal probes for FISH range between 15 and 25 nucleotides to provide high specificity and stringency during the hybridization process, resulting in greater discrimination between closely related organisms. The number of copies of the target molecule(s) present in the cell determines the detection limit of the target probe, with $\geq$ 1,000 ribosome molecules needed for successful hybridization. There are currently no DNA-in situ hybridization assays that are commercially available.

PNA-FISH

Peptide nucleic acid (PNA) probes are fluorescent probes attached to PNA molecules that have a non-charged polyamide (peptide) backbone as opposed to the negatively charged sugar-phosphate backbone of DNA and RNA. PNA probes have the same nucleotides (adenine-A, guanine-G, cytosine-C, and thymine-T) and follow the same base-pairing rules allowing them to bind normally to complementary sequences of nucleic acid. There are several advantages to the structure of PNA probes over nucleic acid probes: (1) PNA probes bind stronger in the hybridization reaction than the DNA probe; PNA chemistry hybridizes more readily to the target of interest due to very small dye-labeled probes, approximately 12–20 nucleotides in size. (2) The neutral charge of the PNA backbone reduces natural repulsion with negatively charged backbones (i.e., DNA), and therefore, they bind tightly. (3) Greater specificity as a result of more precise binding to the target and exquisite base-discrimination. (4) PNA probes provide greater sensitivity; they add very low background with better visualization of the fluorescent signal.

Advandx, Inc. (Woburn, MA) commercially markets FDA-cleared PNA-FISH kits for clinical diagnostic use and identification of *Staphylococcus aureus*, *Enterococcus* spp., *Mycobacterium* spp., *Streptococcus agalactiae*, *E. coli*, *Klebsiella pneumoniae*, *and Pseudomonas aeruginosa*, among other targets (Chapin and Musgnug 2003; Stender 2003; Forrest 2007; Forrest et al. 2008; Hensley et al. 2009; Morgan et al. 2010, 2011). These kits are relatively easy to use and are now widely used as a method for rapid identification with an approximate turnaround time of about 1.5–3.5 h. A current disadvantage to the use of PNA-FISH is the ability to rapidly infer resistance to antimicrobials, based on identification of the organisms and local antibiograms.

For the identification of fastidious, non-cultivatable, and slow-growing pathogens, PNA-FISH is a quick and easy method. This method relies solely on the availability of nucleic acid targets rather than phenotypic expression of markers that are usually targeted by more extensive and time-consuming immunological methods. More cost-effective than PCR techniques, PNA-FISH does not require extensive knowledge or experience in molecular techniques. The chemistry of FISH probes makes this technology just as sensitive but more specific than gram staining and direct fluorescence staining, and will conceivably propel PNA-FISH to a more routinely used diagnostic method.

Emerging Technologies

Mass Spectrometry

Promising new broad-based techniques have merit, based on their ability to identify difficult-to-culture organisms or newly emerging strains, as well as their capacity to track disease transmission. Both techniques revolve around the mass spectrometry technology: matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) and polymerase chain reaction electrospray ionization mass spectrometry (PCR-ESI/MS). The use of mass spectrometry methods is challenged by the high costs of instruments, yet there is potential for these technologies to supplant the foundation of clinical microbiology, replacing most of biochemical testing as we know it. Overall, both methods show promise for both routine and, in some cases, epidemiological use in hospital settings. These two techniques, in a direct comparison, show no statistically significant differences in their performance, but PCR-ESI/MS is substantially more expensive and has greater capabilities.

Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Spectrometry

Mass spectrometry methods leverage their high sensitivity with links to large microbial databases, which enable users to obtain species-specific spectra that can be used to reproducibly identify microorganisms. MALDI-TOF/MS requires only media to culture the organism and a small quantity of matrix (typically less than 1 μg/sample), the cost of which is negligible.

Analysis of whole cells was first proposed in 1975 to study the biomarker profile of various bacterial species following pyrolysis-mass spectrometry for low molecular weight products (Anhalt and Fenselau 1975). However, it was not until 1996 that the first MALDI-TOF/MS experiment was successful in identifying bacteria directly from whole colonies based on protein biomarkers (Claydon et al. 1996; Holland et al. 1996). A great number of developments have been made over the last decade on whole organism MALDI-TOF/MS. The protein biomarkers that are measured in mass spectrometry of microorganisms are highly expressed proteins responsible for housekeeping functions, such as ribosomal components, chaperones, and transcription/translation factors.

Relying on identification of protein profiles, MALDI-TOF/MS derives its information from profiles of highly conserved bacterial proteins. For MALDI-TOF, the protein profiles are generated from direct ionization of an intact colony or a bacterial protein extract, after manual extraction. Identification occurs after correlating a protein's spectral signature to a database of spectra collected from reference strains. In its current form, MALDI-TOF/MS requires subculture prior to identification. An advantage of MALDI-TOF/MS is that it does not require batching.

MALDI-TOF/MS is widely used, for its high accuracy, low consumable cost, and speed of analysis. A typical experiment

consists of outgrowth of bacteria, colony selection and placement on a target, addition of matrix, and analysis with MALDI-TOF/MS. Mass spectrometry identification is broadband, such that it can measure multiple analytes simultaneously, does not require prior knowledge about the organism, and is both fast and sensitive by not requiring a pre-fractionation step. It generally measures all *m/z* between 2 and 20 kDa. Several reviews are available on this topic for a more in-depth overview (Fenselau and Demirev 2001; Lay 2001; Demirev and Fenselau 2008).

Limitations of Mass Spectrometry–Based Technologies

The major barrier to implementing mass spectrometry–based technologies to the clinical laboratory is the cost associated with capital purchase of a dedicated mass spectrometer (Wolk and Dunne 2011). Thus, as is true for most newly emerging technologies, these methods may be limited to larger reference laboratories until the cost-benefit is fully evaluated or until smaller more affordable benchtop options are developed. Nevertheless, the potential for high analytical sensitivity, accuracy, and broad-based applicability of mass spectrometry–based platforms have the potential to change the practice of clinical microbiology in the future and offers the promise of off-setting some portion of the complexity and workload in clinical microbiology.

Fig. 5.6
The Bruker MicroFlex Mass Spectrometer

Bruker MALDI Biotyper

Commercial mass spectrometry systems exist that can integrate with traditional antimicrobial susceptibility systems. Among them, the MALDI Biotyper from Bruker Daltonics (*Fig. 5.6*) is among the most widely tested and has proven accuracy in the identification of bacteria (Maier and Kostrzewa 2007). The system is partnered with Becton Dickenson Phoenix System, which supplies antimicrobial susceptibility results that pair with bacterial identification. Evaluations of the system show high accuracy, even for rare or fastidious bacteria (Alanio et al. 2010; Saffert et al. 2011), which represent a challenge to clinical laboratories using phenotypic identification evaluation due to their limited biochemical reactivity. Identification of yeast has also been reported (Marklein et al. 2009). The method finds common use in Europe where clinical microbiologists are using it to rapidly identify microbial colonies isolated from culture.

A few reports show promise for the identification of microorganisms using MALDI-TOF/MS without subculture (Ferreira et al. 2010; Moussaoui et al. 2010; Prod'hom et al. 2010; Stevenson et al. 2010). Recent evaluations show promise, but the methods were limited by the need for a large number of cells—adaptations showed successful identification for only approximately 80 % of blood cultures. A promising new technology, the MALDI Sepsityper system (Bruker Daltonics), is under evaluation and aims to identify bacteria and yeast directly from positive blood culture bottles. If successful, the MALDI Sepsitype system has the potential to reduce the identification time for many different bacterial species.

It is important to note some of the current limitations associated with MALDI-TOF/MS. The need for outgrowth of organisms from potentially contaminated material is still required in order to obtain isolated colonies of organisms as the technique's ability to resolve mixtures is lacking. Additionally, a high number of bacterial cells are required for identification, such that a whole intact colony is typically used for analysis, limiting the ability to rapidly identify microorganisms directly from biological fluids where the bacterial count is expected to be relatively low. Research is currently being done in the research field to mitigate some of these requirements. Finally, until accurate determinations of resistance factors can be made, parallel culture-based recovery of positive blood cultures will most certainly be required for the foreseeable future for antimicrobial susceptibility testing.

Vitek MS-Biomerieux/Shimadzu AXIMA Microorganism Identification System

Another MALDI-TOF system, the bioMerieux Vitek MS (*Fig. 5.7*), shows similar capabilities to the Bruker system; no statistically significant difference was identified between the two platforms for general bacteria (Cherkaoui et al. 2010). While

Fig. 5.7
The bioMériuex, Vitek MS with disposable array

fundamentally equivalent, the Vitek MS differs in its instrumentation scoring algorithm and databases. The mass spectrometer, designed by Shimadzu, is partnered with bioMerieux systems for antimicrobial susceptibility testing. The system is supported with a bacterial database from Anagnsotic Biosystems.

PCR Mass Spectrometry: Abbott IBIS

Brief History of PCR-ESI/MS

Emerging new technology, like Polymerase Chain Reaction Electrospray Ionization Mass Spectrometry (PCR-ESI/MS), sold by Abbott Molecular (DesPlaines, IL) has the potential to identify nearly all known human pathogens directly from clinical specimens and the potential to identify genetic evidence of undiscovered pathogens (Taylor et al. 2001) or genetic changes to diagnostic targets. Therefore, if a novel microbial genetic sequence is uncovered, it is reported and accompanied by information that describes its relationship to closely related organisms. The Ibis technology was originally developed for biodefense and public health safety, the first use of PCR-ESI/MS was reported to be in 2005, and the technology was further advanced in 2008 (Hofstadler et al. 2005).

The T5000™ Biosensor System was the PCR-ESI/MS prototype instrument (Ecker et al. 2008). Originally created by Ibis Biosciences (Carlsbad, CA), the system couples equipment that desalts PCR products to a time-of-flight (TOF) analyzer, providing mass accuracy of sufficient resolution to discern the base compositions of the amplicons produced in multiplex PCR. The Ibis technology has advanced the prototype to the refined current version of the instrument, now called the PLEX-ID (*Fig. 5.8*). Abbott Molecular currently supports reagents that are classified as "not-for-diagnostic-use" (NFDU), but the system is being evaluated in public health and military laboratories as well as several large clinical reference laboratories.

The PLEX-ID enables rapid identification (approximately 6–8 h, depending on nucleic acid type) and characterization of bacterial, viral, fungal, and other infectious organisms as well as analysis of human DNA, achieving a wide breadth of pathogen identification. As the PLEX-ID is relatively new, the published literature summarized in this review describes research performed on its prototype the Ibis T5000 (Hofstadler et al. 2005; Ecker et al. 2008).

Fig. 5.8
The Abbot PLEX-ID system for PCR/ESI-MS

Principles of PCR-ESI/MS

1. Sample Preparation
   As with other molecular sample preparation methods, nucleic acids must be extracted, either directly from clinical specimens or from cultivated microbial isolates. A wide variety of DNA extraction methods, both manual and automated, were successfully used to effectively isolate nucleic acid from bacteria prior to the upstream PCR-ESI/MS assays (Hannis et al. 2008; Baldwin et al. 2009; Ecker et al. 2009; Hall et al. 2009; Wolk et al. 2009a; Crowder et al. 2010; Emonet et al. 2010; Endimiani et al. 2010; Eshoo et al. 2010; Whitehouse et al. 2010; Kaleta et al. 2011; Massire et al. 2011). Sample types include bacterial colonies and bacterial cultures, clinical samples (throat swabs, nasal swabs, nasopharyngeal swabs, nasal washes, sputum, and skin swabs), etc.
2. Multiplex PCR Prior to PCR-ESI/MS
   After sample preparation, nucleic acids are dispensed into wells of a microtiter plate for downstream multiplex amplification. Each well contains one or more pairs of broad-range or target-specific primers (depending on the assay type) and other PCR components, to support amplification

via multiplex PCR. The PCR-ESI/MS assays contain a variety of purposefully designed primer sets that interrogate common conserved and variable sequences among various classes of organisms. Small amplicons (80–150 bp) of various sizes are created, depending on the species of microbes present in the original sample. Amplification produces genetic products, unique within a group of organisms, microbial domain or microbial division.

Proper amplification relies on the genetic similarities in microbial genomes. For example, bacteria have highly conserved sequences in a number of chromosomal locations, including the universally conserved regions of ribosomal, other noncoding RNAs, and essential protein-encoding genes. These conserved sequences, intercalated with regions of sequence diversity, serve as priming sites for broad-range primers to amplify sequences of various sizes and compositions.

A typical PCR-ESI/MS assay uses a master-mix of reagents, optimized for use in an amplification reaction using multiple primers. Multiplex amplification generally occurs under conditions of low stringency to allow for nonspecific primer annealing, a parameter that supports the mismatch amplification required to identify unknown genomic mutations within the targeted regions. Amplification typically requires a two-step PCR protocol to enrich amplification.

3. Desalting and Electrospray Ionization-Time of Flight
After amplification, products undergo an extensive desalting process prior to injection into a mass spectrometer. Negative ion-mode electrospray ionization is used for analysis. Electrospray ionization moves charged amplicon into the mass spectrometer, via processes optimized to detect negatively charged oligonucleotide ions. By definition, mass spectrometry (MS) is a sensitive analytical technique that measures the *mass/charge* (*m/z*) ratio of charged particles, and can do so with high mass-accuracy. To optimize the process, the electrospray ionization separates double-stranded amplicon, and intact strands are pulsed into the flight tube of the mass spectrometer under high vacuum and are separated based on *m/z* where lower *m/z* ions travel faster and reach the detector earlier than ions with higher *m/z*.

The resulting mass spectrum, which consists of a distribution of peaks corresponding to different charge states, is then "deconvoluted," a process by which the spectra are mathematically simplified. The mass of the intact amplicon is calculated and signals from low molecular weight chemical noise are eliminated on the ESI-TOF instrument. Once signals are digitally processed, the results are listed as highly accurate molecular weights (masses) of the forward and reverse strand of each amplicon.

Once the amplicon masses are established, software algorithmically predicts their base composition. Calculations rely on the known masses of the four nucleic acids (adenine, guanine, cytosine, and thymine bases) present in the amplicon and the knowledge of DNA strand complementarity. A joint least-square algorithm correlates potential organism identifications across multiple genetic regions to reveal the unique identity of the microbe. Since several genetic regions are amplified, multiple genetic compositions are compared to a curated database to narrow the genetic possibilities to one unambiguous genetic identity. The regions amplified vary by organisms and are assay dependent.

4. Curated Amplicon Reference Database
Undoubtedly, a key element of the PLEX-ID system is the Ibis Biosciences-curated database of genomic information that associates base composition with the identity of thousands of organisms. Despite the enormous class diversity of organisms, an Ibis database containing carefully selected and curated genetic sequences allows the interrogation to produce relevant and accurate results for surveillance, epidemiology, forensic, and biological research. The database is regularly updated with the latest information for newly identified microorganisms, based on input from users and review of web-based genomic databases.

For sequence matching to occur, the software bases its deductions on several known assumptions: (1) The potential genetic targets are known and present in each multiplex primer set. (2) Base compositions of forward and reverse amplicon strands must be complementary; therefore, for a particular *m/z* ratio, there are a limited number of possibilities. (3) For a single, unique base composition, only a small number of possible base compositions must be consistent with each measured mass. Finally, the unique base composition of an amplicon is compared to the Ibis database of over 750,000 entries, which links the base composition for each primer pair to a small list of candidate microbes.

Workflow Overview

The PLEX-ID Rapid Bioidentification System provides complete information management for instrument control, tracking, mass spectral signal processing, and analysis from original samples to organism identification. After the extraction and amplification step, the PLEX-ID process is fully automated with regard to sample tracking, instrument control and robotics, data processing and analysis, and comparison to the curated database. The entire PLEX-ID process requires approximately 6 h per batch of specimens tested, including 4 h for amplification. Processing events, which are the time-limiting factors, such as PCR amplification and sample dispensing, are scheduled in parallel to maximize the throughput of the instrument system. Post-PCR on-instrument throughput is approximately 1.25 h, such that approximately 20 plates or more can be analyzed each day, and, depending on the assay, can process between 6 and 12 patient samples per plate, resulting in 120–240 samples per day maximum. During the PLEX-ID testing process, robotics and sample tracking register and verify plate barcodes to ensure accurate tracking of the physical transfer of samples during the spray and data acquisition phases. The system also automatically triggers spectral processing and data analysis of the raw data. Coupled together, the instrument enables a continuous, automated workflow from physical sample to resulting organism identification.

Although the PLEX-ID system is in its infancy, it is a technology that could shift the way microbiologists think about the diagnosis of infections. It appears accurate and flexible, and has potential for implementation in large reference laboratories or in public health surveillance.

DNA Sequencing

Sequence-based identification, strain typing, and comprehensive isolate fingerprinting can be utilized for the tracking and control of pathogenic organisms; therefore, molecular methods like sequencing have been a widely used tool for epidemiological fingerprinting of isolates important to public health. However, in 1995, the first two complete DNA sequences of the bacterial genomes *Haemophilus influenzae* and *Mycoplasma genitalium* were revealed by scientists at the institute for genomic research (TIGR), followed in 1996 by the first complete genome sequencing of an archaea, *Methanococcus jannaschii*. Since then automated sequencing using fluorescent chain-terminating nucleotides during elongation of a DNA template (referred to as Sanger sequencing) has made it possible to sequence the genomes of many pathogens and environmental microorganisms rapidly both in research and diagnostic settings. A major advantage of sequencing is that it allows for the comparison of genomes identifying differences between virulent and avirulent medically important pathogens. The comparison data generated at the genomic level of a pathogenic species can provide information about host or tissue specificity and antimicrobial resistance patterns.

Short Read Pyrosequencing

Pyrosequencing is a DNA sequencing method that detects released pyrophosphate as DNA polymerase acts on a target sequence. Pyrosequencing assays have been developed for several applications, including genotyping, single nucleotide polymorphism (SNP) detection, and microorganism identification. Pyrosequencing is real-time sequencing and thus can be used for not only microbial species identification but for the discrimination of point mutations that confer antimicrobial resistance. There are several major advantages of pyrosequencing: it is sensitive, rapid, simple, customizable, highly specific and also provides high throughput (Quiles-Melero et al. 2011; Jordan et al. 2005, 2009; Zhang et al. 2011).

The principle of the pyrosequencing reaction relies upon annealing of a target primer to a single stranded template. After binding, DNA polymerase acts to elongate the sequence, much like PCR. As the polymerase adds deoxynucleotides, in a predefined dispensation pattern, the primers extend and release inorganic pyrophosphate (PPi) as part of the synthesis. Inorganic pyrophosphate is converted into ATP by ATP-sulfurylase. The accumulating ATP is utilized by firefly luciferase in a reaction to produce light. The pattern of light signals, correlating to the added nucleotide, is a true representation of the sequence that is being analyzed. The amount of light generated by the reaction is directly proportional to the PP_i produced or the number of nucleotides that were incorporated into the newly generated sequence. Any nucleotides that are not incorporated into the new sequence are enzymatically degraded. The sequence generated by pyrosequencing is quantitative in nature. Select pyrosequencing methods utilize a solid-phase template preparation of streptavidin beads that interact with biotinylated 5' ends of single-stranded DNA as opposed to the enzyme-based template preparation.

The reaction is rapid, as it takes less than 2 h to result, and DNA extracted from multiple microbial species in a single specimen can be sequenced in the same assay batch. Still, disadvantages to the technology consist of the method being relatively hands-on, labor intensive, and open to potential contamination events. In addition, the sequencing reaction is only able to produce sequence for short targets, in some cases <100 nucleotides. Qiagen Pyromark (Qiagen Inc., Valencia, CA), a commercial system, is currently available in three system formats—Q24, Q96, and Q96MD.

Next-Generation Sequencing

Traditionally, sequencing methods were based on slab gel electrophoresis that separated DNA fragments produced from in vitro Sanger sequencing reactions. These methods involved laborious molecular cloning and PCR techniques. Several instruments have recently been introduced that utilize molecular cloning techniques (minus any bacterial vectors), PCR amplification, and capillary electrophoresis to produce sequencing data. Next-generation sequencing takes amplified templates isolated by molecular cloning techniques within the instrumentation and discriminates the incorporation of nucleotides (adenosine, thymine, cytosine, or guanine) on two-dimensional arrays. Digital imaging monitors the nucleotides as they are incorporated on a cycle-by-cycle basis. Next-generation sequencing provide high throughput results, and these DNA sequencers can synthesize >100 megabases of DNA sequences per assay, drastically shortening the time required to sequence an entire bacterial genome. Though the principles are similar, the currently available systems use sequence synthesis chemistries that are distinct to each system.

The Illumina MiSeq (Illumina Inc., San Diego, CA) system uses fluorophore-labeled nucleotides with terminating 3' ends that are added to the end of a primer one at a time. The addition of the fluorophore-labeled nucleotides results in laser excitation of the fluorophore and detection by the imaging system; subsequently the 3' end terminators and fluorophore are cleaved, allowing for the addition of the next nucleotide. The nucleotides equally compete for incorporation resulting in superior strain specificity. MiSeq permits ~150 bp paired sequence reads and results can be achieved in less than 8 h. Any genome can be sequenced using this technology with the ability to uncover genetic variants and antimicrobial resistance mechanisms.

The ABI SOLiD (Applied Biosystems, Foster City, CA) utilizes a similar reaction chemistry of labeled nucleotides with blocked 3' termini that are enzymatically attached to the end of a primer; however, the central dinucleotide is flanked with universal and degenerated nucleotides. This provides added specificity to the sequencing chemistry. The nucleotides are fluorophore labeled and added in blocks of eight nucleotides or octamers that specify the sequence for two nucleotides. Once added and the fluorophore is detected, the entire octamer is removed. The subsequent ligation reactions use smaller and smaller primers until ultimately a 35 bp sequence is read. The ABI SOLiD sequencers can sequence multiple whole bacterial genomes in less than 2 weeks as opposed to months.

Roche 454 instruments (Roche Diagnostics Corp, Branford, CT) are high-throughput solid-phase pyrosequencers, with long sequence reads of approximately 400 bp with up to 99 % accuracy. For microbiology, the Roche GS Junior benchtop system can be used for whole genome sequencing. In these systems, beads are loaded onto a nanoscale well device with a single capture bead per well and all the required enzyme reagents. A camera detects chemilumenescent light signals as complementary nucleotides, which flow sequentially across the wells and are incorporated into the template strand attached to the beads. Applications for the 454 pyrosequencers include, but are not limited to, pathogen detection, direct amplicon sequencing, de novo microbial genome sequencing, and metagenomic characterization of environmental samples.

The Ion Torrent Personal Genome Machine (Life Technologies, Grand Island, NY) is centered on semiconductor technology as opposed to light chemistry to acquire genomic sequences (*Fig. 5.9*). Chemical signals are translated into digital signals which are then detected by the proprietary ion sensor. The chemical signals are generated as nucleotides are added to the nucleic acid strand. When a complementary nucleotide is incorporated the release of hydrogen ions results in a pH change that is ultimately read as a voltage change. The chip is flooded sequentially with nucleotides and if the nucleotide is not a match, no change in voltage occurs. If two or more nucleotides are complementary to the strand, the voltage change is multiplied proportionally to the number of correctly added nucleotides. Recording of sequence synthesis by the ion sensor is analogous to a small pH-meter and is extremely rapid since laser scanning and cameras are not necessary for synthesis detection. Thus sequence acquisition occurs rather quickly, in about 2 hours time.

Fig. 5.9
Ion Torrent sequencer (Life Technologies)

Summary

Although molecular and protein-based testing is becoming commonplace, and offers the prospect for major improvements in speed and accuracy of clinical microbiology methods, in the short term there remains a strong need to continue culture-based testing. Culture is still required for most antimicrobial susceptibility testing and will still be advantageous for identifying genotypes and mutated strains that may not be detected by molecular or protein-based techniques. While there are many advantages of rapid broad-range testing, the cost of implementation of molecular methods and mass spectrometry methods will keep traditional culture and phenotypic and biochemical characterization methods available in the clinical laboratories. Nevertheless, the trend toward multiplex and broad base testing is primed to continue and should greatly impact the practice of clinical microbiology in the future.

Specifically, today's molecular diagnostic platforms are providing unparalleled clinical value, especially for diagnosis of infectious diseases. Moreover, these molecular diagnostic technologies are smaller, less expensive, and more user-friendly than their predecessors, driving the adoption and utilization of new innovative tests. Through prudent and evidence-based use of new technology, we can improve both the care and the economics of patient care. Laboratory tests that streamline diagnostic laboratory processes can significantly impact our ability to advance clinical practice.

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6 Bacterial Adhesion

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Abstract
Although bacteria adhere to many different types of surfaces present in their habitat, this review focuses on bacterial adhesion to animal cells and tissues as a first step in the ability of pathogens to colonize and subsequently cause tissue damage. Accordingly, basic principles that govern the interaction of bacterial adhesins to their cognate receptors on animal cells are presented, such as fimbriae as adhesin structures. Significantly, we discuss the types of receptor-adhesin relationship, the phenomenon of multiple adhesins each specific for distinct receptors produced by pathogenic clones, the identity of glycoconjugates as receptors for lectins that serve as adhesins, and the interaction of bacterial adhesins with the extracellular matrix on animal tissues. Finally, a specific section is devoted to recent developments in preventing or treating infections by blocking bacterial adhesion to animal cells. In this context, a review of the different approaches of antiadhesion therapy is discussed, including the use of receptor and adhesin analogs, dietary constituents, sublethal concentrations of antibiotics, and adhesin-based vaccines. A discussion and summary of these topics focus on the in vivo data, including human trials, whereby plant extracts are used as a source of antiadhesion agents to prevent or treat urinary tract infections caused by *Escherichia coli* and infectious gastritis or peptic ulcer diseases induced by *Helicobacter pylori* and to maintain oral health.

Introduction

Although it is now well established that virtually all bacteria that inhabit the planet adhere to target substrata in order to colonize a specific niche, this chapter will focus on adhesion of pathogenic bacteria to tissues and animal cells. Nevertheless, many of the basic concepts that govern the adhesion process are common. The process of adhesion occurs in distinct steps. Because both the surfaces of both the bacteria and its target animal cell are negatively charged at pHs encountered at sites of infection, the bacteria must first overcome the repulsive forces that separate the two surfaces. This is thought to be accomplished by hydrophobic determinants, also known as hydrophobins, that bind the bacteria to hydrophobic domains on host cells and which lead to effectively weak and reversible adhesion interactions (Rosenberg and Doyle 1990; Hasty et al. 1992). This reversible adhesion is followed by a firmer type of adhesion, which involves specific surface molecules that bind in a complementary manner (Ofek et al. 2003a). It is now well established that to initiate infection at a particular site, bacteria must adhere to host cells or to extracellular material covering these cells (Ofek and Doyle 1994f; Ofek et al. 2003a). The mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts are the most common portals by which infectious bacteria enter the deeper tissues of a mammalian host. Thus, adhesion to the epithelial cells of these mucosal surfaces followed by colonization of the mucosal tissue is considered to comprise the first stages of the infectious process. In numerous cases, the firm adhesion by bacteria is mediated by special protein molecules (known as adhesins) associated with proteinaceous organelles (known as fimbriae or pili). These adhesins, which are on the surface of the infectious bacteria, combine with complementary structures on the mucosal surfaces known as receptors. Adhesion to mucosal surfaces offers the infectious agent a number of advantages: It provides the bacteria with a mechanism (1) to firmly attach and resist dislocation by the hydrokinetic forces from fluids that regularly wash these surfaces, (2) to better access nutrients released by the host cell, and

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(3) for increased protection from deleterious effects of antimicrobial agents in the surrounding milieu (Zafriri et al. 1987).

In this chapter, we review the current state of knowledge of bacterial adhesins and their mucosal cell receptors. We will then discuss how the knowledge gained provides a basis for the development of antiadhesion agents that can block and even reverse bacterial colonization of mucosal surfaces before tissue damage is initiated.

Bacterial Adhesins and Their Cognate Receptors

In order for a molecule to function as an adhesin that promotes binding of pathogenic bacteria, it must be presented at the surface in such a manner that the binding domain is capable of docking with its complementary host cell receptor. In most cases, the adhesins comprise assembled structures that are tightly anchored onto the bacterial surface. The adhesins of gram-positive bacteria are anchored on the surface by a number of different mechanisms (Ofek et al. 2003c). One prominent mechanism involves the sortase-mediated anchoring of the assembled adhesin through covalent linkage via its LPXTG motif to the cell wall peptidoglycan. One example of this type of anchoring process is the F protein of *S. pyogenes* (see *Table 6.2*). Another mechanism is the association of adhesins with surface proteins, as is the case of lipoteichoic acid (LTA) that forms firm complexes with LTA-binding proteins (e.g., M protein), which together bind the streptococci to fibronectin on the animal cell surface (*Table 6.2*, Ofek et al. 2003c). The morphological appearance of gram-positive surface adhesins can vary from short filaments, commonly called "fuzz" (e.g., surface fuzz layer of *S. pyogenes*, composed of proteins complexed with LTA), to long fibrils and filaments. Although a number of gram-positive bacterial species, including streptococci, corynobacteria, and bacilli, express fibril structures reminiscent of fimbriae of gram-negative bacteria (see below), there is no firm evidence that these structures in gram-positive bacteria function as adhesins (Vengadesan and Narayana 2011)

Fimbriae or pili (the two terms are used interchangeably in this chapter) are the most common adhesive structures expressed by gram-negative bacteria. They are usually classified based on their morphological appearance into four major categories. One category is made up of fimbriae, comprised of rigid filamentous structures measuring approximately 7 nm in diameter with various lengths. An example is the type 1 fimbriae of enterobacteria and P fimbriae of uropathogenic *E. coli* (*Tables 6.1* and *6.2*). Studies employing quick-freeze, deep-etch electron microscopy have shown that the rigid fimbriae are composite structures comprising fibers with a distinct fibrillar tip. The 7-nm fiber, also known as the fimbrial shaft, is composed of the main subunits (e.g., PapA and Fim A subunits of P and type 1 fimbriae, respectively), arranged in a right-handed helix with an external diameter of approximately 7 nm, an axial hole of approximately 2 nm, and 3.2 subunits per turn of the helical cylinder. The tip fibrillum is composed of three subunits, only one of which is the adhesin, which exhibits lectin activity (e.g., PapG and FimH adhesins of P and type 1 fimbriae, respectively). During the fimbriated phase, a typical gram-negative bacterium expresses up to 200–500 peritrichously arranged fimbriae on its surface.

The second category of fimbriae is comprised of thin, flexible structures, 2–3 nm in diameter (e.g., F17 fimbriae, *Table 6.2*). The third category comprises even thinner fimbriae that are very flexible. The best example of this category is curli, which are curly filamentous structures shared by many strains of *E. coli* and Salmonella. Curli are highly aggregated structures composed of a number of different subunits that promote bacterial binding to fibronectin molecules that coat the host cells. The fourth category of fimbriae is known as the type IV fimbriae, expressed by a number of different bacterial species, including *Neisseria*, *Vibrio*, and enteropathogenic *E. coli*. These fimbriae are usually flexible structures, 6–4 nm in diameter, and often found in bundles. They are typically comprised of two or more subunits, one of which, the major subunit, contains a positively charged leader sequence with a modified amino acid, *N*-methylphenyl alanine, at the N terminus. These above-listed categories of fimbriae are by no means inclusive, and several other types of fimbriae are not described here. For further details on the chromosomal operons involved in the genetic expression

Table 6.1
Molecular features of adhesin-receptor interactions in bacterial adhesion to host cells

Type of interaction	Bacterial ligand (and example)	Receptor on host cell (and example)	References
Lectin-carbohydrate	Lectin (type 1 fimbriae)	Glycoprotein (uroplakin on bladder cells)	Wu et al. (1996)
	Polysaccharide (Klebsiella capsule)	Lectin (mannose receptor of macrophages)	Ofek et al. (1995)
Protein-protein	Fibronectin-binding proteins (F protein of *S. pyogenes*)	Fibronectin (fibronectin on respiratory cells)	Hanski and Capron (1992); Hanski et al. (1996)
Hydrophobin-protein	Glycolipid (lipoteichoic acid of *S. pyogenes*)	Lipid receptors? (lipid-binding region of fibronectin on epithelial cells)	Courtney et al. (1990)
			Hasty et al. (1992)
	Lipid-binding proteins (surface protein of *Campylobacter* spp.)	Membrane lipids (phospholipids and sphingolipids of cells)	Szymanski and Armstrong (1996)
			Sylvester et al. (1996)

Table 6.2
Examples of carbohydrates as attachment sites for bacteria colonizing mucosal surfaces

Organism	Target tissue	Carbohydrate structure	Form
E. coli type 1[fim]	Urinary tract	*Manα3 [Manα3(Manα6)*	Glycoprotein
P[fim]	Urinary tract	Galα4Gal	Glycolipid
S[fim]	Neural	Neu5Ac(α2–3) Galβ3GalNAc	Glycolipid
CFA/1	Intestinal	Neu5Ac(α2–8)-	Glycoprotein
F1C[a]	Intestinal	GalNAcβ4Gal	Glycoprotein
F17	Intestinal	GlcNAc	Glycoprotein
K1	Endothelial	GlcNAc(β1–4)GlcNAc	Glycoprotein
K99 [fim]	Intestinal	Neu5Gc(α2–3)Gal (β1–4)Glc	Glycolipid
H. pylori	Stomach	*Neu5Ac(α2–3)Gal*	Glycolipid
		Fuc(α1–2) Gal(β1–3) (Fuc α4)Gal	Glycoprotein
N. gonorrhea	Genital	*Gal(β1–4)GlcNAc*	Glycoprotein
N. meningitidis	Respiratory/ meninges	*Neu5Ac(α2–3)0,1-Gal (β1–4)GlcNAc(β1–3) Gal(β1–4)GlcNAc*	Glycolipid
P. aeruginosa	Intestinal	*Gal(β1–3)GlcNAc (β1–3)Gal(β1–4)Glc*	Glycoprotein
H. influenza	Respiratory	*Neu5Ac(α2–3)0,1-Gal (β1–4)GlcNAc(β1–3) Gal(β1–4)GlcNAc*	Glycolipid
S. pneumoniae	Respiratory	*GlcNAcβ3Gal*	Glycoprotein
S. suis	Respiratory	*Gal(α1–4)Gal*	Glycoprotein
K. pneumoniae	Respiratory and enterocytes	*Man*	Glycoprotein
C. jejuni	Intestinal	*Fuc(α1–2) Gal(β1–4) GlcNAc*	Glycoprotein

[fim]Fimbrial lectins based Sharon and Ofek (2007)

and chaperone-mediated assembly of various fimbriae of gram-negative fimbriae, the reader is referred to other reviews on the subject (Ofek and Doyle 1994d; Kline et al. 2010).

In summary, the strategy of presenting the adhesin as a complex of assembled molecules whereby the adhesin moiety is located on the tip of a structure appears to be conserved in both gram-positive and gram-negative bacteria. The adhesin per se is sometimes referred to as "tip adhesin." Such a strategy allows the bacterium to bind to a target cell and maintain an effective distance that overcomes repulsive forces. In a way, this approach is reminiscent of a street lamp, which consists of a lamp and a lamppost, for maximum efficiency.

Table 6.3
Selected bacterial clones expressing multiple adhesins

Bacterial clone	Source of isolation	Adhesin	Characteristics
E. coli	*Pyelonephritis*	*Type P*	*Fimbrial lectin*
		Type 1	Fimbrial lectin
S. saprophyticus	*Urinary*	*Gal-GlcNAc*	*Peripheral lectin*
		Lipoteichoic acid	Fibrillar hydrophobin
N. gonorrhea	*Urogenital*	*Pilus*	*Pilin adhesin*
		Opa protein	Outer membrane
S. sanguis	*Dental plaque hydrophobin*	*Protein*	*Peripheral*
		Fimbriae	Fimbrial adhesin
		Protein	Peripheral lectin
		Lipoteichoic acid	Fibrillar hydrophobin

Adapted from Ofek and Doyle (1994b)

Types of Adhesin-Receptor Interactions

The adhesive interactions of over 100 bacterial pathogens of humans and farm animals have been studied (Ofek and Doyle 1994a; Karlsson 1995). Based on these studies, three main types of adhesin-receptor interactions can be distinguished (*Table 6.1*). The first type, probably shared by the majority of bacterial pathogens, is attributable to lectin-carbohydrate recognition. Many bacterial adhesins are lectins, a class of sugar-binding proteins that link bacteria to carbohydrate moieties of glycoproteins or glycolipids on the mammalian host cell (*Table 6.2*). In some cases, the converse is true, where bacterial surface polysaccharides on either the capsule or the outer membrane lipopolysaccharides bind to cognate lectins on the host cell (e.g., macrophage) surface (Ofek et al. 1995). The second type involves recognition of a protein on the bacterium by a complementary protein on the mucosal cell surface. The third type, and the least characterized, involves binding interactions between hydrophobic moieties of proteins on one cell with lipids on the other cell or between lipids on either cell type (Ofek and Doyle 1994b).

Multiple Adhesins

A number of common themes have emerged regarding the interactions between bacteria and mucosal cells. The most notable is the concept that pathogenic bacteria attach to mucosal cells typically through multiple adhesive interactions. Thus, a bacterial cell may express several adhesin moieties, each one specific for a distinct receptor molecule on the epithelial cell surface (for examples see *Table 6.3*). Interactions may be

mediated by multiple bacterial adhesins that are structurally similar but may exhibit different binding specificities, such as the type 1 and P fimbriae of uropathogenic *E. coli* (Table 6.4). Alternatively, adhesins may be structurally and chemically dissimilar, as is the case with the lipoteichoic acid (LTA) and proteinaceous adhesins of *Streptococcus sanguis*. Some pathogens (e.g., *Neisseria gonorrhoeae*) simultaneously express two surface lectins, each specific for distinct carbohydrate structures, one found in glycolipids and the other in glycoproteins.

In many instances, different subpopulations of a bacterial clone express these distinct adhesins. By generating several phenotypic variants, each expressing adhesins of distinct specificities, a given bacterial clone will increase the repertoire of its target tissues and perhaps also acquire antigenic variability that will enhance its ability to withstand the multifaceted defenses of the host (Ofek and Doyle 1994b). This notion is exemplified by pyelonephritic isolates of *E. coli*, which express either the P fimbrial or type 1 fimbrial adhesin at any given time. Because transmission from one host to another is via the fecal-oral route, it is postulated that the pyelonephritogenic isolates may need the type 1 fimbriae mainly to transiently colonize the gastrointestinal tract. Such colonies might then provide a constant source of bacteria entering the stomach and thus increase the chances for the incoming bacteria to colonize the intestine (Bloch et al. 1992). Once in the urinary tract, the bacteria seem to need the P fimbrial adhesins to adhere to the upper urinary tissues (Roberts et al. 1994; Winberg et al. 1995). In fact, the diverse types of fimbrial adhesins carried by various enterobacteria may be determined by virtue of their distinct receptor specificity which are colonized onto the unique niches along the intestine (Edwards and Puente 1998).

Recently, a theory has been proposed whereby pathogenic bacteria use a common adhesin to mediate the initial interaction with the host cell to initiate colonization, which then triggers the pathogen to express a species-specific adhesin (Krachler et al. 2011). Many gram-negative bacteria but not gram-positive bacteria express an outer membrane protein termed multivalent adhesion molecule 7 (Man7) which is expressed constitutively and has a key role in the initial attachment of bacteria to some host cell types during the early stages of infection. It is possible that LTA has a similar role in gram-positive bacteria (Hasty et al. 1992).

In those instances where multiple adhesins are expressed simultaneously on the same organism, each adhesin appears to complement the other functionally. For instance, the cell surface LTA and the M protein co-expressed on the surface of *Streptococcus pyogenes* have both been implicated in mediating bacterial binding to Hep-2 cells (Hasty et al. 1992). Adhesion of *S. pyogenes* appears to involve a two-step process. The first step is mediated by the interaction of LTA with fibronectin molecules on the host cells (Hasty et al. 1992) and the second by binding of the M protein to an as yet unidentified receptor on these same cells.

Table 6.4

Types of receptor-adhesin relationship in bacterial adhesion to animal cells

Type[a]	Receptor molecule	Animal cell	Adhesin molecule	Bacteria (source)
A.				
	Dr blood group antigen	Erythrocytes	Dr fimbriae	*E. coli* (UTI)
		Erythrocytes	AFA II	*E. coli* (ETEC)
		Erythrocytes	F 1845 fimbriae	*E. coli* (pigs)
B.				
	Fibronectin (NH_2 terminal)	Epithelial cell	Lipoteichoic acid	*S. pyogenes*
			Fibronectin-binding protein	*S. aureus*
C.				
	Glycolipid (Galα1–4Gal)	Uroepithelial cell	P fimbriae, FsoG	*E. coli* (pyelonephritis)
	Fibronectin	–	P fimbriae, FsoF/H	–
D.				
	66 kDa Gp	Erythrocytes	Type 1 fimbriae	*E. coli* (mannose sensitive)
	CD11/18 Gp	Neutrophils	Type 1 fimbriae	–
	CD48 Gp[b]	Macrophages	Type 1 fimbriae	–
	Uroplakin[c]	Uroepithelial cell	Type 1 fimbriae	–

Adapted from Ofek and Doyle (1994c)

Gp Glycoprotein

[a]A. Target host cell expresses one receptor molecule that contains three attachment sites for three different adhesins produced by three clones of bacteria. B. Two bacterial species express two distinct adhesins that bind the bacterium to the same receptor molecule on target host cell. C. The same bacterial clone produces a fimbrial structure comprised of two subunits, each of which binds the bacterium to a distinct receptor on the target host cell. D. The same adhesin binds the bacterium to similar attachment sites contained in different receptor molecules (isoreceptors) expressed by various target host cells

[b]Baorto et al. (1997)

[c]Wu et al. (1996)

Lectins as Adhesins

Many microorganisms, including viruses, parasites, fungi, and bacteria, employ lectins, usually located on their surface, to bind to cognate receptors expressed by tissues colonized by the microbes. The structure of these lectins and their sugar specificity can be found in reviews on this topic (Sharon and Ofek 2007)

The bacterial lectins are specific for diverse sugars, which are found on the cell membrane of host cells either as glycoproteins or as glycolipids. Because these receptors are found on the surface of erythrocytes, bacteria-mediated hemagglutination assays are now routinely employed to monitor bacterial expression of particular lectins. Although the same bacterial clone (e.g., *E. coli*) can express multiple lectins that act as adhesins, it is not readily known whether individual bacteria co-express multiple lectins or if each lectin is confined to a distinct subpopulation of the bacterial clone. *Table 6.2* presents a list of bacterial lectins, their sugar specificities, as well as their animal and organ specificities. There are several methods currently available for the detection and identification of sugar specificities of lectins on various bacteria (Goldhar 1994, 1995; Sharon and Ofek 1995). For further details, the reader is referred to other reviews (Cassels and Wolf 1995; Karlsson 1995; Ofek and Doyle 1994c; Ofek et al. 2003b).

Bacterial lectin-mediated adhesion can be inhibited both in vitro and in vivo by either simple or complex carbohydrates that compete with the binding of the lectins to host cell glycoproteins or glycolipids. In general, the affinity of adhesins or lectins for simple sugars (e.g., mono- or disaccharides) is low (in the millimolar range), thus representing the primary sugar specificity. Affinity can be increased by several orders of magnitude either by using suitable chemical derivatization or by employing complex oligosaccharides containing the sugar, which, in its monomeric form, inhibits the lectin activity (Firon et al. 1987). For example, Manα3[Manα3(Manα6) shows 30-fold increase in affinity as compared to the monomeric mannose. Such derivatives are called the fine sugar specificity. Increased affinity also can be obtained by attachment of the mono- or disaccharides to polymeric carriers, to form multivalent ligands (Lindhorst et al. 1997; Sharon 1996; Sharon and Lis 1997).

Some bacterial lectins recognize not only terminal sugars but internal sequences as well. For example, the tip adhesin PapG of P fimbriae recognizes internal Galα(1–4)Gal sequences on cell surface glycolipids (*Table 6.2*). When the bacterial adhesin binds the pathogen to a cognate glycolipid, the ceramide group of the latter may, in some cases, contribute to the affinity of the interaction (e.g., *Helicobacter pylori*; *Table 6.4*).

Although the sugar specificity of a considerable number of bacterial lectins has been established, very few of them have been studied with respect to their detailed structure, biosynthesis, and sugar specificity (Kline et al. 2010). In the following, we will focus on the sugar specificity of two of these fimbrial lectins: type 1 fimbrial adhesin, common in many enterobacterial species, and P fimbrial adhesin expressed by uropathogenic *E. coli*. These two fimbrial adhesins have been studied in detail including biosynthesis, crystallography of the lectin-sugar complexes, as well as their role in infections (Kline et al. 2010). Here, we will discuss the relationship of the minor fimbrial subunit containing the lectin activity and the major fimbrial subunit or the fimbrial shaft with respect to the fine sugar specificity and pathogenicity.

The study of bacterial lectins or adhesins, especially when these molecules are associated with fimbriae that are multisubunit structures, has been hampered by difficulties in obtaining sufficient amounts of lectins in pure soluble form. To overcome this obstacle, fusion proteins, comprising the adhesion molecule, were generated. For example, fusion proteins, composed of the ZZ polypeptide of staphylococcal protein A and the amino terminal region of either PapG I, PapGII, or PapGIII of the P fimbrial adhesin of uropathogenic *E. coli*, have been prepared (Hansson et al. 1995). Each of the three fusion proteins exhibited distinct fine sugar specificities that corresponded to those of the parent fimbriae. This is, however, not always the case. For example, the fine sugar specificity of the isolated FimH lectin of type 1 fimbriae of *E. coli*, *K. pneumoniae*, and *Salmonella typhimurium* strains appears to be influenced, at least in part, by the fimbrial shaft on which these adhesins are presented. For example, genetically engineered hybrid fimbriae, in which the *fimH* of *E.coli* was presented on the shaft derived from *K. pneumoniae* fimbriae, exhibit binding specificities distinct from that of *E. coli* type I fimbriae but instead appear to resemble that of *K. pneumoniae* type I fimbriae (Madison et al. 1994). Thus, although classified together as mannose-specific or mannose-sensitive on the basis of their monosaccharide (or primary) specificity, the FimH lectin of *E. coli* and *K. pneumoniae* differs in their fine sugar specificity, which appears to be influenced by the association of their FimH with their respective fimbrial shafts. That the shaft influences the fine sugar specificity of FimH is supported by studies in which the sugar specificity of soluble hybrids of the lectin domain of FimH and maltose-binding protein was compared to the native type 1 fimbriae of S. *typhimurium* and *E. coli*. The fine sugar specificity exhibited by the fimbriated bacteria was not retained by the soluble fused lectins, which bound a wide range of mannosylated glycoconjugates (Duncan et al. 2005).

Bacterial Glycoconjugates as Adhesins

Mammalian macrophages express lectins, which recognize complementary carbohydrate structures on bacterial surfaces and mediate nonopsonic phagocytosis of bacteria. The macrophage lectins include the mannose/*N*-acetylglucosamine (GlcNAc)/fucose receptor (MR), DC-SIGN, and Dectin-1. All of these proteins are members of the C-type lectin family and show calcium-dependent interactions with a variety of carbohydrate ligands (McGreal et al. 2004; Endo et al. 2007). They recognize complementary glycoconjugates on bacterial surface capsules or lipopolysaccharides. It has been reported that the MR

and DC-SIGN molecules on macrophages recognize specific glyconjugates on certain capsular serotypes of *K. pneumoniae* and *S. pneumoniae* (Sahly et al. 2008). Since such recognition events lead to phagocytosis and killing of these capsular serotypes, it was postulated that their low frequency of isolation among clinical samples could partially be the result of early clearance by macrophages.

The lipo-oligosaccharide/lipopolysaccharide (LOS/LPS) on the outer membranes of gram-negative bacteria can mediate adhesion to nonprofessional phagocytes (including mucosal cells) as well as to mucus components coating these surfaces (Jacques 1996; Nassif and Magdalene 1995). Although not conclusive, the evidence for this is based on the following observations: (1) Parent bacteria but not mutant derivatives lacking the O side chain of LPS bind avidly to epithelial cells, and isolated LPS inhibits this binding; (2) LPS isolated from *Vibrio mimicus* can readily agglutinate rabbit erythrocytes (Alam et al. 1996); (3) the heptose-3-deoxy-D-manno-2-octulosonic acid disaccharide present in the inner core of LPS is bound by a lectin-like molecule on the plasma membrane of rat hepatocytes (Parent 1990); and (4) the binding and internalization of *Pseudomonas aeruginosa* by corneal epithelial cells requires the intact inner-core of LPS with a terminal glucose residue (Zaidi et al. 1996). In a few cases, the interaction between a lectin on one bacterium and lipo-oligosaccharides on another can promote bacterial aggregation (Blake et al. 1995). The animal lectin galectin-3 was found to recognize and bind lipopolysaccharides of gram-negative bacteria (Mey et al. 1996). In no case, however, has there been definitive proof of a mucosal cell lectin binding carbohydrate residues on pathogenic bacteria. Nevertheless, it is noteworthy that the hyaluronic acid capsule of *Streptococcus pyogenes* has been reported to bind CD44 receptor on epithelial cells (Cywes et al. 2000). This receptor normally binds hyaluronic acids of the host extracellular matrix. Interestingly, application of hyaluronic acid in the oral cavities of mice prevented subsequent adhesion and colonization by streptococci. Under some circumstances, however, the hyaluronic acid capsule was reported to mask the adhesins, thereby blocking their access to cellular receptors by steric hindrance. Further studies are therefore needed to resolve the contradictory roles of the bacterial surface-based hyaluronic acid capsule in the adhesion process.

Adhesin-Receptor Relationship

The adhesins of a number of bacterial pathogens and their cognate receptors on host cells have been characterized for a large number of pathogenic organisms (reviewed in Ofek and Doyle 1994b; Sharon and Lis 1997). Several general features are notable (*Table 6.2*). The same molecule may serve as a receptor to multiple adhesins where different regions of the molecule serve as attachment sites to different adhesins. This is illustrated by the Dr blood group glycoprotein, which acts as receptor on host cell membrane for three different clones of *E. coli*, each of which produces a distinct adhesin that binds to a different region of the Dr group molecule (Ofek and Doyle 1994e).

Another general feature of these adhesins is that two different pathogens, each expressing structurally distinct adhesins, can exhibit the same receptor specificity. This is the case with *S. aureus* and *S. pyogenes*, both of which bind the amino terminal region of fibronectin on mucosal cells. The adhesin on *S. aureus* is a fibronectin-binding protein, whereas that of *S. pyogenes* is lipoteichoic acid (*Table 6.4*). The finding that several different respiratory tract pathogens recognize the disaccharide GalNAcβ4Gal is yet another example of the above (*Table 6.2*). It has been suggested that the GalNAcβ4Gal sequence is preferentially accessible in glycolipids of the respiratory epithelium, and this permits firm binding of a diverse group of respiratory pathogens bearing appropriate adhesins. In some cases, however, distinct adhesins share the same specificity but are carried by different bacteria that colonize different tissues and animal hosts, as is the case for the Galα(1–4)Gal-specific lectins of the uropathogenic P-fimbriated *E. coli*, the pig pathogen *Streptococcus suis* (Tikkanen et al. 1995), and the respiratory/enteropathogenic P-like fimbriated *K. pneumoniae* (Prondo-Mordarska et al. 1996).

Conversely, the same bacterial adhesin can bind to several distinct receptors on different cell types; such receptors are called isoreceptors. For instance, several glycoproteins, ranging in size from 45 to 110 kDa, have been described as receptors for type 1 fimbriae on different cell types (*Table 6.2*). All these isoreceptor glycoproteins share a common oligomannose-containing attachment site for FimH, the adhesin subunit of type 1 fimbriae. Another feature is where an adhesin molecule contains multiple domains, each with distinct receptor specificity as is the case of the filamentous hemagglutinin adhesin of *Bordetella pertussis*. This hemagglutinin, which has been cloned and sequenced, contains at least three domains: (1) an arginine-glycine-aspartate (RGD)-containing sequence which binds the bacteria to a CR3 integrin present on pulmonary macrophages (Relman et al. 1989), (2) a carbohydrate-binding domain specific for galactose (Tuomannen et al. 1988), and (3) a carbohydrate-binding domain specific for sulfated sugars (Menozzi et al. 1994).

Interaction of Bacterial Adhesins with Extracellular Matrix

Mucosal cell surfaces are often coated by an extracellular matrix (ECM), which is a heterogeneous assembly of proteins, mainly glycosylated but to different extents. Included in this matrix are structural glycoproteins that are typical constituents of the ECM, such as collagens, elastin, fibronectin, fibrinogen, laminin, chondroitin sulfate proteoglycans, and heparan sulfate proteoglycans. Many mucosal colonizers express adhesins that specifically recognize one or more of these substances. These adhesins are also referred to as microbial surface components, which recognize adhesive matrix molecules (MSCRAMMs) (Vengadesan and Narayana 2011). The same three categories of adhesin-receptor interactions, presented in *Table 6.1*, occur between bacteria and ECM components. Thus, there may be

interactions between proteins only, between lipids and proteins, or between lectins and carbohydrates. A more thorough discussion of ECM-bacteria interactions may be found in other reviews (Patti and Hoök 1994; Hasty et al. 1994; Patti et al. 1994; Wadström et al. 1994). Among the various ECM components, fibronectin has been studied the most at both the molecular and cellular levels. Because this multifunctional glycoprotein is found on the surface of many types of cells, including mucosal cells, fibronectin has been shown to serve as a major receptor for bacterial adhesion and colonization. The adhesion of bacteria to ECM components other than fibronectin is now becoming known (Table 6.5). A remarkable feature is that many of the bacterial species found to bind ECM express on their surfaces at least two proteins that specifically bind an ECM component. For example, *Helicobacter pylori* expresses a 25 kDa sialic-acid-binding lectin that binds sialyl residues of laminin as well as a lipopolysaccharide which recognizes another as yet unidentified region(s) in laminin (Valkonen et al. 1994, 1997). Many studies have established fibronectin as an important receptor for *S. pyogenes* and other bacteria on mucosal surfaces (Ofek and Doyle 1994e; Courtney et al. 1990). At least six different molecules on *S. pyogenes* surfaces are known to recognize fibronectin, including LTA, protein F/Sfb, a 28 kDa fibronectin-binding protein, glyceraldehyde-3-phosphate dehydrogenase, serum opacity factor, and a 54 kDa fibronectin-binding protein (FBP54; reviewed in Hasty and Courtney 1996). It is not clear whether all these fibronectin-binding entities mediate the adhesion of streptococci to mucosal surfaces.

Antiadhesion Therapy

One of the major goals for the study of bacterial adhesion to animal cells is to provide knowledgeable approaches to prevent and/or treat infections. Indeed, since the discovery four decades ago that adhesion of pathogens to soft and hard tissues is essential for initiating infection, much research has focused on developing effective antiadhesion therapy for treating or preventing infectious diseases (Kahane and Ofek 1996; Ofek et al. 2003e; Shoaf-Sweeney and Hutkins 2009). Unlike the use of bactericidal agents (e.g., antibiotics), utilization of antiadhesion agents does not result in the development of resistant strains as the viability of both "resistant" and "sensitive" strains are not affected. In the following, we review different approaches of antiadhesion therapy, including the use of receptor and adhesin analogs, dietary constituents, sub-lethal concentrations of antibiotics, and adhesin-based vaccines.

Table 6.5
Examples of MSCRAMMs mediating bacterial binding to ECM glycoproteins

Bacteria	Bacterial adhesin	ECM component	References
Borrelia burgdorferi	*19 and 20 kDa proteins*	*Proteoglycan decorin*	Guo et al. (1995); Leong et al. (1995)
	Protein A (Osp A) and 70 kDa protein	Plasminogen	Hu et al. (1995)
H. influenzae	*P2 and P5 outer membrane proteins*	*Respiratory mucin*	Davis et al. (1995); Reddy et al. (1996); Kubiet and Ramphal (1995)
N. gonorrhea	*Opa protein*	*Proteoglycan*	Van Putten and Paul (1995)
P. aeruginosa	*57 and 59 kDa outer membrane proteins*	*Laminin*	Plotkowski et al. (1996)
	42–48 and 77–85 kDa outer membrane proteins and flagellar 65.9 kDa FLi F (MS ring)	Respiratory mucins	Scarfnman et al. (1996); Akora et al. (1996)
Staphylococcus aureus	*138 and 127 surface proteins*	*Nasal mucin*	Shuter et al. (1996); Foster and Höök (1998)
	Cna protein (55 Kda domain)	Collagen	–
	ClfA (clumping factor)	Fibrinogen	–
	FnBPA and FnBPB	Fibronectin	–
Mycobacterium bovis	*28 kDa protein*	*Heparan*	Menozzi et al. (1996)
E. coli	*Gaf D protein of G fimbriae*	*Laminin*	Saarela et al. (1996)
Bordetella pertussis	*Filamentous hemagglutinin (N-terminal region of FHA)*	*Heparan*	Hannah et al. (1994)
H. pylori	*Lipopolysaccharide and 25 kDa protein*	*Laminin*	Valkonen et al. (1994), (1997)
Listeria monocytogenes	*ActA outer membrane protein*	*Heparan*	Alvarez-Domínguez et al. (1997)
Enterococcus faecalis	*ACE (N1 and N2 domain)*	*Collagen*	Ponnuraj and Narayana (2007); Liu et al. (2007)

Receptor Analogs as Antiadhesive Agents

There is evidence that receptor analogs can be used for antiadhesion therapy against pathogens that bind to animal cells via carbohydrate-specific adhesins (i.e., lectins). In these cases, the receptor analogs are saccharides that are structurally similar to those on the glycoprotein or glycolipid receptors on the host cell for the bacterial adhesin and, therefore, act by competitive inhibition (see *Table 6.2*). One of the first examples of this approach was reported almost three decades ago where D-mannose was used to competitively inhibit binding of type 1 fimbriated enterobacteria to animal cells (Ofek et al. 1977). Since then, the sugar specificities of many bacteria have been determined, resulting in the use of receptor-like carbohydrates to inhibit the adhesion of pathogens to host cells and tissues (*Table 6.6*). Because the affinity of the saccharides employed for inhibition for the bacterial lectins is low, the concentrations of the carbohydrates required for effective inhibition of bacterial adhesion in vitro are usually high, in the millimolar range. Interestingly, the affinity of the inhibiting saccharides for the adhesion can be increased several orders of magnitude by covalently linking a hydrophobic residue (e.g., phenyl or methyl umbelliferyl or heptyl to the saccharide) (Firon et al. 1987; Bouckaert et al. 2005). The affinity of the saccharide can also be increased by generating many copies of the saccharide on a suitable carrier, yielding a multivalent adhesin inhibitor, as demonstrated for *H. pylori* (Simon et al. 1997; Mulvey et al. 2001) and for type 1 fimbriated *E. coli* (Almant et al. 2011; Lindhorst et al. 1997).

The feasibility of using modified saccharides to protect against experimental infections by bacteria expressing adhesive lectins was first demonstrated more than two decades ago (Aronson et al. 1979). Co-administration of methyl α-mannoside with *E. coli* expressing the mannose-specific type 1 fimbrial lectin into the bladders of mice reduced bladder colonization by uropathogenic *E. coli* by about two-thirds compared to animals that had received the bacteria alone or with methyl α-glucoside, a sugar that does not inhibit the mannose-specific bacterial lectin. Subsequently, many other studies have confirmed the ability of saccharides to prevent experimental infections caused by different pathogenic bacteria in a variety of animals including nonhuman primates with *H. pylori* infections (*Table 6.6*).

In view of the successes of animal models described above, a number of clinical studies have been undertaken where specific oligosaccharides were employed to prevent or treat infections caused by lectin-expressing pathogens, but these have had limited success. In one of these studies, children 10–24 months old were treated for 3 months with a nasal spray containing the antiadhesive pentasaccharide α sialyl-3′-lacto-*N*-neotetraose [NeuAc α(2–3) Galβ(1–4)GlcNAcβ(1–3)Galβ(1–4)Glc] (Ukkonen et al. 2000). However, this treatment not only failed to reduce the incidence of nasopharyngeal colonization with *S. pneumoniae* and *H. influenzae* but it also failed to prevent acute otitis media commonly caused by these pathogens. In another trial, orally administered NeuAc α(2–3)Galβ(1–4)GlcNAc, which inhibits the sialic acid-specific adhesin of *H. pylori*, failed to eradicate the pathogen from the gastric mucosa in human patients (Parente et al. 2003). It is possible, therefore, that, as suggested above, targeting only one of several adhesins that a pathogen is capable of expressing may frequently be insufficient to prevent colonization and subsequent infection. This conclusion is supported by a clinical trial of 58 patients with acute otitis externa, 36 of whom were found to be culture positive for *Pseudomonas aeruginosa* (Beuth et al. 1996). The patients were treated by local administration of gentamicin or a mixture of galactose, mannose, and *N*-acetylneuraminic acid, sugars specific for two *P. aeruginosa* lectins, PA-I and PA-II, (Wentworth et al. 1991). The gentamicin-treated patients recovered more rapidly than did the patients treated with the sugars, but recovery of the latter was more rapid than that of the untreated control patients.

Table 6.6
Inhibitors of carbohydrate-specific adhesion prevent infections in experimental animals

Organism	Animal (site)	Inhibitor
Campylobacter jejuni	Mouse (GIT)	Milk oligosaccharides
Escherichia coli (type 1 fimbriated)	Mouse (UT)	MeαMan
	Mouse GIT	Mannose
E. coli (P fimbriated)	Mouse UT	Globotetraose
	Monkey UT	Galα1,4GalOMe
E. coli K99	Calf GT	Glycopeptides
K. pneumoniae (type 1 fimbriated)	Rat UT	MeαMan
Helicobacter pylori	Piglet GT	Sialyl3′LacNAc
	Monkey GT	Sialyl3′Lac
Shigella flexneri (type 1 fimbriated)	Guinea pig eye	Mannose
Streptococcus pneumoniae	Rabbit lungs	Sialyl3′Galβ1 − 4
	Rat lungs	LacNAc

For reference see Ofek et al. (2003)
GT gastrointestinal tract, *UT* urinary tract

Adhesin Analogs as Antiadhesive Agents

The strategy of using adhesin analogs to prevent infections is based on the notion that the isolated adhesin molecule, or an active synthetic or recombinant fragment, can bind to the receptor on host cells and competitively blocks adhesion of the bacteria (Ofek et al. 2003e). So far, it has been impractical to use analogs of adhesins for antiadhesion therapy because they are typically macromolecules that are not readily available and because they must be employed at relatively high concentrations. In addition, careful consideration must be given to their potential toxicity and immunogenicity. Nevertheless, modern advances in proteomics and recombinant biotechnology have

permitted the development of select types of relatively small peptides for antiadhesion therapy, as reported by Kelly et al. (1999). In these studies, the inhibitor employed was a synthetic 20-residue peptide mimicking the sequence of a *S. mutans* cell surface adhesin which mediates the binding of the bacteria to a salivary protein on dental surfaces. In vitro, this peptide inhibited the binding of the streptococci to the immobilized salivary receptor (i.e., an artificial tooth pellicle). Application of the peptide to teeth that had been pretreated with chlorhexidine gluconate to reduce normal microbiota and eliminate *S. mutans* significantly retarded re-colonization of the teeth with these bacteria but had no effect on re-colonization by *Actinomyces naeslundii*. Re-colonization by *S. mutans* was observed in control volunteers receiving saline or a control peptide. These results are promising, especially because the peptide employed was small. While conceptually encouraging, the above results must, nevertheless, be interpreted with some degree of caution because the adhesion of *S. mutans* may also be mediated by other adhesins unrelated to the one on which this peptide sequence was based. For example, these bacteria utilize sucrose to synthesize a secreted glucan, which can become immobilized on tooth surfaces. Adhesion to immobilized glucan is then mediated via a glucan-binding protein expressed on the bacterial surface. Thus, a mixture of peptides corresponding to the multiple adhesins that are known to be expressed by the streptococci could prove to be more effective. The premise that peptides corresponding to a protein adhesin can serve as potent inhibitors of adhesion is supported by data from other experimental systems. For example, synthetic peptides mimicking a fragment of the fimbrillin adhesin of *Porphyromonas gingivalis* were found to inhibit adhesion of the organisms to hydroxyapatite (Lee et al. 1992, 1995).

Non-proteinaceous adhesins may also be useful for antiadhesion therapy, as shown in studies of the lipoteichoic acid (LTA)-mediated adhesion of groups A and B streptococci. In one such study, LTA was applied to the oral cavity, perineum, and nape of 5-day-old mice (Cox 1982). The mothers were painted with a suspension of group B streptococci in their oral cavity and vagina or on the nipples. None of the experimental pups were culture positive after 3 days, whereas 47 % of control pups were culture positive. In another study, LTA was instilled into the nasal cavities of mice, followed by administration of a group A streptococcal suspension (Dale et al. 1994). The incidence of colonization and death of the LTA-treated mice was significantly reduced as compared to that of the control mice. Although this proves a principle, the use of LTA in antiadhesive therapy would probably not be advisable because of its inherent toxicity and/or proinflammatory properties.

As indicated above (section ❯ "Bacterial Glycoconjugates as Adhesins"), group A streptococci binds the CD44 receptor on epithelial cells via their hyaluronan capsule (Cywes et al. 2000). CD44 is also a host cell receptor which binds to the hyaluronan of the host extracellular matrix to maintain the integrity of the tissue (Underhill 1992). Application of hyaluronan to the oral cavity of mice prevented adhesion and colonization by group A streptococci. The hyaluronan capsule of *S. pyogenes* may also act indirectly to block adhesion, by masking adhesins and blocking their access to cellular receptors by steric hindrance. The capsule also acts as chelator of cations, some of which may also be required for proper function of other adhesins (Goh et al. 2000).

Dietary Inhibitors of Adhesion

Some of the most effective antiadhesion compounds identified thus far are present in foods. Foods containing either a mixture of inhibitors or a single inhibitor with a broad spectrum of activity are likely to be especially effective. While it may be possible to find appropriate inhibitors for particular pathogens, it is unlikely that it will be possible to match every individual or group of pathogens with specific diets that contain complementary adhesin inhibitors. Empirical observations over the years have suggested that certain dietary constituents may have beneficial effects on bacterial infections. These dietary constituents represent good candidates for antiadhesion studies. However, caution should be used because some dietary components may also have bactericidal and/or bacteriostatic properties, and the selective pressures imposed by such compounds are undesirable and should be avoided (Cowan 1999). Most of the studies in this regard have focused on human milk and plant extracts as a source for antiadhesion molecules with potential for human use to treat infections.

Human Milk

Human milk has long been known to be beneficial in preventing certain bacterial infections (Ashkenazi 1994; Kunz and Rudloff 1993; and Newburg 2000). This property has been associated with the presence of large amounts of oligosaccharides and related compounds to which many bacteria can potentially bind (❯ *Table 6.7*). It was not until the key role of lectin-carbohydrate interactions in microbial adhesion was demonstrated that investigators began to explore the therapeutic effects of milk oligosaccharides and glycoproteins. ❯ *Table 6.7* lists glycoconjugate compounds in milk that act as inhibitors of bacterial adhesins. In one study, it was found that fucosylated oligosaccharides in milk blocked infection in mice caused by *Campylobacter jejuni* (Ruiz-Palacios et al. 2003). Subsequently, the first clinical evidence demonstrating the close association between the relative quantity of oligosaccharides in maternal milk and protection of breast-fed infants against diarrhea was made (Morrow et al. 2004, 2005). This correlation was based on the results of a study of a cohort of breast-feeding mother-infant pairs up to 2 years postpartum, with feeding and illness data collected weekly. In addition, the concentrations of fucosylated oligosaccharides were determined in maternal milk samples collected 1–5 weeks postpartum. It was found that the infants had a significantly lower risk of moderate to severe diarrhea due to *Campylobacter* spp. if the milk was rich in fucosyl-2′lactose.

Table 6.7
Glycoconjugates and saccharides from human milk that interact with bacteria

Glycoconjugate	Bacterium
Caseinoglycopeptides	*H. pylori*
	S. sobrinus and *Streptococcus mutans*
Fucosylated saccharides	*Escherichia coli*
Glycoprotein	*S. mutans* (40 kDa)
Glycoprotein	*Staphylococcus aureus*
Gal β(1–4)GlcNAc and Gal β(1–3) GlcNAc	*Pseudomonas aeruginosa*
Neutral oligosaccharides	*S. pneumoniae* and *Haemophilus influenzae*
Sialylated glycoproteins	*Mycoplasma pneumoniae*
Sialyl 3′Lac	*H. pylori*
Sialylated poly (*N*-acetyl lactosamine)	*Mycoplasma pneumoniae*
Sialylated poly (*N*-acetyl lactosaminoglycans)	*Streptococcus suis*
Sialyl 3′Lac and sialylated glycoproteins	*E. coli* (S fimbriae)
Sialylgalactosides	*E. coli* (S fimbriae)

For reference see Ofek et al. 2003e

Table 6.8
Antiadhesin activity of plant constituents

Plant	Constituent	Bacterium affected	References
Azadirachta indica (neem stick)	ND	*Streptococcus sanguis*	Wolinsky et al. (1996)
Camillia sinensis (green tea)	(−) Epicatechin gallate, (−) gallocatechin gallate	Porphyromonas	Sakanaka et al. (1996)
(oolong tea)	Polyphenol	*S. mutans* and *S. sobrinus*	Ooshima et al. (1993)
Galanthus nivalis (snowdrop)	Mannose-sensitive lectin	*E. coli*	Pusztai et al. (1993)
Gloiopeltis furcata and *Gigartina teldi* (seaweeds)	Sulfated polysaccharides	*S. sobrinus*	Saeki (1994)
Hop bract	Polyphenols (36–40 kDa)	*S. mutans*	Tagashira et al. (1997)
Melaphis chinensis	Gallotannin	*S. sanguis*	Wolinsky et al. (1996)
Persea americana (avocado)	Tannins	*S. mutans*	Staat et al. (1978)
Legume storage proteins	Glycoprotein	*E. coli*	Neeser et al. (1986)

ND Not determined, *NT* Not tried

Other constituents capable of binding to pathogenic bacteria and inhibiting adhesion are present in human milk. The glycoprotein, lactoferrin, a common constituent of milk, has been shown to inhibit adhesion of *Actinobacillus actinomycetemcomitans, Prevotella intermedia*, and *P. nigrescens* to fibroblast monolayers and reconstituted basement membranes via its protein moiety (Alugupalli and Kalfas 1995, 1997). Lactoferrin was also found to inhibit binding of *E. coli* to HeLa cells and to red blood cells (Longhi et al. 1993; Giugliano et al. 1995). Another secretory component, often found in human milk in considerable concentrations, inhibited adhesion of CFA-I-expressing enterotoxigenic *E. coli* to epithelial cells (Giugliano et al. 1995). Mucins in human milk are known to inhibit adhesion of S-fimbriated *E. coli* to buccal epithelial cells (Schröten et al. 1993).

Milk contains fat globules, which can carry glycoconjugates specific for bacterial adhesins. For example, adhesion of S-fimbriated *E. coli* to buccal epithelial cells was inhibited by sialylated fat globules (Schröten et al. 1992). Colostrum lipids inhibited adhesion of *H. pylori* and *H. mustelae* to immobilized glycolipids (Bitzan et al. 1998). Beta-lactoglobulin was shown to bind to *Listeria monocytogenes* (al-Makhlafi et al. 1994). In addition to the various compounds mentioned, human milk contains antimicrobial agents such as lysozyme, lactoperoxidase, and immunoglobulins (Newman 1995). A well-designed study is needed to define the individual contributions of each of these antimicrobial and antiadhesive components to the overall beneficial effects of human milk in preventing infections.

Plant Extract

Because of their ready availability, plant materials possessing antiadhesion activities are attractive candidates to combat pathogenic bacteria. Furthermore, the identified active components can be used as food supplements, thereby negating the necessity to adhere to a particular diet. Perhaps the most important advantage of searching dietary agents in food is that approval of clinical trials is easier to obtain, as toxicity is not an issue. Among the plant extracts exhibiting antiadhesion activity and listed in *Table 6.8*, are those observed in cranberry (*Vaccinium macrocarpon*) and which are the most thoroughly studied with respect to their antiadhesion activities in vitro as well as in clinical trials. In the following, we will focus on the in vivo and supporting antiadhesion activity in vitro studies, which suggest beneficiary effects of cranberries or its constituents for treating or preventing bacterial diseases. In particular, cranberry effects on urinary tract infections caused by *Escherichia coli*, infectious gastritis or peptic ulcer diseases induced by *Helicobacter pylori*, and oral health will be discussed.

Table 6.9
In vitro and ex vivo studies showing antiadhesion activity of cranberry juice and cranberry constituents against uropathogenic *E. coli*

Activity	References
Cranberry juice but not other juices inhibits hemagglutination and uroepithelial adhesion of P-fimbriated uropathogenic *E. coli*	Ofek et al. (2003d), Gupta et al. (2007)
A-type proanthocyanidins composed of epicatechin oligomers, with at least one A-type linkage (double bonds between two epicatechin: 4 > 8 and 2 > O > 7 interflavan bonds) and a high molecular weight nondialyzable material (also known as NDM) that contains polyphenols, probably of A-type epicatechin polymers, all of which were obtained from cranberries or cranberry juice, inhibit adhesion and hemagglutination of uropathogenic *E. coli* but not diarrheal *E. coli*. B-type proanthocyanidins with one B-type linkage (one bond between epicatechin units: 4 > 8 interflavan bond) obtained from other plants which was without effect	Ofek et al. (1991), Ofek et al. (2003d), Howell (2007)
Urine from individuals or rodents consuming cranberry exhibits antiadhesion activity of uropathogenic *E. coli*	Howell (2007), Liu et al. (2010)

Urinary Tract Infections

During the last three decades in vitro studies have confirmed that cranberry constituents inhibit adhesion of P-fimbriated uropathogenic *E. coli* to their cognate Gal-Gal receptor present on epithelial cells as well as on other cells. As the P-fimbrial receptor is also present on certain erythrocytes, mixing of P-fimbriated *E.* coli with particular erythrocytes in the presence of extracts of cranberry is a convenient assay to screen for functional activity. Indeed, inhibition of hemagglutination has become the test of choice in assays for antiadhesion activity of cranberry constituents. *Table 6.9* summarizes the in vitro and ex vivo studies showing that cranberry or high molecular weight nondialyzable material (NDM) or proanthocyanidin (PAC) constituents inhibit selectively the adhesion of P-fimbriated uropathogenic *E. coli*, the most common cause of UTI.

Attempts were made to substantiate the in vitro observations on the antiadhesive effects of cranberry components by a number of controlled clinical trials. They were summarized by Jepson et al. (2008) based on Cochrane Database meta-analysis of studies up to 2008. Juice formulation was used in six studies, tablet formulation in three studies, and both formulations were used in one study. The clinical trials support the potential use of cranberry products in the prophylaxis of recurrent UTIs in young and middle-aged women. However, the efficacy of cranberry in other groups (i.e., elderly, pediatric patients, patients with neurogenic bladder, or chronic indwelling urinary catheters) is questionable. The high withdrawal rates (up to 55 %) in these studies suggest that these formulations may not be acceptable for prolonged use. More recently, it was found that consumption of 27 % cranberry juice cocktail for 6 months (240 mL twice per day of low calorie juice) by young college females (mean age 21 ± 3) was ineffective in preventing UTI as compared to placebo (Barbosa-Cesnik et al. 2011). The lack of "adequately powered placebo-controlled trials" was proposed by the authors to explain the unexpected failure of cranberry treatment compared to studies in Cochrane review. However, there could be a number of other explanations, one of which is that in previous studies, the cranberry juice cocktail was sweetened with fructose, while in the young females study, low calorie, sucralose-sweetened juice was used. It was shown that the fructose inhibits the type 1 fimbrial adhesin expressed by all P-fimbriated uropathogenic *E. coli*, emphasizing the notion that the beneficiary effect of cranberry juice cocktail may be due to two inhibitors (fructose and PAC) each specific for a distinct adhesin (Zafriri et al. 1989; Ofek et al. 1991). Another explanation may be related to the study population, that is, young females who experience sexual intercourse 2–3 times a week (Barbosa-Cesnik et al. 2011). It was reported that urinary *E. coli* counts increased transiently approximately tenfold after sexual intercourse in nearly half of the volunteers (Buckley et al. 1978). Sexual activities decline with increasing age (Kotz 2003). Thus, it is possible that the young females require either fructose-sweetened juice or a higher dose of low calorie juice to cope with the observed increase in vaginal *E. coli* counts. However, consumption of higher doses of cranberry juice over a long period "may not be acceptable" as suggested by low compliance (Jepson et al. 2008; Guay 2009). To overcome this, there is a need to isolate the most active of the antiadhesion cranberry constituents and use it as a supplement to other juices for the control of UTI. Such an approach may now be feasible with the isolation of highly active fractions from cranberries (*Table 6.8*).

Oral Health

There are numerous in vitro studies showing that cranberry components such as PAC or NDM inhibit adhesion of oral bacteria to teeth surfaces and to epithelial cells as well as to each other (e.g., intergenera/species coaggregation) (Bodet et al. 2008). NDM and PAC were found to inhibit key bacterial enzymes involved in adhesion and in biofilm formation on teeth surfaces such as glucosyl and fructosyl transferases as well as tissue-damaging bacterial enzymes such as gingipain of the periopathogenic *P. gingivalis* bacteria. In addition, the cranberry components inhibit acid production by cariogenic bacteria. Of special interest are the observations that NDM inhibits host enzymes, such as MMP-3 and MMP-9 and elastase, that participate in periodontal diseases. Induction of proinflammatory cytokines, such as IL-1_{β}, TNF_{∞}, IL-6, IL-8, and RANTES, are also affected. In spite of these intriguing in vitro studies, there are only two that involve cranberry components. In a clinical

trial, the effect of mouthwash supplemented with NDM on oral hygiene of healthy volunteers was assessed. Following 6 weeks of daily usage, the salivary mutans streptococci count as well as the total bacterial count was reduced significantly compared to the placebo control group, although no clinical changes in the plaque and gingival indices were observed (Weiss et al. 2004). Recently, Koo et al. (2010) showed in an animal study that topical application (twice daily) of PAC onto rat teeth surfaces decreased incidence and severity of *S. mutans*-induced smooth surface as well as sulcal surface caries.

Gastric, Duodenal, and Peptic Ulcers

In vitro studies indicated that the high molecular weight nondialyzable material (NDM) obtained from cranberry juice inhibits adhesion of *H. pylori*, which triggers in susceptible individuals gastrointestinal diseases including gastric, duodenal, and peptic ulcers, as well as gastric cancer, and gastric lymphoma. Significantly, NDM inhibited the sialyllactose-specific (S fimbriae) adhesion of *H. pylori* strains to immobilized human mucus, erythrocytes, and cultured gastric epithelial cells (Burger et al. 2000, 2002; Vattem et al. 2005). The adhesion of two-third of 83 *H. pylori* clinical isolates to gastric cell line was inhibited by 0.2 mg/mL of cranberry NDM (Shmuely et al. 2004). There was no relationship between the antiadhesion effect of the cranberry and bacterial resistance to metronidazole. These data suggest that a combination of antibiotics and a cranberry preparation may improve *H. pylori* eradication. This additive effect was assessed in a recent double-blind clinical study, in which one group of 170 patients with elevated ^{13}C-urea breath test (e.g., infected with *H. pylori*) was treated with antibiotics for 1 week and randomly allocated to receive 250 mL of either cranberry juice or placebo (Shmuely et al. 2007). An additional control group consisted of 712 patients who were treated with antibiotics alone. Analysis by gender revealed that for female subjects, the eradication rate was significantly higher in the cranberry-antibiotic arm reaching 95 % compared with the placebo-antibiotic arm and also significantly higher than in the antibiotic group (80 % and 83 %, respectively). These results suggest that the addition of cranberry to triple therapy improves the rate of *H. pylori* eradication in females. A double-blind placebo-controlled clinical trial employing patients with elevated ^{13}C-urea breath test supported this conclusion. In this study, the patients were randomized in two groups, one of which was asked to drink cranberry only and the other placebo juice only, consisting of 500 mL daily for 90 days (Zhang et al. 2005). Although the overall rate of eradication of helicobacter was low in the range of 5–17 %, the eradication rate in the cranberry group (14.3 %) was significantly higher as compared to the placebo group (5.2 %). Experimental infection in mice treated with cranberry revealed that although the organisms were not eradicated, there was a marked reduction in the total helicobacter mass after consumption of cranberry alone (Xiao and Shi 2003). It thus appears that cranberry consumption alone may reduce the total helicobacter mass rather than eradication of all bacteria. Such an outcome may especially benefit pregnant women where antibiotic treatment is not recommended.

Sub-lethal Concentrations of Antibiotics

Sub-inhibitory concentrations of antibiotics have been shown to reduce the ability of pathogens to adhere to various substrata (Ofek and Doyle 1994c). There is, however, little evidence that sub-lethal concentrations of antibiotics are beneficial in the therapy of infections in vivo. This apparent contradiction may be due to a number of factors, including the paucity of pilot clinical trials or well-designed in vivo animal experiments. Nevertheless, this is an important issue to study, if for no other reason that there is a high likelihood that a significant fraction of antibiotic-treated patients undergo periods in which the concentration of the drug in their system is sub-lethal, at least transiently, due to poor compliance.

Interestingly, the potential antiadhesion activity of antibiotics has become a factor of promotional consideration by at least one pharmaceutical company. In an advertisement presenting a new antibiotic (Monurol®, Rafa Laboratories, Ltd), the inhibition of bacterial adhesion to uroepithelial cells was cited (Patel et al. 1997).

Adhesin-Based Vaccines

Prevention of infectious diseases by blocking adhesion using adhesin-based vaccines can be achieved either by active or passive means. Although active antiadhesin immunity, evoked when the vaccine comprising isolated adhesin is applied at the mucosal surface, is expected to prevent infection by stimulation of secretory IgA on mucosal surfaces, significant amounts of serum IgG also appear to reach these surfaces, such as the gut, oral cavity, and even the urinary tract (Robbins et al. 1995). For passive immunity, the target host is treated with antiadhesin antibodies made in another host. The best example of this is where the K88 fimbriae and related adhesins of farm animal pathogens were used to vaccinate the mothers so that the suckling piglets passively acquire milk-secreted antibodies that functioned to prevent infection by the pathogen (Moon and Bunn 1993). It has been assumed that these antibodies prevented infection by inhibiting adhesion.

Antigenic variability of protein adhesins may compromise the efficacy of antiadhesin vaccines, especially when one considers that each of the multiple adhesins produced by a particular pathogen can undergo allelic variations, giving rise to so-called iso-adhesins (e.g., adhesins that maintain their receptor specificity but differ antigenically). For example, there are numerous allelic variants of the FimH adhesin subunit of type 1 fimbriae of *E. coli* (Sokurenko et al. 1998). It has been suggested that a conserved region of an adhesin may serve as a vaccine especially if this region contains the receptor-binding domain (Langermann et al. 1997; Wizemann et al. 1999; Thankavel et al. 1997).

Although it has been known for more than three decades that antiadhesin antibodies effectively inhibit adhesion, there are no reports on herd immunizations with purified adhesins or with recombinant strains containing the DNA encoding for the adhesin. It has been suggested that mucosal immunity, as opposed to systemic, IgG-mediated immunity, can improve the protective effect of adhesin-based vaccines (Mestecky et al. 1997, Wizemann et al. 1999). In some cases, whole attenuated or inactivated bacteria that carry the adhesin have been employed and the antiadhesin antibodies that were generated functioned in concert with antibodies against other virulence factors to protect the vaccinated host from symptomatic infection. For example, the inactivated *Bordetella* vaccine contains the hemagglutinin adhesin. Vaccinated individuals developed anti-hemagglutinin antibodies which were shown to inhibit adhesion of *Bordetella* to epithelial cells (Relman et al. 1989).

In animal models, active immunization with adhesins provides IgG- and/or secretory IgA-mediated protection against infection (reviewed in Ofek and Doyle 1994b). The choice of animal model for the study of the role of immunoglobulins in mucosal infections is of paramount importance and may be a major drawback in attempts to extrapolate the results obtained to humans (Wizemann et al. 1999). Active immunization with a complex of the FimH adhesin and its periplasmic chaperone, FimC, was shown to protect against urinary tract infection caused by *E. coli* in both mice and nonhuman primates (Langermann et al. 1997, 2000).

In contrast to active immunization, the passive administration of anti-adhesin antibodies may be more useful to prevent infection. The success of antibodies in milk in protecting against diarrheal infections in suckling piglets and calves is one example (Moon and Bunn 1993). In another study, anti-*S. mutans* monoclonal antibodies directed against the bacterial SA I/II adhesins were applied onto the tooth surfaces of human volunteers that had been treated with chlorhexidine to eliminate the *S. mutans* microbiota prior to the passive application of the antibodies (Ma et al. 1998). The re-acquisition of *S. mutans* in the placebo group receiving irrelevant monoclonal antibodies occurred within 2–3 months, whereas the passively immunized subjects remained virtually free of *S. mutans* for at least a year. However, because the antiadhesin antibodies were no longer detectable a day after application, it is difficult to explain these findings. One very reasonable speculation is that transiently inhibiting *S. mutans* adhesion enabled other non-pathogenic oral bacteria to colonize the tooth surfaces, therefore preventing *S. mutans* from re-colonizing the occupied surfaces (Kelly and Younson 2000). Nevertheless, these studies are promising and, in the future, consumption of milk from immunized cows might be especially useful in the prevention of infections in targeted populations.

Concluding Remarks

The alarming increase in drug-resistant bacteria makes the search for novel means of fighting bacterial infections imperative. An attractive approach is the use of agents that interfere with the ability of the bacteria to adhere to tissues of the host since such adhesion represents the initial stages of the infectious process. The validity of this approach has been unequivocally demonstrated in experiments performed in a wide variety of animals, from mice to monkeys, and, recently, also in humans. Bacteria can adapt to many noxious or deleterious agents, either by mutation, by acquisition of new genetic material via horizontal transfer, or by phenotypic variation (Ofek et al. 2003e). Bacteria resistant to antiadhesion agents may also be expected to emerge, but because these agents do not act by killing or arresting growth of the pathogen, as, for example, antibiotics do, it is reasonable to assume that strains resistant to antiadhesion agents will be diluted by the sensitive bacteria whose adhesion is inhibited and are expelled from the host. It follows that spread of bacteria resistant to the antiadhesion agent is expected to occur at significantly lower frequencies than that of bacteria resistant to antibiotics. This would potentially allow sensitive and resistant organisms to propagate and be transmitted at equivalent rates, dramatically slowing the emergence of a predominantly resistant population.

The major drawback of antiadhesion therapy is that most pathogens possess genes encoding for more than one type of adhesin, so that during the infectious process the population of pathogens may express more than one of these adhesins. Adhesion may also involve factors other than just adhesin-receptor interactions such as hydrophobic and other nonspecific interactions under different shear-forces. For antiadhesion therapy to be effective, it will probably be necessary to use multiple agents specifically inhibiting each type of adhesin of the infecting pathogen or a single agent that exhibits a broad spectrum of antiadhesion activity.

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7 Cholera

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Abstract
Cholera is a bacterial infection that has sickened and killed millions of individuals since ancient times. It is caused by the Gram-negative bacterium *Vibrio cholerae* and is transmitted by the fecal-oral route, mainly through contaminated drinking water. Colonization of the human small intestine by *Vibrio cholerae* requires presence of the toxin-coregulated pilus (TCP) and allows for multiplication of the bacterium. As it multiplies, *V. cholerae* secretes cholera toxin (CT), which targets heterotrimeric G proteins and induces production of cyclic AMP. Buildup of this molecule leads to secretion of chloride ions into the intestinal lumen, which in turn prompts massive fluid loss in the form of secretory diarrhea. In some cases, patients can lose a liter of fluid every hour and quickly become severely dehydrated, often resulting in death. Over the past several decades, cholera fatality rates have dropped significantly due to the introduction of oral rehydration therapy, an inexpensive and effective method of treatment. Two vaccines, Dukoral and Shanchol, are currently recommended by the World Health Organization (WHO) for prevention of cholera. Both are oral heat-killed whole-cell vaccines that provide >50 % protection when administered correctly. However, cholera is still a grave problem in areas where water sanitation is insufficient, such as developing countries and regions where conflict or natural disasters have damaged infrastructure.

Cholera: Past and Present

Cholera is a waterborne gastrointestinal illness that has afflicted humans throughout recorded history (*Fig. 7.1*). This disease is characterized by massive fluid loss through secretory diarrhea, which contains high levels of mucus and resembles rice water. If untreated, the severe dehydration caused by cholera is often fatal. While cases of cholera have been documented since 500–400 B.C.E., these were mainly limited to sporadic outbreaks in isolated areas prior to the early nineteenth century (Lacey 1995). Cholera is endemic to the Indian subcontinent and has spread worldwide to cause seven pandemics. The exact dates of the pandemics are somewhat controversial; in many cases, historical records are incomplete and tracking down the first and last case of cholera in a particular pandemic is impossible.

It is widely accepted that the first cholera pandemic started in 1817 with the advent of international trade (Sack et al. 2004). After a season of unusually heavy rainfall and massive flooding in India, cholera sickened hundreds of thousands, with a focus in the province of Bengal, which included the city of Calcutta (Kolkata). British soldiers stationed in this area fell victim to the illness and subsequently transmitted it to Nepalese soldiers against whom they were fighting. From there, the disease reached the countries of China, Malaysia, and Japan via merchant ships. This first pandemic lasted until 1824 when an abnormally cold winter halted the spread of the disease (Pollitzer 1954).

The second pandemic, spanning from 1829 to 1851, also originated in India. In this instance, cholera was spread by Polish and Russian soldiers who carried the disease into their home countries. By 1831, cholera had reached Austria and England, and the following year, Ireland, France, and Belgium all experienced epidemics. In the same year, an outbreak of cholera in Mecca sickened thousands of pilgrims, who brought the illness back to Syria, Palestine, and Egypt. Increased travel and immigration further facilitated explosion of the disease, which reached Scandinavia and crossed the Atlantic Ocean in 1832, entering North America through the cities of Quebec and New York (Pollitzer 1954). In the course of the second pandemic, cholera reached every continent except Antarctica (Lacey 1995).

After a brief respite, pandemic cholera returned in 1852, following a severe outbreak of the disease in India. The first years of this pandemic were disastrous, with the United States, Canada, Mexico, the West Indies, England, France, Greece, and Turkey being the most hard-hit countries. The spread of cholera to the latter two was largely blamed on the Crimean War (1853–1856), as infected troops traveled from southern France to these countries (Pollitzer 1954). Meanwhile, in England, an outbreak of the disease in a neighborhood of London led John Snow, a physician, to link the transmission of cholera with ingestion of contaminated water (Snow 1855), a pioneering observation in public health epidemiology. This important discovery had lasting effects; from that point on, England suffered far fewer cholera cases than it had in the past, even as the disease

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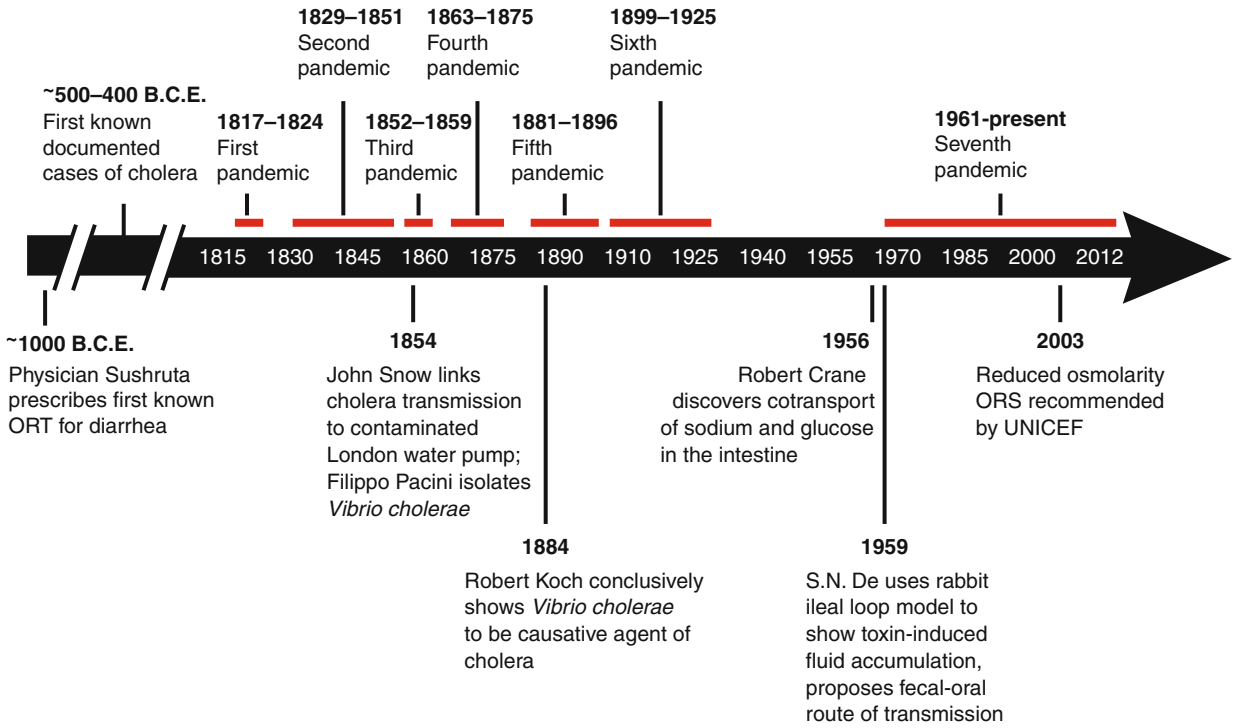

Fig. 7.1
Timeline depicting cholera pandemics and advances in disease treatment and prevention

was repeatedly brought into the country. The causative agent of cholera, the Gram-negative bacterium *Vibrio cholerae*, was isolated in the same year by Filippo Pacini, though Robert Koch is widely credited with making this breakthrough in 1884 (Lacey 1995). Within the next few years, the number of cases worldwide dwindled, and the third pandemic was largely over by 1859 (Pollitzer 1954).

The fourth pandemic, lasting from 1863 until approximately 1875, marked a shift in the routes of transmission of cholera. New trade routes had been established at this time, providing a means for the disease to be spread even more efficiently. In particular, 1865 was an especially important year, as it was a jubilee year in Mecca, where scores of pilgrims gathered. An outbreak in Mecca led to massive numbers of cases, and many of these infected people carried cholera to their homelands in Egypt, Syria, Palestine, Mesopotamia, and others. The spread of disease to Egypt was especially fateful, as cholera took hold in Alexandria, a major shipping center on the Mediterranean Sea. This facilitated transmission to other port cities and reintroduced cholera to Europe, where it ravaged several countries, including Austria, Hungary, Germany, the Netherlands, Belgium, and Russia. Later in the decade, the disease gripped Northern and Eastern Africa, concomitant with the rise of Mozambique and Zanzibar as shipping centers (Pollitzer 1954).

The fourth pandemic also affected North America, reaching New York City by sea in 1865 or 1866. It spread rapidly once there, traveling by the ever-expanding rail system and reaching as far west as Kansas. In response to the rampant spread of the disease in New York, the nation's first Board of Health was founded in 1866 (Duffy 1971). While some measure of disease control was taking place in the United States, cholera was spreading deeper into South America at that time, reaching Argentina, Peru, Brazil, and Uruguay. From a global standpoint, the disease was still very much a problem, even as the fourth pandemic waned in the 1870s.

In 1884, as the fifth pandemic raged on, Robert Koch conclusively linked the cause of cholera to infection with the bacterium *Vibrio cholerae* (Koch 1884). This discovery did not immediately lead to preventative measures, as France, Italy, and Spain saw epidemics throughout the mid-1880s. Of note, cholera was reintroduced to both England and New York City several times during this period, but there were no new outbreaks due to proper quarantine techniques and improved water sanitation. Other areas of the world were not so lucky; the fifth pandemic claimed hundreds of thousands more lives and persisted until 1896 (Pollitzer 1954).

As the twentieth century began, severe outbreaks of cholera in Calcutta and Bombay sparked the beginning of the sixth pandemic, which lasted until approximately 1923. This pandemic was marked by increased spread of the disease due to World War I and saw massive numbers of cases in Europe and Russia, with a peak of infection in 1921. In the following years, fewer infections were noted, with Europe being largely free of cholera after 1925 (Pollitzer 1954).

The seventh pandemic, according to most sources, began in 1961 and continues today (WHO 2011). This pandemic differed from the first six in several ways. While prior pandemics had originated in severe outbreaks in India, the seventh began in

Indonesia and spread from there. Additionally, in contrast to the previous six pandemics, which were caused by *Vibrio cholerae* of the classical biotype, the bacteria responsible for the current pandemic belong to the El Tor biotype, which is generally considered less virulent than its classical relatives. Nonetheless, cholera claimed hundreds of thousands of lives as it spread through Asia, Europe, and Africa, eventually reaching Peru by freighter in 1991. In the late 1980s, studies by the EPA linking chlorination by-products with cancer had led Peruvian officials to stop chlorinating some of the wells in Lima. Thus, when wastewater contaminated with *Vibrio cholerae* entered the main water supply, the disease spread easily and made this epidemic especially devastating (Anderson 1991). Recent studies have suggested that while adequate chlorination of the water supply would not have prevented the epidemic entirely, it would have saved thousands of lives (Tickner and Gouveia-Vigeant 2005).

While cholera remains a severe public health problem, it is now limited to developing countries with poor infrastructure, especially those that have recently experienced military conflicts and natural disasters. Cholera remains endemic in India and Bangladesh, and cases generally appear in waves centered around the monsoon season. These waves of cases are often limited by the presence of bacteriophages, or bacterial viruses, that prey on *Vibrio cholerae* (Faruque et al. 2005).

In the last few years alone, Zimbabwe, Iraq, Afghanistan, and Sudan, among others, have seen deadly epidemics of cholera (WHO 2011). Most recently, Haiti, a country that had not recorded cases of cholera for more than a century, suffered a severe outbreak of disease following a devastating earthquake in January 2010 (Piarroux et al. 2011). Sequence analysis of bacteria isolated from patients indicated that the disease had likely been imported into Haiti by Nepalese troops who arrived as part of post-earthquake humanitarian relief efforts (Chin et al. 2011) and had spread through improperly treated drinking water. While the intensity of the epidemic has waned somewhat, it is expected that the disease will linger in Haiti until water sanitation can be improved (Butler 2011).

Vibrio Cholerae and Mechanism of Infection/Transmission

Ingestion of water contaminated with *Vibrio cholerae* gives the bacterium access to the gastrointestinal tract of the human host. The infectious dose of *V. cholerae* is quite high because the majority of the organisms are killed by the acidity of the stomach. Bacteria able to survive passage through this acidic environment reach the upper small intestine, where the production of toxin-coregulated pilus (TCP) aids in adherence to the mucus layer. While in close association with the intestinal mucosa, *V. cholerae* multiplies rapidly and secretes cholera toxin, which is responsible for the actual disease symptoms. Cholera toxin (CT) is an AB_5 toxin consisting of six subunits: an activity (A) subunit and five binding (B) subunits. Upon binding to GM1 ganglioside on the surface of a host epithelial cell, the toxin is internalized by receptor-mediated endocytosis and transported to the endoplasmic reticulum (ER) via retrograde transport. In the ER, the catalytic (A1) portion of the A subunit is cleaved from the toxin complex; identified by the cell as a misfolded protein, it is then retrotranslocated to the cytosol for degradation (Tsai et al. 2001). Usually, misfolded proteins in the cytosol are polyubiquitinated on lysine residues, triggering proteasomal degradation. However, the CT A1 subunit contains few lysine residues and refolds quickly in the cytosol, effectively avoiding this fate (Rodighiero et al. 2002).

Once free in the cytosol, cholera toxin ADP-ribosylates the s subunit of heterotrimeric G proteins, promoting adenylate cyclase activity, which leads to production of cyclic AMP (cAMP). cAMP is a potent second messenger, and its buildup in epithelial cells sparks phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) and activation of chloride channels in the plasma membrane, among other events (De Haan and Hirst 2004). This triggers high levels of chloride secretion and mucus shedding into the intestinal lumen; recent studies have provided evidence that *V. cholerae* associates with these secreted clumps of mucin (*Fig. 7.2*) (Ritchie et al. 2010). To maintain osmotic balance of the luminal fluid, water is also released, and the overwhelming abundance of fluid produces the secretory diarrhea experienced by cholera patients (De Haan and Hirst 2004). Shedding of this stool, which is rich in aggregates of mucin and mucin-associated bacteria, thus promotes transmission of *V. cholerae* to environmental reservoirs and new hosts (*Fig. 7.3*).

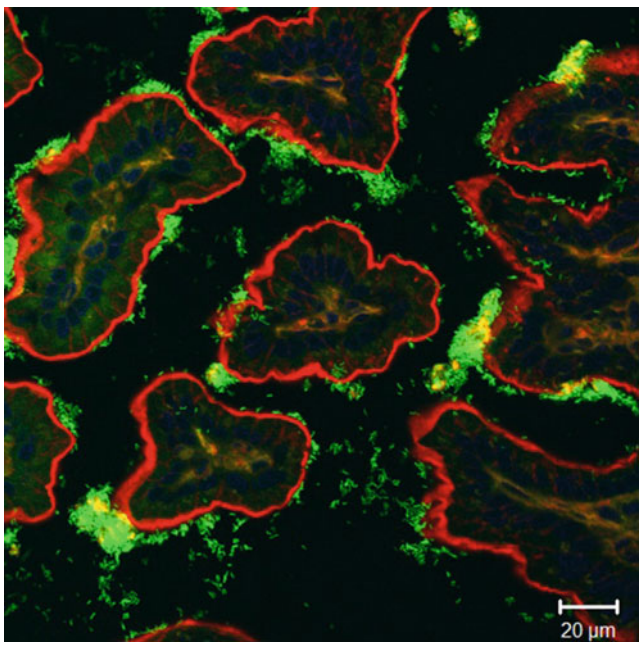

Fig. 7.2
***Vibrio cholerae* in the infant rabbit intestine. Confocal micrograph showing *Vibrio cholerae* (*green*) adhering to mucus (*yellow*) in a cross section of an infected rabbit intestine. Host cells are stained for actin (*red*) and DNA (*blue*) (Image courtesy of Jennifer Ritchie and Matthew Waldor)**

Fig. 7.3
Rice-water stool collected from a cholera patient in Kolkata, India (Image courtesy of Jeffrey Withey)

In order to efficiently colonize its host and promote spreading, *V. cholerae* tightly controls production of its virulence factors. This regulatory cascade has been extensively reviewed in the literature (Matson et al. 2007). Briefly, the inner membrane-bound proteins ToxR and TcpP coordinately activate transcription of the gene encoding ToxT, an AraC/XylS-like transcriptional regulator (Higgins et al. 1992). ToxT then binds toxboxes (consensus binding sequences) upstream of virulence genes, activating transcription at these sites (Withey and DiRita 2006). Targets of ToxT include *ctxAB*, the *tcp* locus, and small regulatory RNAs (Richard et al. 2010; Bradley et al. 2011), among others. *V. cholerae* strains lacking *toxR*, *toxT*, or *tcpA* display severe colonization defects in both suckling mouse and human volunteer studies (Herrington et al. 1988; Taylor et al. 1987; DiRita et al. 1991), while *ctxA* mutants are able to colonize both human and infant rabbit hosts but do not induce secretory diarrhea (Ryan et al. 2006; Rui et al. 2010).

A spatially and temporally regulated pattern of gene expression is crucial for successful *V. cholerae* infection and transmission. Studies using recombination-based in vivo expression technology (RIVET) have identified several genes induced during early and late infection of both human and infant mouse hosts that are required for colonization and disease spread, respectively (Merrell and Camilli 2000; Lombardo et al. 2007; Schild et al. 2007). As one might expect, gene products promoting colonization, such as toxin-coregulated pilus and the accessory colonization factors, are produced in the initial stages of infection. Later, once the bacteria have robustly colonized the bowel, the focus shifts to metabolic functions, such as storage of carbon in the form of glycogen (Bourassa and Camilli 2009), in preparation for exit to the environment. Vibrios freshly shed from the host are hyperinfectious compared to those grown in vitro, suggesting that the genes expressed in vivo help equip the bacterium not only to survive in nutrient-poor environmental reservoirs but also to colonize its next host effectively (Merrell et al. 2002).

To date, over 200 different serotypes of *Vibrio cholerae* have been isolated, but only two of these (O1 and O139) have been found to cause disease. The serotypes are classified based on the specific O antigen found on the lipopolysaccharide (LPS), which is a component of the bacterial outer membrane. Within the O1 serotype, there are two subtypes, Ogawa and Inaba, and two biotypes, classical and El Tor. The Ogawa and Inaba isolates are distinguished by one specific part of the O antigen, while the two biotypes differ at both genetic and phenotypic levels. Classical strains of *V. cholerae* caused the first six pandemics of cholera; the current pandemic features an El Tor strain. While these distinctions are helpful in the laboratory, recent research has suggested that there is significant genetic fluidity between the two biotypes of *V. cholerae*. In fact, hybrid or "El Tor variant" strains have been isolated from cholera patients in Bangladesh, Haiti, India, Thailand, and Malaysia, among other countries (Son et al. 2011; Goel et al. 2011; Na-Ubol et al. 2011; Ang et al. 2010). These strains are constantly evolving through horizontal gene transfer, leading to a diverse population of bacteria in the environment. One mechanism of gene acquisition in the environment may be natural competence, which is induced by chitin, a common component of marine species with which *V. cholerae* associates in nature (Meibom et al. 2005). Acquisition of virulence traits by horizontal gene transfer is well appreciated in *V. cholerae*. Several pathogenicity islands, including VPI-I, encoding the *tcp* genes and *toxT*, have been described (Karaolis et al. 1998). The cholera toxin genes themselves are encoded on the genome of a filamentous phage CTX-phi, which uses the TCP as its receptor for entry into the cell (Waldor and Mekalanos 1996). The most recently identified genetic elements, VSP-I and VSP-II, or Vibrio seventh pandemic islands I and II, were discovered in the last decade (Dziejman et al. 2002) and contain several yet-to-be-characterized genes that may have roles in the pathogenesis of these strains. These regions, specifically VSP-II, are highly variable and appear to undergo frequent genetic rearrangements (Taviani et al. 2010). In sum, delineation of *Vibrio cholerae* into two distinct biotypes may soon be, if it is not already, an antiquated concept.

Symptoms and Treatment

Cholera is characterized by the profuse watery diarrhea induced by secretion of cholera toxin into the intestinal lumen. This diarrhea, while painless and noninflammatory, can lead to severe dehydration and death in a matter of hours, with patients losing up to a liter of fluid an hour. Vomiting is less common but does

occur, contributing to the observed dehydration. In the absence of treatment, indicators of severe dehydration, such as sunken eyes, loss of skin elasticity, and barely detectable blood pressure, manifest within hours. Untreated patients can lose up to 10 % of their body weight within 24 h of the first symptoms, the period in which most deaths from cholera occur (Sack et al. 2004). In many clinics, cholera cots are utilized, which allow for the measurement of lost fluid for treatment purposes.

Currently, treatment of cholera is quite effective and focuses on administration of oral rehydration therapy, or ORT, the goal of which is to replace the massive amount of fluid lost by cholera patients. The composition of the fluid given is important, as patients lose both electrolytes and water to the secretory diarrhea of cholera; if only water is given, the patient will suffer severe hyponatremia. As glucose and sodium are cotransported across the intestinal epithelium, sodium absorption by the patient is much more efficient if glucose is present in the solution. While the most commonly used oral rehydration salts mixture contains specific quantities of glucose and salts, the term ORT can be used for most liquids given to a cholera patient.

The idea of replacing fluids lost to diarrhea is not new; in fact, it dates back 3,000 years to the Indian physician Sushruta, who prescribed a mixture of water, rock salt, and molasses as a treatment for diarrhea (da Cunha Ferreira and Cash 1990). While ORT in some form, be it rice water, coconut juice, carrot soup, or another fluid, has been used to treat cholera in India for the past three millennia, this development did not catch on in other countries until the mid-1900s (da Cunha Ferreira and Cash 1990). The formulation most commonly used today (reduced osmolarity ORS) consists of 75 millimolar (mM) sodium chloride, 75 mM glucose, 20 mM potassium chloride, and 10 mM trisodium citrate; this specific combination has been most efficacious at treating severe dehydration (WHO 2006). In the past, intravenous fluid replacement has also been used to treat cholera, while today it is only used for patients too ill to drink.

In most cases of cholera, antibiotics are ineffective because the disease symptoms are caused by cholera toxin, which is rapidly produced upon infection. Antibiotics target *Vibrio cholerae* itself, and by the time they are administered, high levels of toxin production have already occurred. Similarly, antibiotic treatment does not lessen transmission of disease, as the onset of diarrhea has already occurred, triggering shedding of the bacteria into wastewater. As such, antibiotics are now rarely used to treat cholera and generally only lessen the severity and duration of disease in some cases (Lindenbaum et al. 1967).

Cholera Vaccines

While the discovery of *Vibrio cholerae* as the causative agent of cholera prompted research into pathogenesis of the disease, progress was quite slow at the outset. In fact, it was not until 1959 that the transmission of cholera via the fecal-oral route was proposed by S. N. De (1959). Previously, investigators had tried injecting *Vibrio cholerae* into animals via virtually every other route and had not been able to recapitulate disease symptoms. De observed massive fluid accumulation after injecting *V. cholerae* culture filtrates into a ligated rabbit ileal loop. He proposed that this bacteria "multipl(ies) in the small bowel and produce(s) an exotoxin which acts upon the mucosal cells of the small bowel, causing them to secrete large quantities of isotonic fluid. . . and the result is a watery isotonic diarrhea"(De 1959). This was later confirmed by Finkelstein, who purified cholera toxin and recapitulated disease after orogastric inoculation of rabbits (Finkelstein and LoSpalluto 1969). Prior to this work, immunization studies focused on injectable vaccines with limited success. The most widely used of these preparations was a killed whole-cell vaccine developed in the late 1800s. This fairly expensive vaccine was extensively tested in Bangladeshi children in the 1960s and was found to provide limited protection while causing undesirable side effects (Mosley et al. 1972). Given its poor efficacy and side effects, the vaccine is not recommended by the WHO.

Recently, focus has shifted to oral vaccination strategies, which have been more successful. Vaccines given by this route are especially valuable because they present antigen to the immune system in the same manner that an infection would. While both live attenuated vaccines and killed whole-cell preparations have been developed, the latter have been more fruitful, with greater protection and fewer side effects (for review, see Bishop and Camilli 2011). Several trials of oral live attenuated vaccines have been conducted in the USA and abroad; one of the largest studies tested the strain CVD 103-HgR on nearly 70,000 volunteers in Indonesia. This trial was largely considered a failure because while only very few subjects developed cholera, the numbers of cholera cases were similar between the placebo and vaccine groups (Richie et al. 2000). Other live attenuated vaccine strains that have been developed, such as Peru-3, have produced reactogenic diarrhea in patients (Taylor et al. 1994). Recent work with the infant rabbit model of cholera disease has pinpointed the presence of flagellins as the cause of this inflammatory side effect (Rui et al. 2010).

Dukoral, an oral vaccine consisting of recombinant cholera toxin B subunits and killed *V. cholerae* of the Ogawa and Inaba serotypes and classical and El Tor biotypes, was developed in the 1980s. While relatively expensive and requiring two doses (three doses for young children), this vaccine was shown to be quite effective, providing 85 % protection 6 months after administration in one study (Clemens et al. 1986). Dukoral was licensed in 1991 and has been used in refugee camps with success (Legros et al. 1999). Another oral vaccine, Shanchol, has recently been approved for use by the WHO. Shanchol lacks the recombinant cholera toxin B subunit, making it cheaper than Dukoral and thus more attractive for use in developing countries. While Shanchol also requires two doses, a third dose is not required for children under the age of 6 (WHO 2010). It is a killed whole-cell formulation containing *V. cholerae* of both the O1 and O139 serotypes.

Though there are currently two viable vaccines on the market, research in this area continues in hopes of creating a preparation that demonstrates good protection in children, is cost-effective, and is easy to administer to large populations. Studies utilizing outer membrane vesicles from *V. cholerae* have shown particular promise, with these preparations providing

immunity to neonatal mice when given to pregnant mice (Bishop and Camilli 2011). Additionally, the recent development of an infant rabbit model of disease has provided an important tool for testing of new vaccine candidates (Rui et al. 2010). The field of cholera vaccine research is thus still very active, with many recent and exciting developments toward a safe, effective vaccine.

Future Perspectives

While cholera remains an enormous public health problem that kills an estimated 100,000 people per year in the developing world, there is hope on the horizon (WHO 2011). Research over the past century and a half has pinpointed the mode of transmission of the disease, and much of the mechanism behind the pathogenesis of *Vibrio cholerae* has been elucidated. Vaccine studies have produced two fairly effective vaccines, and the development of oral rehydration therapy has drastically cut the mortality of this illness. However, until infrastructure improvements make access to clean water a reality in developing nations, these countries will continue to suffer massive cholera outbreaks.

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8 Tuberculosis

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Abstract
Mycobacterium tuberculosis is an obligate human pathogen of immense importance, infecting up to one third of the human population and causing 1.4 million deaths per year. Despite a long-established vaccine and initially effective antibiotics, *M. tuberculosis* continues to be a scourge of mankind through a combination of factors: related to the organism, host, their interaction, co-infections such as with HIV, and societal issues. The hallmark of *M. tuberculosis* as a pathogen is its intracellular growth and ability to evade killing by macrophages, including latent infection for many years. Here we consider distinctive characteristics of the organism, pathogenesis, epidemiology, clinical syndrome, diagnosis, treatment, and prevention of tuberculosis. Pressing challenges include combating drug resistance, designing better vaccines, improved diagnostics, and simplified treatment regimes. Molecular techniques are increasingly being transferred from bench to bedside and the coordinated efforts of microbiologists, immunologists, epidemiologists, clinicians, and policy-makers are required to turn the tide against tuberculosis.

Introduction

Mycobacterium tuberculosis is an obligate human pathogen of immense importance, infecting up to one third of the human population and causing 1.4 million deaths per year (World Health Organization 2011b). Targeting tuberculosis is one of the major aims of the World Health Organization, with the goal of halving tuberculosis deaths and prevalence by 2015, through its Stop TB Strategy and the Global Plan to Stop TB (The World Health Organization Stop TB Partnership 2010). These are ambitious plans, for *M. tuberculosis* has been a scourge of mankind for many thousands of years. The common origin of the *M. tuberculosis* complex is thought to have arisen in East Africa 15,000–35,000 years ago, and historical, archaeological, and microbiological evidence from ancient Egypt, India, China, and Peru confirm that it caused death and disease to our ancestors (Daniel 2006).

It was in the nineteenth century that revolutionary advances in medicine and bacteriology heralded the start of a greater understanding of the disease and its infectious etiology. René Théophile Hyacinthe Laënnec, the inventor of the stethoscope, was also the first to synthesize the disease, bringing together clinical examination and pathological findings in his 1819 book *D'Auscultation Mediate* (Laennec 1962). In 1865, his French compatriot Jean-Antoine Villemin, having noticed high rates of tuberculosis among those housed in close proximity to one another, used samples from patients to infect rabbits (Major 1945). In 1882, Robert Koch espoused his now eponymous postulates to establish an infectious etiology for a disease—namely, isolation of the organism from diseased but not healthy tissue, growth of the organism in culture, disease when the organism is transferred into a healthy host, and re-isolation of the same organism. For his work proving these postulates for tuberculosis (Koch 1932), he was later awarded the Nobel Prize in Medicine or Physiology in 1905.

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The importance of the host immune response to tuberculosis became indirectly apparent through Koch's advocacy of tuberculin, isolated from bacilli, as both a cure and a diagnostic test for tuberculosis. Through the work of Clemens Freiherr von Pirquet, Charles Mantoux, and Florence Seibert, among others, the tuberculin skin test was developed and refined, still a mainstay of diagnosis worldwide over a century on. Tuberculin failed as a treatment, and spas and sanatoria together with surgical pneumothoraces and thoracoplasty were the only therapies until World War II. Para-aminosalicylic acid (PAS) was identified in 1943, streptomycin in 1944 (leading to a Nobel Prize for Selman Waksman), and the oral treatments isoniazid and the rifamycins were developed in 1952 and 1957, respectively (Daniel 2006).

With these new antibiotics, a cure for tuberculosis became both possible and feasible. Yet, as discussed above, tuberculosis remains a pressing global emergency to this day. Why? The answer is multifactorial and lies within the pathogen, the host, the interaction between the two, and societal issues. Many characteristics of the organism itself (such as its slow growth, distinctive cell wall, ability to adapt to biological stresses, and lay dormant for years) contribute to its virulence, difficult diagnosis, requirement for prolonged treatment with several agents, and being a poor target for vaccines. The human immune response to exposure to *M. tuberculosis* ranges from eradication of the pathogen, latent infection with potential for subsequent reactivation, to active disease. It remains unclear why some individuals are able to control the organism while others succumb. *M. tuberculosis* is able to manipulate host immune machinery, inhibiting apoptosis of infected cells, preventing maturation of phagolysosomes, and impairing antigen presentation to the adaptive immune system. The challenges of concordance with a multidrug regime for months combined with the pathogen's versatility has led to the evolution of drug resistance, with accompanying challenges for treatment of the patients and public health prevention of transmission strategies. Coinfection with other, in relative terms, new human pathogens such as human immunodeficiency virus (HIV) illustrates both the vast human cost as well as some of the underlying host defenses to tuberculosis. Increased movement of people around the world, poverty, malnutrition, institutionalized populations, and variable access to healthcare contribute to transmission, vulnerability, and challenges in delivering effective treatment.

Nevertheless, significant advances have been made in recent years, exemplified by the translation of molecular genetic research to the global rollout of a novel automated mycobacterial nucleic acid amplification system that can rapidly diagnose tuberculosis and also detect resistance (Boehme et al. 2010; World Health Organization 2011a). However, practical obstacles still exist and much work remains to be done to improve diagnosis, treatment, and prevention in the areas of the world that need it the most. These challenges can only be met with an in-depth understanding of the pathogen, the host, and their interactions, through the collaboration of scientists and health professionals across many disciplines.

Organism

The *M. tuberculosis* complex is composed of *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canetti*. The complex is grouped due to their highly similar genome sequences and the similar clinical syndromes that result from infections. They are slow-growing obligate pathogen Gram-positive aerobic nonmotile non-spore-forming straight or slightly curved bacilli. They are typically between 3 and 4 μm in length. *M. tuberculosis* lies within the genus *Mycobacterium* of the Mycobacteriaceae family and Actinomycetales order.

The *M. tuberculosis* Genome

The publication of the whole genome sequence of *M. tuberculosis* H37Rv represented a pivotal moment in tuberculosis research (Cole et al. 1998). The genome consists of a single 4.4 Mb circular chromosome, which includes approximately 4,000 genes, and has a very high guanine+cytosine content (*Fig. 8.1*). There is a high proportion of repetitive DNA, especially insertion sequences, with many duplicated housekeeping genes. In keeping with the bacterium's complex cell envelope, 9% of the coding capacity is involved in lipid metabolism with a high level of redundancy. Eight percent of the coding capacity of the genome is devoted to two large previously unidentified unrelated families of acidic, glycine-rich proteins, the Pro–Glu (PE) and Pro–Pro–Glu (PPE) families. Gene expression in *M. tuberculosis* is tightly regulated with approximately 190 transcriptional regulators encoded within the genome. Use of gene annotation and function databases is now part of the basic toolkit of many mycobacterial researchers. Comparison of the sequence of H37Rv to those of nonpathogenic mycobacteria is a powerful tool for identifying drug and vaccine antigen targets and elucidating the molecular and genetic contributions to physiology and virulence, although many genes are still of unknown function. Transcriptomic analysis of *M. tuberculosis* emphasizes that genes alone do not hold all the answers, with more than 25% of non-ribosomal RNA representing noncoding RNA, highlighting the importance of post-transcriptional regulation (Arnvig, Comas et al. 2011).

The role of a substantial number of genes remains unclear. While systematic analyses have revealed which genes are required for growth under a variety of conditions (Murry et al. 2008), even the functions of many of the critical proteins encoded by these loci remain unknown. Recent efforts to apply the principles of systems biology to further comprehensive analyses might shed more light on the functions of individual genes (Young et al. 2008).

Slow growth of *M. tuberculosis* contributes to both scientific and clinical challenges. Doubling time in liquid culture of 24 h means that experimental manipulations are time-consuming and arduous, while diagnosis by means of culture and subsequent resistance testing means conventional results are not

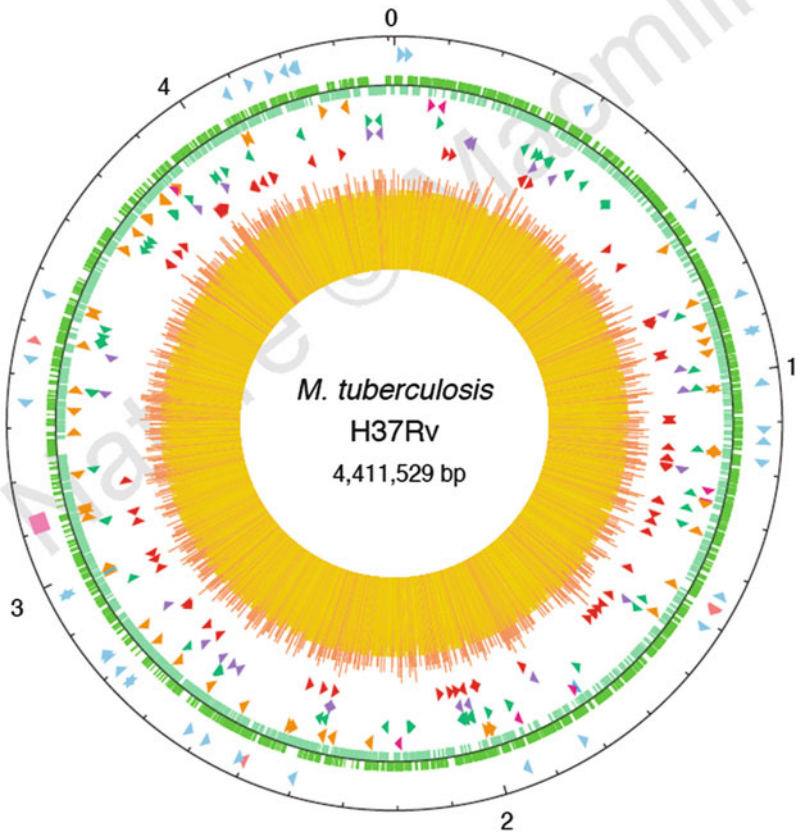

Fig. 8.1
Circular map of the chromosome of M. tuberculosis H37Rv. The *outer* circle shows the scale in Mb, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are *blue*, others are *pink*) and the direct repeat region (*pink* cube), the second ring inward shows the coding sequence by strand (clockwise, *dark green*; anticlockwise, *light green*), the third ring depicts repetitive DNA (insertion sequences, *orange*; 13E12 REP family, *dark pink*; prophage, *blue*), the fourth ring shows the positions of the PPE family members (*green*), the fifth ring shows the PE family members (*purple*, excluding PGRS), and the sixth ring shows the positions of the PGRS sequences (*dark red*). The histogram (*center*) represents G + C content, with <65% G + C in *yellow* and >65% G + C in *red* (Figure and legend reproduced from Cole et al. (1998))

available to guide management until weeks after the patient has presented. The slow rate of replication may be another contributory factor to the challenges of drug treatment of tuberculosis, as many antimicrobial agents target actively dividing bacteria.

Cell Envelope

The cell envelope is one of the distinctive characteristics of *M. tuberculosis*. It comprises a plasma membrane and a lipid-rich cell wall requiring synthesis of long-chain fatty acids known as mycolic acids (Crick et al. 2008; Niederweis et al. 2010). The core cell wall consists primarily of covalently linked peptidoglycan, arabinogalactan, and mycolic acids (*Fig. 8.2*). Molecules such as lipoarabinomannan, other phosphatidylinositol-containing glycolipids, phthiocerol, and trehalose-containing lipids contribute to the soluble component. This cell wall directly protects the bacterium, inhibits drug access to its target, shields antigenic elements from exposure to the host immune system, and also contains immunomodulatory glycopeptolipids. It is also the basis of the microbiological characteristic of acid-fast staining. The bacteria access nutrients through direct diffusion of hydrophobic proteins through the cell wall, via porin proteins spanning the cell wall, and through active transport of molecules such as iron. The cell wall is the target of many existing antituberculous drugs. The precise structure of this cell envelope remains to be elucidated. Redundancy among the components means it is difficult to investigate biological functions of particular constituent compounds. Lipidomics platforms provide a powerful new research tool and also highlight how much more there is to be discovered—only 20% of the ions detected match known structures (Layre et al. 2011).

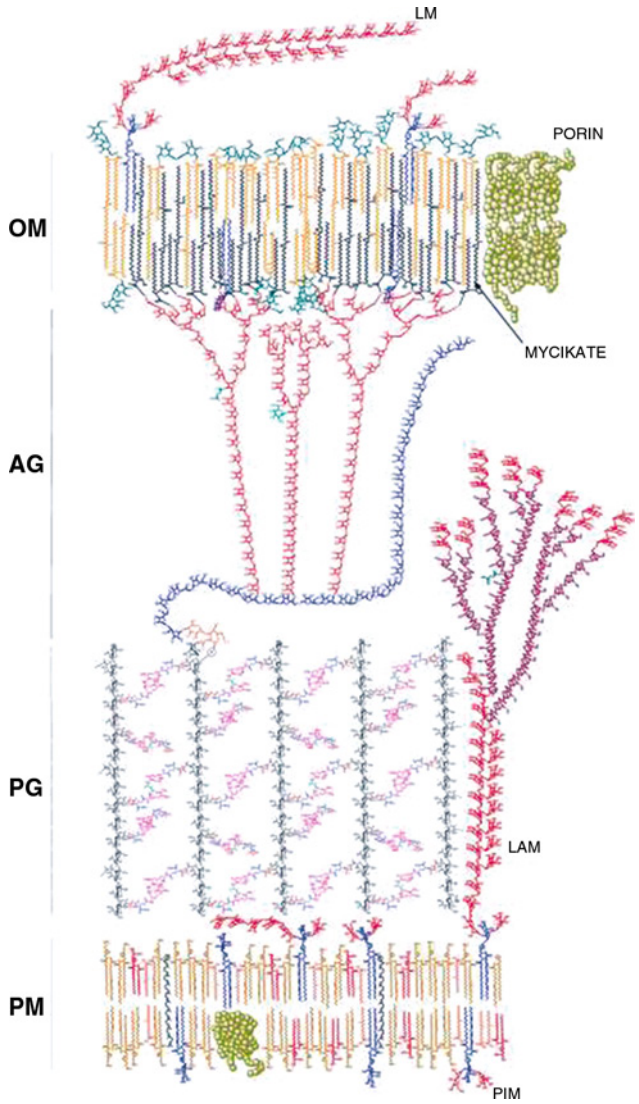

Fig. 8.2
Model of the cell envelope of *M. tuberculosis*. *PM* plasma membrane, *PG* peptidoglycan, *AG* arabinogalactan, *OM* outer membrane, *LAM* lipoarabinomannan phosphatidyl-myo inositol, and *LM* lipomannan (Figure and legend reproduced from Kaur et al. (2009))

Metabolism

One of the key characteristics of *M. tuberculosis* is its ability to adapt to a variety of challenging environments. Metabolic versatility is a key contributor to its resilience within the host (Warner and Mizrahi 2008). Bacilli are able to utilize a range of carbon sources, including those, such as cholesterol, that are relatively energy poor. Not unsurprisingly, given the complexity of the lipid-rich cell envelope described above, lipid metabolism is sophisticated and intricate. *M. tuberculosis* clearly acquires lipids from the host as the transport, and metabolic pathways required for their utilization appear indispensible in animal models (Griffin et al. 2011). The means by which tuberculous bacilli are able to access and transport many other key minerals, cofactors, amino acids, and iron remains elusive. There is significant gene duplication and redundancy within many of these metabolic pathways with large numbers of paralogs for enzymes. This provides a significant challenge to the scientist studying mycobacterial metabolism as manipulating a single component is likely to be compensated for by enzymes with similar function. Accordingly, many of the metabolic pathways employed by *M. tuberculosis* remain to be characterized in detail. Metabolomic (de Carvalho et al. 2010) and proteomic (Kruh et al. 2010) approaches show potential in further unraveling the complexities involved.

Mechanisms of Drug Resistance

M. tuberculosis is resistant to drugs through both inherent and acquired mechanisms (Zhang and Jacobs 2008; Almeida Da Silva and Palomino 2011). Inherent mechanisms range from the previously described cell envelope, including the porins and drug efflux pumps contained therein (Louw et al. 2009). Mycobacteria also possess β-lactamases leading to resistance to β-lactam antibiotics. Unlike many other bacteria that acquire resistance through horizontal transfer of resistance-encoding plasmids or transposons, *M. tuberculosis* does so through spontaneous chromosomal mutations during suboptimal therapy. Isoniazid resistance is most often conferred through mutations in *katG*, a catalase/peroxidase enzyme that activates the isoniazid prodrug, or *inhA*, the gene that encodes a target NADH-dependent enoyl-ACP reductase. Mutations in the *rpoB* gene encoding the β-subunit of the RNA polymerase target of rifampicin lead to conformational changes decreasing drug binding. These mutations generally result in slower growth and less virulence; therefore, bacteria develop compensatory mutations, often in other genes, that decrease the relative fitness cost (Comas et al. 2011). Mutations in *rpoB* are associated with other drug-resistant patterns, especially isoniazid, thereby meaning that such mutations can act as a surrogate marker of multidrug resistance (MDR) in clinical diagnostics (Boehme et al. 2010).

Dormancy

Similarly, elucidation of the "switches" from one metabolic state to another is key to understanding the organism and the disease it causes. Dormancy is a bacterial state of nonreplication that is characterized by long-term viability despite metabolic downregulation (Chao and Rubin 2010). *M. tuberculosis* can become viable but not culturable following incubation in stationary phase for more than 4 months or growth in nitrogen- or carbon-limited media. Conditions such as hypoxia can induce a similar but more easily reversible state called nonreplicating persistence. The changes in gene expression following hypoxia start with a regulon of approximately 50 transcripts called the dormancy survival (*dos*) regulon followed days later by a second set of genes known as the enduring hypoxia response. Dormant

bacteria have the capacity to resuscitate, i.e., metabolically transform from dormancy into an actively dividing state. Dormancy and resuscitation may be the bacterial correlates of the clinical phenomena of latency and reactivation respectively, though how closely these phenomena correlate is unclear.

Pathogenesis

The hallmark of *M. tuberculosis* as a pathogen is its intracellular growth and ability to evade killing by macrophages. *M. tuberculosis* is transmitted through aerosolized droplets by the primary respiratory route from an infected (typically smear positive and therefore high mycobacterial load) patient. Bacilli are then phagocytosed by alveolar macrophages. *M. tuberculosis* is then able to inhibit maturation of the phagolysosome enabling survival and persistence.

The pathological characteristic of tuberculosis is the granuloma (◉ *Fig. 8.3*) (Davis and Ramakrishnan 2009; Rubin 2009; Paige and Bishai 2010). At the center of this structure lie differentiated macrophages containing tubercle bacilli. As a consequence of cytokine release, circulating monocytes, T lymphocytes, and other immune cells are recruited to the focus and incorporated into a surrounding complex network of extracellular matrix. Successful containment of the infection versus local and systemic spread of the bacilli is determined by this host response. Mycobacteria are either confined to the granuloma or a vigorous immune response can cause erosion into the airway with further opportunities for transmission or dispersion locally and to distant organs via hematogenous and lymphatic routes. The importance of CD4+ T lymphocytes, interferon-γ, and type I cytokine response is evident from the increased susceptibility to tuberculosis disease of HIV-infected individuals and families with the Mendelian Susceptibility to Mycobacterial Disease group of genetic disorders. Even when contained within a granuloma, a small population of viable bacilli may persist and in a small proportion are capable of causing reactivation disease decades later in life.

Virulent forms of *M. tuberculosis* inhibit apoptosis of infected macrophages, thereby exiting the macrophage, spreading and impairing optimal presentation of antigens, and priming of the immune response (Behar et al. 2010). Eicosanoid pathways have been implicated in this inhibition of apoptosis (Divangahi et al. 2010). Leukotriene pathways are also involved in susceptibility to mycobacterial disease, and indeed response to anti-inflammatory adjunctive therapy, both in animal models and human tuberculous meningitis (Tobin et al. 2010, 2012).

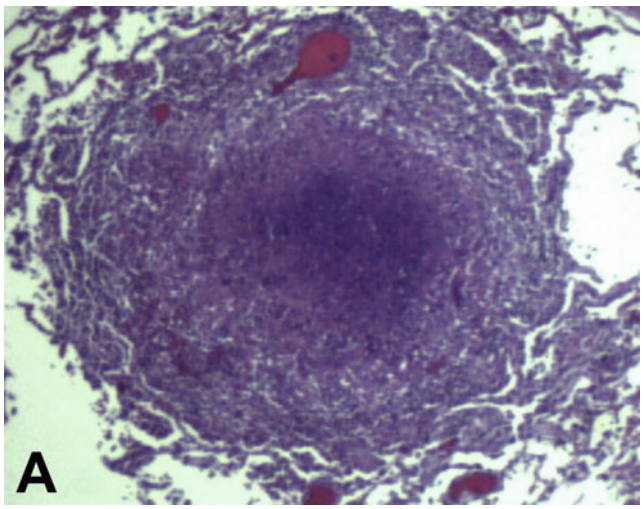

Fig. 8.3
Caseating granuloma of primary tuberculosis showing a central core of necrotic lipid-rich material surrounded by foamy macrophages, epithelioid macrophages, and lymphocytes (Figure and legend reproduced from Hunter (2011))

Epidemiology

M. tuberculosis is a leading cause of death from an infectious disease globally, surpassed only by HIV (World Health Organization 2011b). Recent figures estimate there were 8.8 million new cases of tuberculosis in 2010, and 1.45 million deaths, of whom a quarter were coinfected with HIV. Following on from a concerted Global Health Strategy (see ◉ Prevention), absolute numbers and incidence rates have fallen since 2006 and 2002, respectively, while prevalence and deaths from tuberculosis are predicted to meet the Millennium Development Goal of halving between 1990 and 2015. 3.6% of new incident cases worldwide are estimated to be multidrug resistant, with over half of all cases occurring in China and India (The World Health Organization 2010). There is marked geographical variation in proportion of resistant cases, with areas of Tajikistan reporting up to one in six of new cases of tuberculosis being multidrug resistant.

Although 85% of cases worldwide arise in Asia and Africa, tuberculosis in the United States cannot be ignored (CDC 2011b). 11,182 cases were reported to the Centers for Disease Control and Prevention, representing an incidence rate per year of 3.6 per 100,000 persons. This is actually the lowest number of tuberculosis cases since reporting began in 1953 and represents a 58% decrease since the peak of tuberculosis in the USA in 1992 (◉ *Fig. 8.4*). More than half of the cases arise from California, Texas, New York, and Florida, and foreign-born persons contribute 60% of the national total.

Although the statistics above should rightly be cause for optimism, now is not the time for complacency. Due to the lack of national cohorts, paucity of prevalence and mortality surveys, reliance on accuracy of coding in vital registration systems, and poor documentation of contributory causes in HIV-related deaths in high-burden countries, epidemiological measures can only be best estimates (World Health Organization 2011b). As outlined in this chapter, both tuberculosis disease and particularly infection are true diagnostic challenges, and therefore, public health reporting is likely to be an

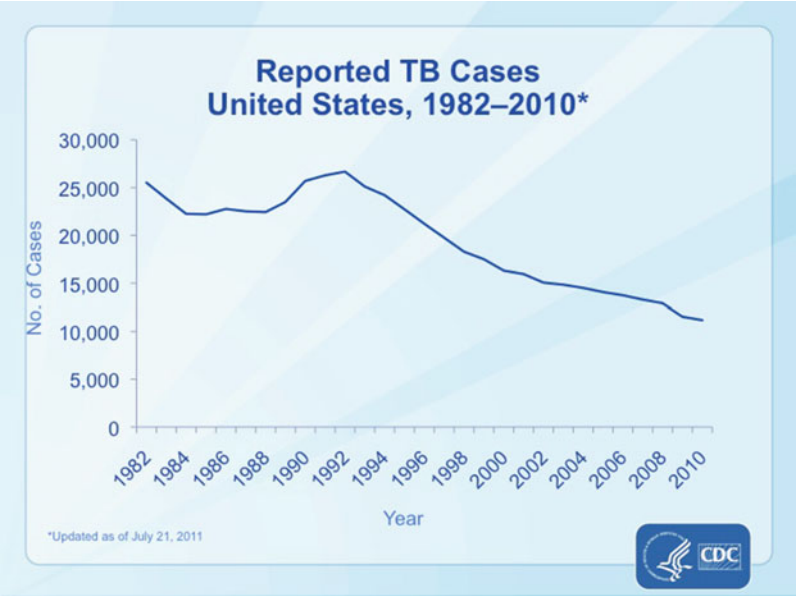

Fig. 8.4
Reported tuberculosis cases, United States, 1982–2010. The resurgence of tuberculosis in the mid-1980s was marked by several years of increasing case counts until its peak in 1992. Case counts began decreasing again in 1993, and 2010 marked the 18th year of decline in the total number of tuberculosis cases reported in the United States since the peak of the resurgence (Figure and legend reproduced with permission from CDC (2011b))

underestimate. Furthermore, as detailed below, drug-resistant tuberculosis is becoming an ever increasing challenge, the HIV/tuberculosis coinfection epidemic continues to wreak havoc, and the STOP TB partnership goal of eliminating tuberculosis by 2050 remains an aspiration but likely not an achievable goal given current approaches.

Molecular Epidemiology

Species and strain distinctions can be made by whole genome sequencing, analysis of highly variable genomic sequences (including 16 S ribosomal RNA), or location of mobile genetic elements such as insertion sequences IS6110. Different clinical strain lineages are associated with particular geographic regions and might represent different ecotypes adapted to different human populations and transmission (Borrell and Gagneux 2011). There are six major lineages which, as anticipated, show evolutionary conservation of essential genes compared to nonessential genes but, interestingly, also hyperconservation of mycobacterial epitopes for human T cells (Comas et al. 2010). The Beijing family of strains, including strain W and W-like families in particular, is associated with drug resistance and thereby raises the question of whether genetic background and epistatic mechanisms may play a role in fitness costs associated with resistance mutations (Borrell and Gagneux 2011).

Clinical Syndrome/Presentation

Active tuberculosis can affect any organ and present with a combination of organ-related and constitutional symptoms and signs. It therefore needs to be considered within a broad differential diagnosis, especially in at-risk and immunosuppressed populations. Primary infection with *M. tuberculosis* is usually mild or asymptomatic. The majority of cases presenting clinically are pulmonary tuberculosis in adults representing reactivation disease (Sia and Wieland 2011). Symptoms include cough and constitutional complaints such as fever, weight loss, fatigue, and night sweats. Chest pain, breathlessness, and haemoptysis can also be features.

Immunosuppressed patients and children are at higher risk of extrapulmonary tuberculosis. Presentations include enlarged lymph nodes, pleural disease, and central nervous system disease. Tuberculous meningitis has particularly high morbidity, progressing from malaise, headache, and personality changes through to neck stiffness, fever, stupor, coma, seizures, paralysis, and death. The most common abdominal presentation is peritonitis with ascites, abdominal pain, fever, and weight loss. Pericardial disease can present with signs of effusion, restriction, or a combination thereof. Skeletal disease presents with signs related to the bone or joint affected. Miliary tuberculosis represents disseminated disease with overwhelming infection via lymphatic and hematogenous spread. Clinical suspicion of

drug resistance is raised by contact with a resistant case, areas of high prevalence, previously or partially treated disease, and where there is no response to the intensive phase of empirical treatment.

Diagnosis

In recent years, there have been significant advances in diagnostics and their application in the clinical arena, from refinements to microscopy, to immunological tests for sensitization to *M. tuberculosis* and, most recently, automated molecular techniques for rapid diagnosis and detection of resistance (Wallis, Pai et al. 2010b). However, the mainstay of diagnosis in many high-prevalence tuberculosis areas remains direct sputum smear microscopy, chest radiography, and clinical judgment (Davies and Pai 2008).

Diagnosing Latent *M. tuberculosis* Infection

By definition, latently infected individuals are asymptomatic and therefore a particular challenge to identify. Because bacteria are rare, diagnosis relies on testing for immune response in sensitized individuals. The ability to mount an immune response is considered to be a marker of current infection. The validity of this assumption is unclear. Certainly, individuals with positive tests are at higher risk for subsequently developing reactivation tuberculosis; however, many may be at little or no risk. Thus, there remains no gold standard for diagnosing latent *M. tuberculosis* infection and, in particular, no test to predict which patients will reactivate and develop disease.

Tuberculin Skin Test

More than a hundred years since its first discovery, the most widely used diagnostic test worldwide remains the tuberculin skin test. It relies upon a delayed-type hypersensitivity response to intradermally injected sterile purified protein derivative (PPD) from seven strains of *M. tuberculosis* (Department of Health 2007). The degree of induration is read at 48–72 h. It is cheap and does not require phlebotomy or laboratory facilities. However, shared antigens between *M. tuberculosis*, nontuberculous mycobacteria, and *M. bovis* Bacille Calmette–Guérin (BCG) vaccine compromise its specificity.

Interferon Gamma Release Assays

Newer blood-based interferon-γ release assays (IGRAs) detect interferon-γ (IFN-γ) released by sensitized T cells when reexposed to *M. tuberculosis*-specific antigens in vitro. Two such tests are currently available: the QuantiFERON Gold test (Cellestis, Carnegie, Australia), which relies on ELISA to detect secreted IFN-γ, and T-SPOT.TB test (Oxford Immunotec, Oxford, UK), which uses an ELISPOT assay to count IFN-γ-producing cells. IGRAs are thought to be as sensitive as the TST, more specific in BCG-vaccinated individuals and those with exposure to environmental mycobacteria, and to correlate better than TST to tuberculosis exposure. However, they fundamentally remain a test of sensitization, without the ability to discriminate between latent infection and active disease (Diel et al. 2011; Rangaka et al. 2012).

Diagnosing Active Tuberculosis Disease

The gold standard of tuberculosis diagnosis relies upon the identification of the organism in symptomatic individuals. While awaiting, or in the absence of, microbiological confirmation, a clinical diagnosis is based on likelihood of infection with tuberculosis, symptoms and signs, and imaging.

Microscopy and Culture

As sputum samples are likely to be contaminated with upper respiratory tract flora, decontamination of samples is required to prevent overgrowth of the slow-growing mycobacteria prior to attempts at culture. Furthermore, homogenization is required to free mycobacteria from the mucus, cells, or tissues in which they may be entrapped. A balance is required between preventing contamination versus false-negative cultures due to damage to tubercle bacilli during sample preparation. The mucolytic N-acetyl-L-cysteine rapidly digests sputum enabling lower concentrations of sodium hydroxide to be used to decontaminate the sample, but even this kills approximately 30% of mycobacteria (World Health Organization 1998b).

The previously described cell envelope is the basis of *M. tuberculosis*' greater ability to withstand alkaline decontamination and also prevents staining of bacilli by conventional means. Components of the cell wall form complexes with dyes such as carbol fuchsin, leaving them resistant to decolorization by acid alcohol. Thus, mycobacteria are termed acid-fast bacilli (*Fig. 8.5*). Fluorescent microscopy with dyes such as auramine has increased sensitivity compared to conventional microscopy (*Fig. 8.5*), while the WHO has endorsed a switch to light emitting diode-based equipment.

M. tuberculosis can be cultured on solid Lowenstein–Jensen or Middlebrook 7 H10 or 7 H11 media. Liquid culture in 7 H9 media is more sensitive than solid culture and decreases time to diagnosis. Glycerol is often used as the preferred carbon source, together with oleic acid–albumin–dextrose–catalase (OADC) supplementation. Detergents such as Tween 80 can be added to reduce clumping and to stimulate growth. However, growth is very slow and colonies can take between 3 and 8 weeks to appear. Growth can generally be detected far earlier using broth cultures and sensitive indicators. Positive liquid cultures can be identified by automated systems measuring production of radiolabeled

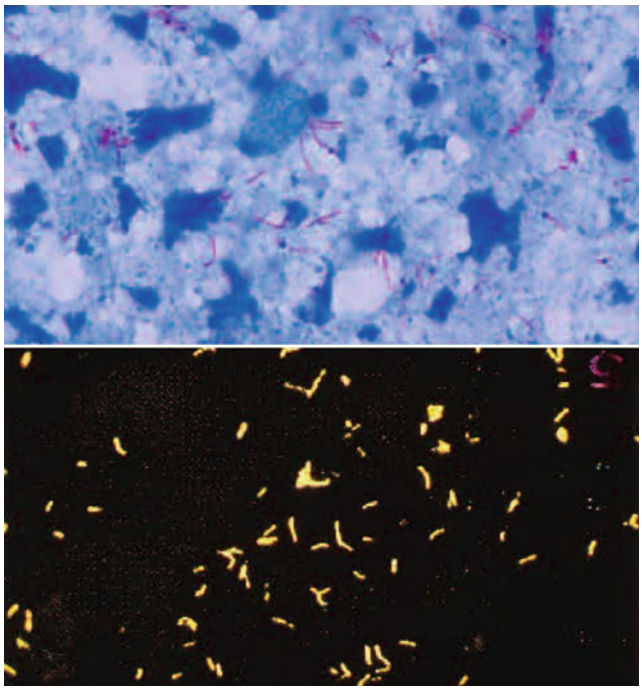

Fig. 8.5
Acid-fast bacilli in sputum appearing as *red* rods against a *blue* background (Ziehl–Neelsen staining) or as *bright yellow* rods against a *dark* background (auramine O fluorescent staining) (Figure and legend reproduced from World Health Organization (1998a))

CO_2 by bacteria growing in media containing ^{14}C-labeled palmitic acid as a carbon source (Bactec460TB, Becton Dickinson Sparks, MD) or by fluorescence from the closed system mycobacterial growth indicator tube (MGIT, Becton Dickinson, Sparks, MD) which contains a plastic resin that fluoresces as oxygen levels are depleted (Hanna et al. 1999). Other recently developed methods might accelerate the time required to detect positive cultures. The Microscopic Observation Drug Susceptibility assay (MODS) involves technicians looking for characteristic microscopic cording of *M. tuberculosis* in liquid culture to detect growth and, through the use of additional wells containing isoniazid and rifampicin, also rapidly tests drug resistance (Caviedes et al. 2006). Bacterial phages that express reporter genes can infect mycobacteria and amplify signals by rapidly growing to high titers (Jain et al. 2011). Sensitive reporters could lead to simple and inexpensive test for both diagnosing disease and detecting drug resistance.

Colonies are classically rough, crumbly, waxy, and nonpigmented (cream-colored). Tuberculous mycobacteria can be distinguished from nontuberculous mycobacteria on the basis of their growth characteristics and biochemical profiles (World Health Organization 1998b). *M. tuberculosis* grows slowly and only at temperatures of 35–37 °C. All mycobacteria produce niacin, which is involved in oxidation–reduction reactions, but *M. tuberculosis* is the most strongly positive because of an inability to convert free niacin into nicotinic acid mononucleotide. *M. tuberculosis* is also one of the strongest reducers of nitrate among mycobacteria, and these tests in combination with the loss of catalase activity when heated to 68 °C form the classical basis of identifying *M. tuberculosis*. Tubercle bacilli also do not grow on LJ medium with p-nitrobenzoic acid. Immunochromatographic detection of a *M. tuberculosis*-specific antigen, MPB64 from culture (Capilia TB Assay, TAUNS Laboratories Inc, Japan), provides an inexpensive speciation result within 15 min with sensitivity and specificity surpassing 99.5% (Muyoyeta et al. 2010).

Molecular Diagnostics

Nucleic acid amplification tests (NAATs) offer the ability to detect *M. tuberculosis*-specific nucleic acids. They rely upon the choice of appropriate target regions that generate species-specific amplicons with a high sensitivity and specificity. These tests have been used in two settings. For several years, NAATs have been used to confirm speciation of cultured organisms in developed world labs. The enhanced amplified *Mycobacterium tuberculosis* direct test (Gen-Probe, San Diego, CA) detects ribosomal RNA, while the Amplicor MTB test (Roche Diagnostics, Branchburg, NJ) detects *M. tuberculosis* DNA. Restriction fragment length polymorphisms can be used to identify species-specific 16S ribosomal RNA, heat shock proteins, *rpoB*, and characteristic insertion sequences (Sankar et al. 2011). Loop-mediated isothermal amplification (LAMP, Eiken Chemical Co Ltd, Tokyo, Japan) involves extraction and purification of DNA from sputum samples followed by amplification and detection using strand displacement reaction, with a turbidity or fluorescence output (Mitarai et al. 2011). As this is a relatively simple manual technique without the need for expensive equipment, there is potential for this to achieve wider geographical coverage than other NAATs.

There are two commercially available line probe assays based on reverse hybridization, where mycobacterial 16S–23S rRNA spacer regions are amplified by PCR and then hybridized to probes on a membrane strip and detected by a colorimetric system. The INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium) detects *M. tuberculosis* DNA and *rpoB* mutations and thereby rifampicin resistance and a surrogate marker for multidrug resistance. It has good sensitivity and specificity from cultures but performed less well with clinical samples (Morgan et al. 2005). The GenoType MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) can detect isoniazid- and rifampicin-associated mutations, *rpoB*, *katG*, and *inhA*. Although it and its predecessors perform well on different specimen types, sensitivity for isoniazid resistance remains variable (Ling et al. 2008).

Recently, a powerful new tool has been added to the diagnostic armamentarium. Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), a fully automated cartridge-based system applying real-time PCR can not only identify *M. tuberculosis* DNA within 2 h, but also *rpoB* mutations associated with rifampicin resistance and a surrogate marker of multidrug resistance (*Fig. 8.6*)

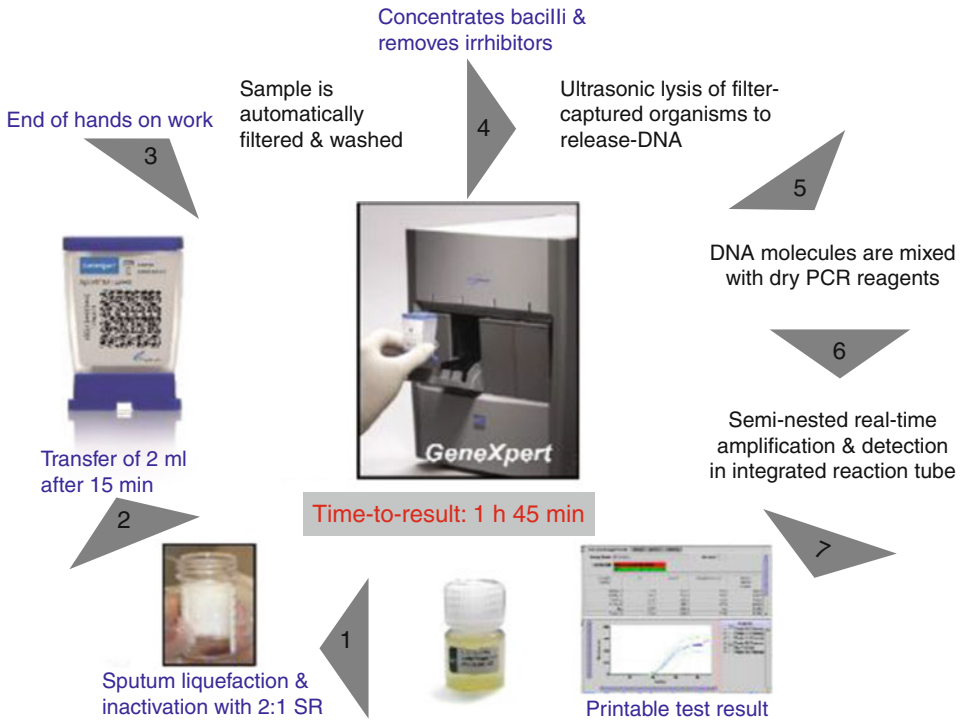

Fig. 8.6

Assay procedure for the Xpert MTB/RIF test. Sample treatment reagent is added to the clinical sample, shaken and incubated. A volume of 2 to 3ml is transferred to the test cartridge. All subsequent steps occur automatically. A printable test result, such as "MTB detected; RIF resistance not detected," is produced (Figure and legend reproduced from World Health Organization (2011a))

(Boehme et al. 2010). For smear-positive culture-positive pulmonary tuberculosis, analysis of a single specimen yielded sensitivity of 99.8% and specificity of 99.2% compared to liquid culture, and analysis of three samples had a sensitivity of 90.2% for smear-negative, culture-positive disease. The opportunity to accurately diagnose not only tuberculosis but MDR-TB within hours of a patient presenting offers the opportunity for early initiation of appropriate treatment and therefore a decrease in spread within the community. Single Xpert MTB/RIF tests on sputum in settings as diverse as urban health centers in India, Peru, and South Africa; drug resistance screening facilities in Azerbaijan and the Philippines; and an emergency room in Uganda have also shown superior sensitivity to microscopy for culture-confirmed disease and decreased time to treatment for smear-negative disease from median 56 to 5 days (Boehme et al. 2011). The endorsement of this method by the WHO heralds a new molecular era in global health, and a strategy to roll out this technology across high-prevalence areas has already begun (World Health Organization 2011a). In pediatric populations, there is again superiority over microscopy in detecting liquid culture–positive cases of pulmonary tuberculosis (Nicol et al. 2011). However, only a quarter of children who were classified as definite or possible tuberculosis were positive using the gold standard of liquid culture. This highlights that in cases of paucibacillary disease, which can occur in pediatric or immunosuppressed populations, molecular microbiological techniques alone cannot provide the diagnostic solution. Furthermore, the reliance on an uninterrupted power supply and annual validation might limit its use to regional centers in developing countries.

Histopathology

As described in the pathogenesis section, the histopathological hallmark of tuberculosis is of the caseating granuloma. This is a localized lesion in tissue consisting of a central area of lipid-rich caseous necrosis surrounded by Langhans giant cells, foamy and epithelioid macrophages, and then a cuff of lymphocytes. Acid-fast bacilli can often be visualized in the center. Granulomas can undergo calcification or fibrotic scarring. In endemic areas, the finding of caseating granulomas is virtually diagnostic of tuberculosis, although, where the disease is less prevalent, other infections, such as those caused by atypical mycobacteria, can be confused with tuberculosis.

Radiology

The range of radiologic appearances reflects the myriad forms of disease that *M. tuberculosis* can cause. Imaging results need to be taken into account with the clinical picture and are not pathognomonic for tuberculosis. When the diagnosis is

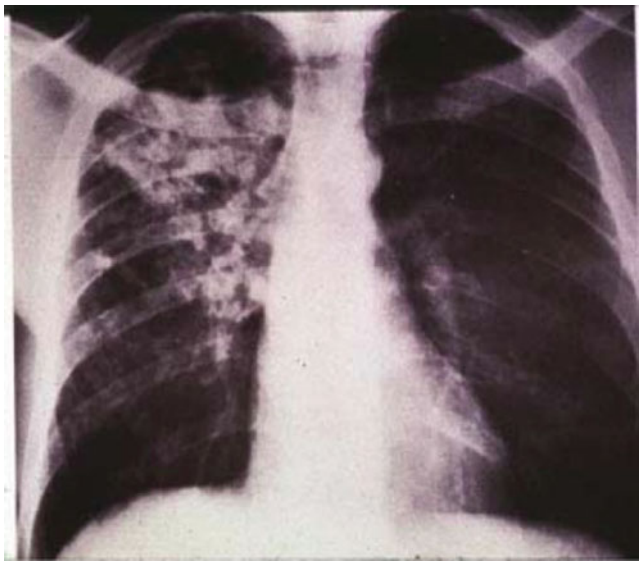

Fig. 8.7
Cavitary pulmonary tuberculosis in a 36-year-old man. *Right upper* lobe streaky infiltrates and nodules are seen; two cavities are also present near the *right* apex (Figure and legend reproduced from Saviola and Bishai (2006))

unclear, it can highlight potential sites from which microbiological samples can be obtained. Furthermore, radiology is able to offer supporting information to make the distinction between infection and active disease when the patient is asymptomatic.

Chest radiographs in pulmonary tuberculosis often show radiological signs of infection, collapse, calcification, cavitation, enlarged lymph nodes, and pleural fluid collections (*Fig. 8.7*) (Burrill et al. 2007). Miliary tuberculosis can be seen on chest X-ray with multiple evenly distributed fine nodules. Depending upon the site and nature of disease, ultrasound, echocardiography, contrast studies, computed tomography (CT), and magnetic resonance imaging (MRI) can be helpful. Recently, positron emission tomography (PET) has been shown to be helpful in research settings (Treglia et al. 2011).

Diagnosing Resistance

Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to at least isoniazid and rifampicin (World Health Organization 2011). Extensively drug-resistant TB (XDR-TB) is resistant to a fluoroquinolone and at least one injectable agent (amikacin, kanamycin, and/or capreomycin) in addition to isoniazid and rifampicin. A group of patients with "totally drug-resistant tuberculosis" has recently been described in India (Udwadia et al. 2011).

Drug resistance can be detected phenotypically through culture of *M. tuberculosis* in media containing antituberculous drugs or genotypically through identification of mutations associated with drug resistance. Traditionally, resistance has been found by growing bacteria on solid medium impregnated with an appropriate antibiotic, a rather cumbersome approach. Other, more rapid, tests of growth can be applied. These include colorimetric redox indicator (CRI) methods (Martin et al. 2007). These rely upon the reduction of a colored indicator when added to the culture medium after *M. tuberculosis* has been exposed to antituberculous drugs. Change in color relates to the number of remaining viable mycobacteria in the culture medium. The nitrate reductase assay similarly detects color changes of reagents associated with reduction of nitrate to nitrite by viable mycobacteria following coculture with antituberculous drugs (Ramos et al. 2012). And, as described above, some tests both diagnose tuberculosis and detect resistance (e.g., phenotypically through MODS or genotypically through Xpert MTB/RIF and other nucleic acid amplification techniques such as the line probe assays).

Diagnostics in Development

Although there have clearly been significant advances in tuberculosis diagnostics, most notably the translation from bench to developing country health-care settings of an automated rapid molecular test both for diagnosis and resistance, many challenges remain. There remains no gold standard for diagnosing latent infection in the absence of disease. Immunological tests of exposure and sensitization rely upon detecting a host immune response, which may be impaired in immunosuppressed or pediatric populations. Similarly, consistently establishing a microbiological diagnosis in such cases of paucibacillary disease remains elusive.

Therefore, the global community continues to pour its efforts into development and evaluation of new biomarkers and diagnostics for both infection and disease. Biomarkers are a particular focus of attention due to their potential to elucidate pathogenesis, improve prognosis, monitor response to treatment, and act as markers of protection in vaccine trials (Wallis et al. 2010b). Discovery of reliable biomarkers could therefore significantly accelerate translation of drug and vaccine research to clinical practice, by reducing the need for prolonged follow-up. The distinction between latent infection and active disease is increasingly being perceived as a spectrum rather than discrete entities, and the complex influence of mycobacterial and host factors on clinical course is also becoming increasingly apparent.

Approaches being explored include discriminatory gene expression signatures derived from transcriptomic approaches (Berry et al. 2010), identifying *M. tuberculosis*-specific lymphocyte subpopulations (Caccamo et al. 2009; Lancioni et al. 2011), proteomic fingerprinting (Agranoff et al. 2006), cytokines (Sutherland et al. 2011), and mycobacterial lipoarabinomannan in the urine (Minion et al. 2011). Mycobacterial reporter phage tests are under development as are breath-based tests, similar to the urease breath test for *H. pylori* (Jain et al. 2011).

Treatment

Treatment for tuberculosis currently requires patients to take multiple medications for months due to the slowly replicating nature of *M. tuberculosis* and the potential for a sensitive strain becoming drug resistant. Not unsurprisingly, at present, concordance with treatment is a significant issue and, therefore, the somewhat optimistically named directly observed treatment short course (DOTS) strategies are a key foundation of treatment, incorporating supervision of medication administration by community health workers to optimize adherence. The goals of drug treatment are to rapidly kill any actively dividing bacilli, sterilize any lesions by killing any semi-dormant bacilli, and prevent emergence of resistance.

Key challenges in treatment therefore include the limited armory of effective medicines for MDR strains and the need for shorter duration treatment options with less frequent dosing. This would improve concordance, decrease expense, and ease logistics. The optimal treatment for HIV-positive patients with tuberculosis remains complex, with a fine balance to be struck between drug interactions and patient pill burden. In addition, one unexpected side effect of effective antiviral treatment has been the immune reconstitution syndrome (IRIS). In IRIS, as the immune system develops the capacity to mount an effective inflammatory response, existing tuberculosis symptoms can paradoxically worsen, or underlying infection can be "unmasked"(Abdool Karim et al. 2011; Blanc et al. 2011; Havlir et al. 2011; Torok and Farrar 2011).

The World Health Organization current recommendations for pulmonary tuberculosis are a total of 6 months treatment, initially with rifampin, isoniazid, pyrazinamide, and ethambutol for the first 2 months, followed by 4 months of rifampin and isoniazid alone (World Health Organization Stop TB Dept 2009). Extrapulmonary tuberculosis involving the central nervous system, bone, or joint requires longer durations of 9–12 months of treatment. Depending upon the patient and health-care system, three times a week dosing is an alternative to daily doses and fixed dose combination tablets can decrease the pill burden. Treatment for MDR and XDR tuberculosis is complex and should be under specialist supervision supported by drug susceptibility testing results. MDR disease requires 18 months of minimum treatment involving at least four drugs including a fluoroquinolone and an injectable antibiotic for at least the first 4 months. If fully implemented, such regimens have treatment success rates of 69% (Orenstein et al. 2009). There are accompanying challenges in terms of the long duration of treatment, likely need for hospitalization, monitoring of response, access to and expense of second line agents, prevention of spread of drug-resistant strains, toxicity, interactions, and vascular access.

Drug development requires huge financial investment and resources from the discovery stage through testing in animal models, safety, dosing, and clinical effectiveness trials in patients. These also take many years. Despite the absence of a large developed world market for antituberculous medicines, there has been considerable activity in developing new drugs with funding mainly coming from academic–industrial partnerships, philanthropic foundations, and global health initiatives. There are many approaches to new tuberculosis drug development (Koul et al. 2011). Conventional screening of small compound libraries remains a labor-intensive and, thus far, low yield strategy. Identification of essential mycobacterial genes with consequent rational drug design is an appealing approach, although such targets are not necessarily "druggable" and there is still the obstacle of the cell envelope to be overcome. A more holistic approach, phenotypic screening for compounds that can kill bacteria without focusing on specific targets, has produced one of the most promising novel compounds, TMC207 (bedaquiline), a diarylquinoline that targets ATP synthase (Diacon et al. 2009). Several other promising compounds have arisen from similar whole cell screens PA-824 (Singh et al. 2008) and OPC-67683 (Diacon et al. 2011), repurposing of existing drugs (such as the fluoroquinolones moxifloxacin and gatifloxacin (Rustomjee et al. 2008) and the oxazolidinone linezolid (Cox and Ford 2012; Lee et al. 2012)), and the development of new agents within existing classes (such as PNU-100480 (Wallis et al. 2010a)).

An alternative approach to fighting infection might be to influence the host response to infection. Steroids are already recommended by the WHO for pericardial and meningeal drug-susceptible tuberculosis to decrease the swelling and consequent damage within these tightly enclosed structures. With increasing evidence of the role for vitamin D, supplementation might help, particularly for those with a particular *TaqI* vitamin D receptor polymorphism (Martineau et al. 2011). In addition, other modulators of the host response might play a role. For example, studies in animals suggest that some protein kinase inhibitors can substantially modify the course of an infection (Napier et al. 2011).

Prevention

WHO declared tuberculosis a public health emergency in 1993 and concerted efforts focused on directly observed treatment short course (DOTS), a multifaceted strategy comprising political commitment and financing, early case detection and diagnosis through quality-assured bacteriology, standardized treatment with supervision, an effective drug supply, and monitoring and evaluation of impact. The STOP TB Strategy also includes scaling up tuberculosis/HIV and MDR-TB activities, laboratory strengthening and research into basic science, diagnostics, drugs, vaccines, and operational areas (World Health Organization Stop TB Partnership 2010). Between 1995 and 2008, 43 million people were treated under DOTS, 36 million cured, mortality rates halved from 8% to 4%, and an estimated six million deaths averted (World Health Organization 2009).

Public Health Measures

One of the cornerstones of an effective strategy for prevention of tuberculosis is to decrease the spread of disease from an

infectious individual. Key to this is early diagnosis, early treatment, identification of contacts, and minimizing the spread of disease. The most infectious patients with tuberculosis are those with pulmonary tuberculosis, a cough, cavitating changes on chest X-ray, and who are smear positive on microscopy (CDC 2005). Those at highest risk of infection and/or progression to disease include household contacts, those originating from high-prevalence areas, children, health-care workers, immunocompromised individuals (including HIV-positive patients and those on steroid or antitumor necrosis factor (TNF) therapy), and those with chronic diseases such as diabetes mellitus and chronic renal failure and drug-abusing populations. Therefore, early diagnosis, isolation in negative pressure rooms when hospital admission is indicated, airborne pathogen precautions, identification and evaluation of household contacts for disease, and latent *M. tuberculosis* infection are all key to preventing transmission from an index case (Sia and Wieland 2011). Prevention and control in the context of HIV infection relies upon a multipronged strategy incorporating intensified case finding, isoniazid preventative therapy, infection control, and antiretroviral therapy (Harries et al. 2010).

Treatment of Latent *M. tuberculosis* Infection

Treating patients with latent *M. tuberculosis* infection in low-prevalence countries offers the opportunity to prevent reactivation disease later in life and therefore contribute to a reduction in disease burden. When deciding to treat patients with latent *M. tuberculosis* infection, the individual's lifetime risk of developing disease, patient concordance, side effects, and interactions need to be considered (Horsburgh and Rubin 2011). Treatment options include 6–9 months of isoniazid, 4 months of rifampicin, or 3 months of both rifampicin and isoniazid. The CDC has recently endorsed a 3-month course of weekly isoniazid and rifapentine with equal efficacy and obvious appeal due to the short duration of treatment and decreased dosing frequency (CDC 2011a), though, as approved, this regimen requires direct observation.

Vaccines

The Bacille Calmette–Guérin (BCG) vaccine is a live-attenuated form of *Mycobacterium bovis* following multiple passages in culture and was first given in humans in 1921. It protects children from severe forms of tuberculosis disease (Trunz et al. 2006) although only offers variable protection against adult pulmonary disease (Colditz et al. 1994). Although BCG has a good safety record, there is the risk of disseminated BCG disease in HIV-infected individuals. Vaccine research therefore focuses on improved ability to prevent infectious cases of pulmonary tuberculosis, either through primary prevention or boosting of immunity following exposure, while improving safety in HIV-positive populations.

There are at least 12 vaccines in the various stages of clinical trials at present (Kaufmann 2011). Approaches include modification of BCG, attenuation of *M. tuberculosis*, and booster strategies such as viral-vector vaccines and fusion-protein adjuvant vaccines. The recombinant vaccine rBCG30 for instance overexpresses the *M. tuberculosis* secreted antigen Ag85b (Hoft et al. 2008). An alternative strategy employed in the rBCGΔureC:hly (VPM 1002) vaccine (Grode et al. 2005) uses expression of listeriolysin, a *Listeria*-derived membrane-perforating protein, to increase phagosomal escape and therefore induce apoptosis of infected host cells leading to improved antigen presentation. A urease deletion optimizes the phagosomal pH for listeriolysin action. Attenuated vaccinia virus (Scriba et al. 2012), replication-deficient adenovirus vectors expressing *M. tuberculosis* antigens (Abel et al. 2010), and fusion-protein adjuvant vaccines (Skeiky et al. 2010) are also in early clinical trials. Attenuated *M. tuberculosis* or auxotrophic *M. tuberculosis* remains in preclinical stages at present, although clinical trials are likely to follow soon (Cardona et al. 2009).

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9 Legionnaires' Disease

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Abstract

Legionella pneumophila, the etiological agent of Legionnaires' disease was first recognized in 1976, during an outbreak of severe pneumonia at the convention of the American Legion in Philadelphia. Since then the genus *Legionella* has expanded, and to date more than 50 different species are described. However, *L. pneumophila* remains the major cause of human disease, as it is responsible for over 90 % of legionellosis cases worldwide. This chapter starts with a description of the genus *Legionella* and the ecology of these bacteria. We then highlight the epidemiology, clinical features, and the different diagnostic assays and methods available. In the second part, we focus on the pathogenesis, the virulence factors, and the immune response of the host. Special emphasis is placed on the implication of the literally hundreds of different effector proteins secreted by the Dot/Icm type IV secretion system. In the third section we discuss

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recent knowledge acquired on genomics, transcriptomics, and the metabolic features of *Legionella*, and, particularly, we present new insight on comparative genomics, evolution, horizontal gene transfer, and the regulation of the life cycle of *L. pneumophila*.

Introduction, Taxonomy, and Ecology

Introduction and History

Legionnaires' disease is an acute pneumonia caused by bacteria of the genus *Legionella*. Pontiac fever is a febrile, nonpneumonic, systemic illness that is associated with, if not caused by, species of *Legionella*. Legionellosis refers to all diseases caused by the *Legionella* bacteria. Legionnaires' disease accounts for >99 % of legionellosis cases (CDC 2011).

Legionnaires' disease was first recognized as a clinical entity when it caused an epidemic of pneumonia at an American Legion convention in Philadelphia in 1976 (Fraser et al. 1977). In that outbreak, 221 people were affected, and 34 died. There was enough national concern to prompt two congressional investigations of the outbreak. An intense epidemiologic review determined that the disease was likely airborne and focused primarily at one convention hotel. About 6 months later, Joseph McDade and Charles Shepard of the United States Centers for Disease Control and Prevention discovered the etiologic agent, a fastidious Gram-negative bacterium (McDade et al. 1977). Because of the association with the American Legion convention, this disease is now called "Legionnaires' disease," and the etiologic agents belong to the family *Legionellaceae*, with *L. pneumophila* (type strain Philadelphia 1) being the agent responsible for the 1976 epidemic (Brenner et al. 1979). Retrospective serologic studies indicated that unsolved outbreaks of pneumonia in 1957, 1965, 1973, and 1974 had been Legionnaires' disease (Brenner 1987; Winn 1988). Unsolved outbreaks of a nonpneumonic illness in 1968 and 1973 were also determined to be due to exposure to *Legionella* bacteria, and this illness was termed "Pontiac fever," named after the city in Michigan where the 1968 outbreak had occurred (Brenner 1987; Winn 1988). Additionally, stored isolates from 1940s to 1950s were found to be *Legionella* bacteria, including *L. pneumophila* strain OLDA from 1947, *L. micdadei* strains TATLOCK and HEBA from 1943 to 1959, respectively, *L. bozemanae* strain WIGA from 1959, and a strain of *L. lytica* from 1954 (Brenner 1987; Fields et al. 2002; Winn 1988).

Legionella bacteria are Gram-negative rods that occur frequently in natural aquatic habitats, with infection of humans usually occurring via the inhalation of *Legionella*-contaminated water droplets (Fields et al. 2002). The legionellae are aerobic, non-spore forming, and unencapsulated. All but a few *Legionella* species are motile by means of flagella. Amino acids are considered their primary energy source. The pH and temperature optima for in vitro growth are 6.8–7.0 and 25–42 °C, respectively, with optimal growth occurring at 35–37 °C. L-cysteine is required for growth, and iron is needed for initial isolation from the environment or clinical specimens. Buffered charcoal yeast extract (BCYE) agar that is supplemented with L-cysteine, iron, and α-ketoglutarate is the preferred growth medium for culturing *Legionella* bacteria. As will be detailed below, *L. pneumophila* and probably all *Legionella* species are facultative intracellular parasites of eukaryotic cells. In the environment, legionellae persist within species of free-living amoebae, and in the human lung, the bacteria primarily parasitize the alveolar macrophages (Newton et al. 2010).

The *Legionella* Genus

Legionella is almost universally regarded as the sole genus in the family Legionellaceae within the γ-2 subdivision of Proteobacteria (Benson and Fields 1998; Fry et al. 1991; Ludwig and Stackebrandt 1983). The nearest phylogenetic relative to *Legionellaceae* is the family Coxiellaceae, which includes *Coxiella burnetii*, the etiologic agent of Q fever (Weisburg et al. 1989; Williams et al. 2010). Currently, there are 56 validly published species of *Legionella* (Table 9.1). Besides the named *Legionella* species, there are bacteria that resemble *Legionella* but have not yet been cultured outside of their protozoal host in order to permit further characterization. These organisms are designated as *Legionella*-like amoebal pathogens (LLAP) (Adeleke et al. 1996; Fields et al. 2002; Hookey et al. 1996). There appear to be at least four *Legionella* spp. among the uncharacterized LLAPs (Adeleke et al. 2001; Birtles et al. 1996; Newsome et al. 1998). Genomic techniques are the definitive means of identifying recognized *Legionella* spp. and of differentiating them from novel species. PCR and sequencing of the 16S ribosomal RNA subunit (16S rRNA) gene is the initial step (Fields et al. 2002; Kampfer 2012; Pearce et al. 2012). Strains possessing 16S rRNA sequences that are <97.0 % similar to that of all known species are considered a new species (Tindall et al. 2010). When the level of similarity falls between 97.0 % and 99.9 %, DNA-DNA hybridization is done; strains showing <70 % similarity by DNA-DNA hybridization are deemed novel species (Fields et al. 2002; Pearce et al. 2012; Tindall et al. 2010). To further facilitate identification, additional genes are subjected to PCR and sequence analysis in a method known as multi-locus sequence typing (Kampfer 2012; Tindall et al. 2010). The sequence targets that are used for *Legionella* studies include the macrophage infectivity potentiator (*mip*), RNase P (*rnpB*), DNA gyrase (*gyrA*), RNA polymerase β-subunit (*rpoB*), and the intergenic 16S-23S ribosomal spacer (Edelstein et al. 2011; Feddersen et al. 2000; Ko et al. 2002; Lo Presti et al. 2001; Luck et al. 2010; Pearce et al. 2012; Ratcliff et al. 1998; Rubin et al. 2005). The European Working Group for *Legionella* Infections (EWGLI) has created an on-line database to aid in *mip*-based identification (Fry et al. 2007). Rapid whole-genome sequencing will likely play a large role in defining species in the future (Kampfer 2012). Thus far, the genome sequence has been determined for five strains of *L. pneumophila*, two strains of *L. longbeachae*, and one strain of *L. drancourtii*

Table 9.1

***Legionella* species and their association with disease**

Species	Initial isolation and species definition			Implicated in disease[a]
	Source	Year	Reference	
L. adelaidensis	Environmental	1991	(Benson et al. 1991)	No
L. anisa	Environmental	1985	(Gorman et al. 1985)	Yes (Fallon and Stack 1990; Jones et al. 2003)
L. belairdensis	Environmental	2001	(Lo Presti et al. 2001)	No
L. birminghamensis	Clinical	1987	(Wilkinson et al. 1987)	Yes
L. bozemanae	Clinical	1980	(Brenner et al. 1980)	Yes (Harris et al. 1998)
L. brunensis	Environmental	1988	(Wilkinson et al. 1988)	Yes (Ricketts and Joseph 2007)
L. busanensis	Environmental	2003	(Park et al. 2003)	No
L. cardiaca	Clinical	2012	(Pearce et al. 2012)	Yes
L. cherrii	Environmental	1985	(Brenner et al. 1985)	Yes[b] (Fang et al. 1989)
L. cincinnatiensis	Clinical	1988	(Thacker et al. 1988)	Yes (Gubler et al. 2001)
L. drancourtii	Environmental	2004	(La Scola et al. 2004)	Yes[b] (Marrie et al. 2001)
L. dresdenensis	Environmental	2010	(Luck et al. 2010)	No
L. drozanskii	Environmental	2001	(Adeleke et al. 2001)	Yes[b] (Marrie et al. 2001)
L. dumoffii	Environmental	1980	(Brenner et al. 1980)	Yes (Flendrie et al. 2011)
L. erythra	Environmental	1985	(Brenner et al. 1985)	Yes[b] (Fields et al. 2002; Lieberman et al. 2002)
L. fairfieldensis	Environmental	1991	(Thacker et al. 1991)	No
L. fallonii	Environmental	2001	(Adeleke et al. 2001)	Yes[b] (McNally et al. 2000)
L. feeleii	Environmental	1984	(Herwaldt et al. 1984)	Yes (Lee et al. 2009)
L. geestiana	Environmental	1993	(Dennis et al. 1993)	No
L. genomospecies	Environmental	1996	(Benson et al. 1996)	No
L. gormanii	Environmental	1980	(Morris et al. 1980)	Yes (Towns et al. 1994)
L. gratiana	Environmental	1989	(Bornstein et al. 1989)	Yes[b] (Lieberman et al. 2002)
L. gresilensis	Environmental	2001	(Lo Presti et al. 2001)	No
L. hackeliae	Clinical	1985	(Brenner et al. 1985)	Yes (Wilkinson et al. 1985)
L. impletisoli	Environmental	2007	(Kuroki et al. 2007)	No
L. israelensis	Environmental	1986	(Bercovier et al. 1986b)	No
L. jamestowniensis	Environmental	1985	(Brenner et al. 1985)	No
L. jordanis	Environmental	1982	(Cherry et al. 1982)	Yes (Meyer et al. 2011)
L. lansingensis	Clinical	1992	(Thacker et al. 1992)	Yes
L. londiniensis	Environmental	1993	(Dennis et al. 1993)	Yes[b] (Lieberman et al. 2002)
L. longbeachae	Clinical	1981	(McKinney et al. 1981)	Yes (Whiley and Bentham 2011)
L. lytica	Clinical	1996	(Hookey et al. 1996)	Yes (Marrie et al. 2001)
L. maceachernii	Environmental	1985	(Brenner et al. 1985)	Yes (Chee and Baddour 2007)
L. micdadei[c]	Clinical	1980	(Hebert et al. 1980)	Yes (Fernandez-Cruz et al. 2011; Girard and Gregson 2007)
L. moravica	Environmental	1988	(Wilkinson et al. 1988)	No
L. nagasakiensis	Environmental and clinical	2011	(Yang et al. 2011)	Yes
L. nautarum	Environmental	1993	(Dennis et al. 1993)	Yes[b] (Lieberman et al. 2002)
L. oakridgensis	Environmental	1983	(Orrison et al. 1983)	Yes (Lo Presti et al. 2000)
L. parisiensis	Environmental	1985	(Brenner et al. 1985)	Yes (Lo Presti et al. 1997)
L. pneumophila	Clinical	1979	(Brenner et al. 1979)	Yes
L. quateirensis	Environmental	1993	(Dennis et al. 1993)	No
L. quinlivanii	Environmental	1989	(Benson et al. 1989)	Yes[b] (Berger et al. 2006; La Scola et al. 2003)
L. rowbothamii	Environmental	2001	(Adeleke et al. 2001)	Yes[b] (McNally et al. 2000)
L. rubrilucens	Environmental	1985	(Brenner et al. 1985)	Yes (Matsui et al. 2010)
L. sainthelensi	Environmental	1984	(Campbell et al. 1984)	Yes (Benson et al. 1990; Loeb et al. 1999)

Table 9.1 (continued)

Species	Initial isolation and species definition			Implicated in disease[a]
	Source	Year	Reference	
L. santicrucis	Environmental	1985	(Brenner et al. 1985)	Yes (Tang and Krishnan 1993)
L. shakespearei	Environmental	1992	(Verma et al. 1992)	No
L. spiritensis	Environmental	1985	(Brenner et al. 1985)	No
L. steelei	Clinical[d]	2011	(Edelstein et al. 2011)	No
L. steigerwaltii	Environmental	1985	(Brenner et al. 1985)	No
L. taurinensis	Environmental	1999	(Lo Presti et al. 1999)	No
L. tusconensis	Clinical	1989	(Thacker et al. 1989)	Yes (Doleans et al. 2004)
L. wadsworthii	Clinical	1982	(Edelstein et al. 1982)	Yes (Yu et al. 2002)
L. waltersii	Environmental	1996	(Benson et al. 1996)	Yes (Konig et al. 2005)
L. worsleiensis	Environmental	1993	(Dennis et al. 1993)	Yes[b] (Berger et al. 2006)
L. yabuuchiae	Environmental	2007	(Kuroki et al. 2007)	No

[a]Reference(s) in parenthesis provide information on the clinical cases for non-*pneumophila* species that have occurred subsequent to the initial isolation of the species
[b]Species implicated in disease through serologic studies as opposed to strain isolation
[c]*L. micdadei* and *L. pittsburghensis* (Pasculle et al. 1980) are considered homotypic synonyms (http://www.bacterio.cict.fr/l/legionella.html)
[d]Strain isolated from a human nasal cavity but not implicated in disease

(Cazalet et al. 2010, 2004; Chien et al. 2004; D'Auria et al. 2010; Glockner et al. 2008; Kozak et al. 2010; Moliner et al. 2009b; Schroeder et al. 2010). Phenotypic traits, such as growth characteristics, fatty acid composition, and serology, continue to be important in the definition of the species (Fields et al. 2002; Kampfer 2012; Pearce et al. 2012). *L. pneumophila* contains at least 16 different serogroups (Helbig et al. 2007; 2002; Luck et al. 1995). Eight of the other species contain two serogroups, and the remaining species consist of single serogroups (Benson and Fields 1998; Harrison and Saunders 1994).

Thus far, 37 of the *Legionella* species have been linked to Legionnaires' disease, with 26 of them being isolated from patients and the remaining 11 implicated based upon serologic evidence (*Table 9.1*). It is likely that additional species will prove to be etiologic agents of disease; for example, at least one of the unnamed LLAPs appears pathogenic based upon serologic evidence (Adeleke et al. 2001; Lamoth and Greub 2010; Marrie et al. 2001; McNally et al. 2000). Except for *L. tucsonensis* and the recently described *L. cardiaca* and *L. steelei*, all of the *Legionella* species that have been isolated from clinical sources have also been isolated from the environment (*Table 9.1*) (Buse et al. 2012; Edelstein et al. 2011; Flannery et al. 2006; Graham et al. 2011; Pearce et al. 2012; Thacker et al. 1989). In the United States and Europe, *L. pneumophila* accounts for approximately 90–95 % of Legionnaires' disease cases (Benin et al. 2002b; Joseph 2004; Joseph and Ricketts 2010; Yu et al. 2002). The next most common causes tend to be *L. anisa*, *L. bozemanae*, *L. dumoffii*, *L. longbeachae*, and *L. micdadei*, accounting for approximately 2–8 % of cases (Aurell et al. 2003; Benin et al. 2002b; Joseph and Ricketts 2010; Svarrer and Uldum 2011; Yu et al. 2002). In Australia, New Zealand, and parts of Asia, however, *L. longbeachae* is the most commonly isolated species, representing up to 30–53 % of clinical cases (Gobin et al. 2009; Graham et al. 2011; Whiley and Bentham 2011).

L. pneumophila serogroup 1, which caused the 1976 Philadelphia outbreak, is the cause of 80–95 % of all cases of Legionnaires' disease (Amemura-Maekawa et al. 2010; Aurell et al. 2003; Borchardt et al. 2008; Campese et al. 2011; Doleans et al. 2004; Harrison et al. 2009, 2007; Helbig et al. 2002; Joseph 2004; Joseph and Ricketts 2010; Ricketts et al. 2010; Ricketts and Joseph 2007, 2005; Yu et al. 2002). A single subtype of *L. pneumophila* serogroup 1 is responsible for 64–92 % of cases of Legionnaires' disease due to *L. pneumophila*, and 80–94 % of cases due to *L. pneumophila* serogroup 1 (Amemura-Maekawa et al. 2010; Edelstein and Metlay 2009; Harrison et al. 2009, 2007; Helbig et al. 2002; Kozak et al. 2009). This subtype is defined by its reactivity with a particular monoclonal antibody and is variously designated as the "Pontiac," "Joly monoclonal type 2 (MAb2)," or "Dresden monoclonal type 3/1 (MAb 3/1)" monoclonal subtype. A sequence-based typing (SBT) method has also been developed for characterizing *L. pneumophila* isolates, and once again, a subset of strains (e.g., those belonging to ST1, ST47, ST213, ST222) is responsible for most clinical cases (Amemura-Maekawa et al. 2010; Harrison et al. 2009; Hilbi et al. 2010; Kozak et al. 2009; Vergnes et al. 2011). Interestingly, when environmental isolates are examined, there is not such a predominance of serogroup 1 strains, MAb2- or MAb3/1-positive strains, or those sequence subtypes, implying that these strains have enhanced virulence and/or transmissibility (Cazalet et al. 2008; Doleans et al. 2004; Hilbi et al. 2010; Kozak et al. 2009).

The *Legionella* species that have been linked to cases of Pontiac fever are *L. anisa*, *L. feeleii*, *L. longbeachae*, *L. maceachernii*, *L. micdadei*, and *L. pneumophila* (Cramp et al. 2010;

Huhn et al. 2005; Jones et al. 2003). The most common is *L. pneumophila*, accounting for approximately >70 % of reported cases.

Environmental Ecology of *Legionella*

Legionella bacteria are ubiquitous in natural aqueous environments. Shortly after its discovery, planktonic *L. pneumophila* was detected in virtually all of the 267 freshwater habitats (i.e., lakes, ponds, rivers, creeks, swamps, wet soil) examined in the United States (Fliermans et al. 1981, 1979). The ubiquity of *L. pneumophila* in freshwater has been confirmed throughout the world (Bercovier et al. 1986a; Carvalho et al. 2008, 2007; Castellani Pastoris et al. 1989; Dutka and Ewan 1983; Joly et al. 1984; Lawrence et al. 1999; Lee et al. 2010; Ortiz-Roque and Hazen 1987; Parthuisot et al. 2010; Sheehan et al. 2005; Tobiansky et al. 1986; Verissimo et al. 1991), and, over the years, the organism has also been found in marine and estuarine environments (Heller et al. 1998; Ortiz-Roque and Hazen 1987; Palmer et al. 1993; Paszko-Kolva et al. 1993). In man-made (engineered) water systems, *L. pneumophila* is similarly widespread, existing, in some areas, within the plumbing of 60–85 % of large and small public buildings, as well as in private residences (Alary and Joly 1991; Atlas 1999; Lasheras et al. 2006; Lee and West 1991; Mouchtouri et al. 2007; Ragull et al. 2007). The broad distribution of *L. pneumophila* is partly due to the organism's capacity to survive at 4–63 °C (Atlas 1999; Fliermans et al. 1981, 1979; Heller et al. 1998; Joly et al. 1984; Wadowsky et al. 1985; Wullings and van der Kooij 2006). However, warm water is more likely to contain the bacterium, especially in hot water tanks and heaters and in water-cooled heat rejection devices such as cooling towers. The ability of *L. pneumophila* to survive and grow at lower temperatures has been linked to its type II secretion system (see below), a secreted protein foldase, lipid A modifications, RNase R, and an RNA helicase, among other things (Charpentier et al. 2008; Söderberg and Cianciotto 2008; Söderberg and Cianciotto 2010; Söderberg et al. 2004, 2008). Although fewer studies have focused on assessing the distribution of non-*pneumophila* species, a variety of these bacteria are often easily found in natural and engineered water systems (Buse et al. 2012; Lee et al. 2010; Parthuisot et al. 2010). *L. pneumophila*, *L. longbeachae*, and several other *Legionella* species are also found in soil, potting soil, and compost (Hughes and Steele 1994; Lindsay et al. 2012). Under certain conditions, including low-nutrient environments, oxidative and osmotic stresses, and heat shock, viable but not cultivatable (VBNC) forms of *L. pneumophila* have been observed (Allegra et al. 2008; Delgado-Viscogliosi et al. 2005, 2009; Dusserre et al. 2008; Edagawa et al. 2008; Hay et al. 1995; Hussong et al. 1987; Paszko-Kolva et al. 1992, 1993; Yamamoto et al. 1996). It is unknown if VBNC legionellae are directly pathogenic for mammals.

It is now accepted by most authorities that *L. pneumophila* and probably all other *Legionella* species are facultative parasites of free-living amoebae and that the major replicative form of the organism in the environment is within amoebae (Buse et al. 2012; Fields et al. 2002; Hilbi et al. 2010; Lau and Ashbolt 2009; Pagnier et al. 2009; Taylor et al. 2009; Thomas et al. 2010). Several lines of evidence support this viewpoint. First, waters that contain legionellae, including those sources linked to disease transmission, are always rich in protozoa; indeed, the number of legionellae in a water sample correlates with the number of protozoa (Breiman et al. 1990b; Fields et al. 1989; Lasheras et al. 2006; Moore et al. 2006; Paszko-Kolva et al. 1991; Patterson et al. 1997; Valster et al. 2011; Yamamoto et al. 1992a). Second, amoebae (e.g., hartmannellae, acanthamoebae, naegleriae) isolated from natural and engineered aquatic environments (as well as soil samples) harbor intracellular legionellae, including *L. pneumophila* (Berk et al. 2006; Declerck et al. 2007a; Gast et al. 2011; Harf and Monteil 1988; Iovieno et al. 2010; Michel et al. 1998; Newsome et al. 1998; Singh and Coogan 2005; Thomas et al. 2006). Third, the capacity of a *Legionella*-containing water sample to support bacterial growth is dependent upon the presence of the amoebae (Barbaree et al. 1986; Fields et al. 1989; Kuiper et al. 2004; Nahapetian et al. 1991; Steinert et al. 1998; Wadowsky et al. 1988, 1991). Fourth, in coculture experiments done in the laboratory, *L. pneumophila* replicates in at least 20 different types of amoebae, including representatives from the genus *Acanthamoeba* (8 species), *Balamuthia* (1 species), *Dictyostelium* (1 species), *Echinamoeba* (1 species), *Hartmannella* (2 species), *Naegleria* (6 species), *Vahlkampfia* (1 species), and *Willaertia* (1 species) (Anand et al. 1983; Barbaree et al. 1986; Dey et al. 2009; Fields 1996; Fields et al. 1989; Hagele et al. 2000; Harada et al. 2010; Harf and Monteil 1988; Henke and Seidel 1986; Holden et al. 1984; Michel et al. 1998; Miyamoto et al. 2003; Molmeret et al. 2001; Newsome et al. 1985; Rowbotham 1986, 1980; Shadrach et al. 2005; Solomon et al. 2000; Tyndall and Domingue 1982; Wadowsky et al. 1991). Finally, a recent study of engineered water systems suggests that *L. pneumophila* might also be capable of replicating within *Diphylleia* and *Neoparamoeba* (Valster et al. 2010). In addition to parasitizing amoebae, *L. pneumophila* has the ability to infect and grow in at least 3 species of ciliates belonging to the genus *Tetrahymena* (Barbaree et al. 1986; Fields 1996; Fields et al. 1986, 1984; Kikuhara et al. 1994). Intra-amoebal growth of *L. pneumophila* occurs at temperatures ranging from 22 °C to 37 °C (Buse and Ashbolt 2011; Newsome et al. 1985; Söderberg et al. 2008), whereas replication in *Tetrahymena* occurs at 30–35 °C (Fields et al. 1984; Kikuhara et al. 1994; Steele and McLennan 1996). Other *Legionella* species that have been shown to infect amoebae and/or tetrahymenae are *L. anisa*, *L. bozemanae*, *L. drancourtii*, *L. dresdenensis*, *L. drozanskii*, *L. dumoffii*, *L. fallonii*, *L. feeleii*, *L. gormanii*, *L. hackeliae*, *L. jamestowniensis*, *L. jordanis*, *L. londiniensis*, *L. longbeachae*, *L. lytica*, *L. micdadei*, *L. oakridgensis*, *L. quinlivanii*, *L. rowbothamii*, *L. rubrilucens*, *L. steelei*, *L. steigerwaltii*, and *L. worsleiensis* (Adeleke et al. 2001; Doyle et al. 1998; Edelstein et al. 2011; Fields et al. 1986, 1990; Furuhata et al. 2010; Hsu et al. 2011; Kuroki et al. 2007; La Scola et al. 2003, 2004; Luck et al. 2010; Moffat and Tompkins 1992; Neumeister et al. 1997; Rowbotham 1983, 1986; Steele and

McLennan 1996; Wadowsky et al. 1991). Not all legionellae will grow in the same protozoan host, indicating strain-to-strain variation in the selection of the optimal host cell (Buse and Ashbolt 2011; Dey et al. 2009; Fields et al. 1989, 1990; Rowbotham 1986; Steinert et al. 1994; Wadowsky et al. 1991). Among the various protozoa that are permissive for *L. pneumophila* growth, *Hartmannella vermiformis* amoebae are most often cited as being the critical host cell within environmental water systems, including those linked to disease (Breiman et al. 1990b; Buse et al. 2012; Fields et al. 1989; Hsu et al. 2011; Kuiper et al. 2004; Taylor et al. 2009; Valster et al. 2010, 2011; Wadowsky et al. 1988). Of the remaining types of protozoan hosts, the acanthamoebae and naegleriae are also considered important natural reservoirs (Taylor et al. 2009).

In vitro studies have shown that *L. pneumophila* can infect the soil nematode *Caenorhabditis elegans* (Brassinga et al. 2010; Komura et al. 2010, 2012). However, it is unknown whether legionellae survive and/or grow within nematodes in the natural environment. Based upon antibody-detection methods, *L. pneumophila* has been observed within various types of insects living in aquatic habitats, suggesting that insects may be another natural reservoir for legionellae (Castellani Pastoris et al. 1989).

In addition to surviving within the planktonic phase and protozoan hosts, *Legionella* bacteria also exist within multi-organismal biofilms that cover surfaces within natural and engineered water systems (Colbourne et al. 1984; Declerck 2010; Emtiazi et al. 2004; Flannery et al. 2006; Hsu et al. 2011; Lau and Ashbolt 2009; Marrao et al. 1993; Riffard et al. 2001; Sheehan et al. 2005; Tison et al. 1980; Wingender and Flemming 2011). In fact, the interaction between *Legionella* bacteria and its protozoan hosts likely occurs for the most part within and near complex biofilms. Many in vitro studies have examined the ability of *L. pneumophila* to exist within model biofilms. Physical parameters that have been found to influence the process include the chemical and physical properties of the surface, the flow rate and turbulence of the liquid over the surface, the ambient temperature, organic-carbon content, metal (e.g., iron) concentrations, and the presence of biocides (Bezanson et al. 1992; Cargill et al. 1992; Donlan et al. 2005; Green and Pirrie 1993; Hindre et al. 2008; Lehtola et al. 2007; Liu et al. 2006; Pang and Liu 2006; Pecastaings et al. 2010; Piao et al. 2006; Rogers et al. 1994; Schoenen et al. 1988; Schofield and Locci 1985; Schofield and Wright 1984; Turetgen and Cotuk 2007; Walker et al. 1993; Wright et al. 1989, 1991). Biological factors that influence *L. pneumophila* within biofilms include both the presence of amoebal hosts (e.g., *H. vermiformis*, *A. castellanii*) and the presence of other types of bacteria, some of which promote or are at least compatible with *Legionella* (e.g., certain species of *Acinetobacter*, *Aeromonas*, *Empedobacter*, *Escherichia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Sphingomonas*, and *Stenotrophomonas*) and others which inhibit the legionellae (e.g., *Corynebacterium* and certain other species of *Pseudomonas* (Declerck et al. 2007b, 2009; Donlan et al. 2005; Giao et al. 2011; Guerrieri et al. 2008; Kimura et al. 2009; Kuiper et al. 2004; Mampel et al. 2006; Manz et al. 1995; Messi et al. 2011; Moritz et al. 2010; Murga et al. 2001; Rogers and Keevil 1992; Storey et al. 2004; Temmerman et al. 2006; Vervaeren et al. 2006; Williams and Braun-Howland 2003). Substances released from dead bacteria as well as blue-green algae can promote *Legionella* growth (Temmerman et al. 2006; Tison et al. 1980). *Legionella* factors that are needed for colonization of biofilms include flagella, type IV pili, and Lcl, a surface protein that has collagen-like domains (Duncan et al. 2011; Lucas et al. 2006; Mampel et al. 2006). Some in vitro studies have concluded that *L. pneumophila* replication within biofilms can occur in the absence of amoebal hosts (Keevil 2003; Mampel et al. 2006; Rogers and Keevil 1992; Temmerman et al. 2006). However, others have argued that, although persistence can occur in the absence of amoebae, replication requires the presence of protozoan hosts (Declerck et al. 2007b, 2009; Kuiper et al. 2004; Murga et al. 2001). Clearly, the mechanism of *Legionella* growth within natural biofilms is likely to be variable, depending upon extraneous microbial and environmental factors.

In addition to providing a means for bacterial growth in the environment, *Legionella* infection of protozoa has great significance for the genesis of disease for several more reasons. First, legionellae in amoebae remain viable for long periods of time, increasing the possibility for disease transmission (Bouyer et al. 2007), and residence within the amoebae may protect the bacterium from the harmful effects of aerosolization. Second, ingestion and growth in amoebae "resuscitate" VBNC legionellae, resulting in viable bacteria that are infective (Allegra et al. 2008; Dusserre et al. 2008; Garcia et al. 2007; Hwang et al. 2006; Steinert et al. 1997). Third, the relative chlorine resistance of environmental *L. pneumophila* and other legionellae is explained in part by the protection afforded the bacterium growing within an amoebal cyst and the phenotype change of the bacterium resulting from intra-amoebal growth (Barker et al. 1992, 1993; Kilvington and Price 1990; King et al. 1988). Fourth, legionellae grown in amoebae maintain and in some cases display enhanced infectivity for macrophages and experimental animals (Cirillo et al. 1994, 1999; Harf and Monteil 1988; Neumeister et al. 2000; Tyndall and Domingue 1982; Vandenesch et al. 1990). Fifth, *Legionella*-laden protozoa or protozoan vesicles or cysts containing legionellae might be part of the inoculum that initiates lung infection (Berk et al. 1998; Brieland et al. 1996, 1997a, b; Rowbotham 1986). Finally, it is widely believed that the ability of *L. pneumophila* to parasitize protozoa engendered it with the capacity to infect macrophages and thereby cause disease (Albert-Weissenberger et al. 2007; Cianciotto and Fields 1992; Fields et al. 2002; Hilbi et al. 2011; Lau and Ashbolt 2009; Molmeret et al. 2005; Newton et al. 2010; Shin and Roy 2008; Swanson and Hammer 2000). Indeed, many of the bacterial genes that promote protozoan infection also promote infection of macrophages (Cianciotto and Fields 1992; Gao et al. 1997; Newton et al. 2007; Pruckler et al. 1995; Segal and Shuman 1999). Moreover, the intracellular infection pathway within amoebae is similar to what occurs within macrophages (Abu Kwaik 1996; Bozue and Johnson 1996; Gao et al. 1999; Liles et al. 1999; Newsome et al. 1985). Thus, understanding how *Legionella* bacteria grow in protozoa is critical to both understanding the natural history of

Legionnaires' disease and devising novel ways of minimizing the risk of disease transmission. A large body of literature exists regarding the molecular and cellular pathogenesis of *L. pneumophila* for amoebae and its relationship to pathogenicity for macrophages; this topic is covered in a later section of this chapter.

Epidemiology, Clinical Presentation, Treatment, and Laboratory Diagnosis

Epidemiology of Legionnaires' Disease

Legionnaires' disease is usually transmitted from the environment to humans by the inhalation of aerosols of *Legionella*-contaminated water (Breiman and Butler 1998; Carratala and Garcia-Vidal 2010; Stout and Yu 1997). The waters linked to disease include both potable and non-potable sources (Craun et al. 2010; Fry et al. 2003; Kusnetsov et al. 2010). Microaspiration and direct installation of contaminated water into the lungs are another, albeit less common, mode of spread particularly in nosocomial disease (Blatt et al. 1993; Carratala and Garcia-Vidal 2010; Venezia et al. 1994). Aspiration of large amounts of water during near drowning is a rare but reported mode of disease transmission (Hasselmann et al. 1983; Inoue et al. 2011; Lavocat et al. 1987; Miyamoto et al. 1997; Nozue et al. 2005; Sekla et al. 1982). Person-to-person transmission of legionellosis does not occur (Edelstein and Cianciotto 2010).

The sources of *Legionella*-containing aerosols are usually water-cooled heat rejection equipment such as air-conditioning cooling towers, whirlpool spas, sink taps, and shower heads (Breiman et al. 1990b; Brulet et al. 2008; Campese et al. 2010; Cordes et al. 1981; Den Boer et al. 2002; Ferre et al. 2009; Fields et al. 2002; Garcia-Fulgueiras et al. 2003; Lam et al. 2011; Nguyen et al. 2006; Nicolay et al. 2010; Pagnier et al. 2009; Ricketts et al. 2011). However, virtually, any device that can create an aerosol of water can be a disease source, including ice machines, mist machines (e.g., vegetable misters in grocery stores), decorative fountains and waterfalls, evaporative condensers, industrial air scrubbers, high-pressure power washers, asphalt paving machines, sump pumps, gardening hoses, windshield wipers, and respiratory therapy equipment, including nebulizers, humidifiers, oxygen humidifiers, and ventilator tubing (Arnow et al. 1982; Barrabeig et al. 2010; Breiman et al. 1990a, b; Castor et al. 2005; Cordes et al. 1981; Coscolla et al. 2010; Haupt et al. 2012; Kool et al. 1998; Mahoney et al. 1992; Marrie et al. 1991; Nygard et al. 2008; O'Loughlin et al. 2007; Piso et al. 2007; Schuetz et al. 2009; Wallensten et al. 2010; Woo et al. 1992). *L. longbeachae* infections are more commonly associated with exposure to soil and compost as opposed to the aquatic environments typically inhabited by *L. pneumophila* and the other species (Graham et al. 2011; Lindsay et al. 2012; Whiley and Bentham 2011).

Legionnaires' disease occurs both within the community and in hospital settings, with >90 % of cases being community-acquired pneumonia and <10 % nosocomial pneumonia (Fields et al. 2002; Joseph and Ricketts 2010; Korvick et al. 1987; Ricketts and Joseph 2005, 2007). In both settings, Legionnaires' disease manifests sporadically and as outbreaks (Fields et al. 2002). Overall, however, the vast majority of cases are sporadic, for example, 89 % of cases in the USA in the 1980s (Fields et al. 2002), 73–82 % of cases in England and Wales during the 1990s (Joseph et al. 1994a, 1995, 1997, 1998; Newton et al. 1996), and 73–91 % of cases in Europe in the 2000s (Joseph 2004; Joseph and Ricketts 2010; Ricketts and Joseph 2005, 2007). Based upon a prospective study, it has been estimated that 8,000–18,000 sporadic cases of Legionnaires' disease occur each year among US adults needing hospitalization (Marston et al. 1997). When taking into account an earlier study that focused on community-acquired pneumonia not requiring hospitalization (Foy et al. 1979), the incidence of Legionnaires' disease is estimated as being 18,000–88,000 US cases per year (Edelstein and Cianciotto 2010). Both in Germany and in Spain, the incidence of sporadic *Legionella* pneumonia is projected to be at least 15,000–30,000 cases per year (von Baum et al. 2008). In various studies aimed at assessing the overall etiology of community-acquired pneumonia, the percentage due to Legionnaires' disease has ranged from 0 % to 16 % (Borchardt et al. 2008; Breiman and Butler 1998; Cilloniz et al. 2011; Edelstein and Cianciotto 2010; Fields et al. 2002; Muder et al. 1989; Ruiz et al. 1999; Woodhead 2002; Yu and Stout 2008). When considering only those cases requiring hospitalization, most studies place the percentage due to *Legionella* at 2 % to 9 % (Bohte et al. 1995; Cilloniz et al. 2011; Fields et al. 2002; Ruiz et al. 1999; von Baum et al. 2008; Woodhead 2002; Yu and Stout 2008). Since 2003, there has been an increase in number of cases of Legionnaires' disease reported to the CDC, suggesting that there might be an increasing incidence of the disease in the USA (Carratala and Garcia-Vidal 2010; CDC 2011; Neil and Berkelman 2008; Ng et al. 2008a). Increased notifications in the last decade have been documented elsewhere, including in Australia and Canada (Li et al. 2002; Ng et al. 2008b). However, other countries, such as the Netherlands, have reported a recent decline in disease incidence (Euser et al. 2012).

Although most Legionnaires' disease cases are sporadic, outbreaks continue to occur throughout the world; for example, 51 outbreaks happened in Europe between 1997 and 2001 (Joseph 2002). Some outbreaks have been especially serious in terms of the large number of people involved. ❯ *Table 9.2* lists 13 outbreaks that have occurred since 1999 and resulted in >50 confirmed cases. These outbreaks serve to emphasize four additional points. First, cooling towers continue to be a notorious source for disease transmission. Second, based upon findings obtained during the outbreaks in France and Norway, long-distance spread of the disease agent can be >6 km from a cooling tower source and >10 km from an air scrubber source. Third, disease prevention through the use of proper engineering and construction and of rapid diagnosis by lab testing is important, but knowledge of the factors responsible for disease outbreaks remains lacking.

Host risk factors for community-acquired Legionnaires' disease include administration of glucocorticosteroid medications,

Table 9.2
Recent community-based outbreaks of Legionnaires' disease[a]

Date	Location	Source	Confirmed cases	Case fatality rate (%)	Ref.
March 1999	Bovenkarspel, Netherlands	Whirlpool spas at a flower show	188	11	(Den Boer et al. 2002)
April 2000	Melbourne, Australia	Cooling tower at an aquarium	125	3	(Greig et al. 2004)
July 2001	Murcia, Spain	Cooling towers at a city hospital	449 (800 suspected)	5	(Garcia-Fulgueiras et al. 2003)
April 2002	Barrow-in-Furness, England	Cooling tower in town center	170 (498 suspected)	3	(Telford et al. 2006)
July 2002	Miyazaki, Japan	Recreational spas	295	2	(Okada et al. 2005)
August 2002	Mataro, Spain	Cooling tower in the city	113	2	(Sabria et al. 2006)
Nov 2003–Jan 2004	Pas-de-Calais, France	Cooling tower at an industry plant	86	21	(Nguyen et al. 2006)
May 2005	Sarpsborg, Norway	Air scrubber at an industry plant	56	18	(Nygard et al. 2008)
Sept–Oct 2005	Scarborough, Canada	Cooling tower at a long-term care facility	135	17	(Gilmour et al. 2007)
Oct–Nov 2005	Vic and Gurb, Spain	Cooling tower at an industry plant	55	5	(Ferre et al. 2009)
June 2006	Pamplona, Spain	Cooling tower in the city	146	0	(Castilla et al. 2008)
2007	Verkhnyaya Pyshma, Russia	Town hot water supply	130	4	(Joseph and Ricketts 2010)
Dec 2009–Jan 2010	Ulm and Neu-Ulm, Germany	Cooling tower in the city center	64	8	(Freudenmann et al. 2011)

[a]Only outbreaks that have occurred since 1999 and involved $>$ 50 confirmed cases are listed

anti-TNF-α therapy, and other forms of immunosuppression, organ transplantation, smoking, end-stage renal disease, age greater than 50 years, AIDS, hematologic malignancies, lung cancer, chronic heart or lung disease, diabetes, silicosis, and male gender (Beigel et al. 2009; Broome and Fraser 1979; Carratala et al. 1994; Den Boer et al. 2002; Ginevra et al. 2009; Girard and Gregson 2007; Gudiol et al. 2009; Hofmann et al. 2009; Jacobson et al. 2008; Jinno et al. 2009; Marston et al. 1994; Nguyen et al. 2006; Straus et al. 1996; Tubach et al. 2006). Alcohol abuse may or may not be a significant risk factor (Broome and Fraser 1979; Carratala et al. 1994; Ferre et al. 2009; Lettinga et al. 2002; Marston et al. 1994; Straus et al. 1996). The same host risk factors seem to apply for nosocomial acquisition (Carratala et al. 1994; Haley et al. 1979; Joseph et al. 1994b). Recent surgery, or more probably general anesthesia, has been a risk factor in some nosocomial epidemics (Korvick and Yu 1987; Serota et al. 1981). Legionnaires' disease is rare in children, accounting for $\leq$1 % of pediatric pneumonias. When it does occur, it usually results from nosocomial infection of immunosuppressed children (Edelstein and Cianciotto 2010).

In addition to the host factors named above, activities that increase the chances of exposure to *Legionella* bacteria in water heighten the risks of disease. Activities that increase the risk of community-acquired Legionnaires' disease include recent overnight travel, use of well water, plumbing work in the home, and disruptions in the water supply that result in "brown" water in taps (Alary and Joly 1991; Fields et al. 2002; Joseph et al. 2010; Straus et al. 1996). Additional risk activities include living near or proximity to a cooling tower or other wet cooling systems, using or being nearby whirlpool spas or hot spring baths, being near decorative fountains, working in underground wells, and being a professional (e.g., taxi, bus) driver (Bhopal et al. 1991; Den Boer et al. 2002; Hlady et al. 1993; Jernigan et al. 1996; Miyamoto et al. 1997; Ricci et al. 2010; Ricketts et al. 2011; Sakamoto et al. 2009; Wallensten et al. 2010). Activities that have been more associated with an increased risk of nosocomial disease include the use of respiratory therapy equipment and on rare occasions wound treatments and water birthing (Edelstein and Cianciotto 2010; Lowry et al. 1991; Nagai et al. 2003).

Cases of community-acquired Legionnaires' disease tend to be most frequent during the summer and early fall (CDC 2011; Joseph and Ricketts 2010; Li et al. 2002; Neil and Berkelman 2008). Nosocomial disease does not show this seasonal variation. Wet, humid weather, rainfall, and low winds can increase the risk for disease (Ferre et al. 2009; Fisman et al. 2005; Hicks et al. 2007). Geographical location is also a factor; for example, within the USA, cases are more frequent in the Northeast and Midwest regions (Neil and Berkelman 2008).

Clinical Presentation and Treatment of Legionnaires' Disease

Legionnaires' disease presents clinically as pneumonia, with features indistinguishable from other common forms of bacterial pneumonia, such as pneumococcal pneumonia (Diederen 2008; Edelstein 1993; Edelstein and Cianciotto 2010; Granados et al. 1989; Roig et al. 1991; Sopena et al. 1998; Tan et al. 2000; Tsai et al. 1979). The incubation period of the disease is usually 2–10 days but can be more than 2 weeks (Breiman and Butler 1998; Den Boer et al. 2002). A prodromal illness may occur, lasting for hours to several days, with symptoms such as headache and myalgia. There was some suggestion that a combination of factors such as diarrhea, hyponatremia, and increased serum creatine kinase is more consistent with Legionnaires' disease than other pneumonic diseases, but no study has shown this unequivocally. Recently, it has been suggested that a useful diagnostic might involve high body temperature, absence of sputum production, low serum sodium, high levels of lactate dehydrogenase and C-reactive protein, and low platelet counts (Carratala and Garcia-Vidal 2010; Fiumefreddo et al. 2009). Thus, the currently accepted clinical presentation generally consists of fever, fatigue, often headache or muscle aches, and nonproductive cough. Chest pain, diarrhea, confusion, shaking chills, and shortness of breath also may be seen. The chest roentgenogram usually demonstrates alveolar filling, focal infiltrates, and lung consolidation with or without pleural effusions. Lung abscesses can occur, but rarely (Yu et al. 2009). Extrapulmonary infection occurs rarely as disseminated infection in patients with pneumonia or very rarely as isolated primary infection (Edelstein 1993; Lowry and Tompkins 1993; Stout and Yu 1997). Pleural empyema, myocarditis, pericarditis, endocarditis, meningitis, encephalitis, vascular shunt infections, arthritis, peritonitis, and colitis have all been documented to very rarely occur during the course of pneumonia (Edelstein and Cianciotto 2010; Fernandez-Cruz et al. 2011; Flendrie et al. 2011; Pearce et al. 2011). Other sites of metastatic infection have been the intestines, spleen, liver, bone marrow, and surgical wounds including prosthetic heart valves and aorta. Isolated infections, without pneumonia, include disease of prosthetic heart valves, respiratory sinuses, open wounds, soft tissue abscesses, and cellulitis (Edelstein and Cianciotto 2010; Han et al. 2010; Loridant et al. 2011).

Fatality rates for Legionnaires' disease vary greatly, ranging from 1 % to 80 % (Benin et al. 2002b; Graham et al. 2011; Lam et al. 2011). Factors influencing the rate include host risk factors, time to effective therapy, and whether disease is sporadic vs. outbreak and nosocomial vs. community acquired (Edelstein and Cianciotto 2010). Overall, the lowest fatality rates tend to be associated with large community outbreaks, whereas the highest rates occur with untreated nosocomial infections.

Legionnaires' disease is treated with macrolide, fluoroquinolone, or tetracycline antimicrobial agents (Carratala and Garcia-Vidal 2010; Cunha 2010; Diederen 2008; Edelstein 1998; Edelstein and Cianciotto 2010; Fields et al. 2002). All of these agents concentrate within macrophages and therefore are able to act on the replicating, intracellular legionellae. The drugs of choice to treat mild disease in community-acquired pneumonia include erythromycin, doxycycline, azithromycin, and levofloxacin. For severely ill patients, or immunocompromised ones, either azithromycin or levofloxacin is the drug of choice (Edelstein 1995, 1998). Antimicrobial agents that are ineffective include all ß-lactam agents and penems, aminoglycosides, glycopeptides, and chloramphenicol. The response to treatment depends on the patient's age, underlying diseases, degree of pulmonary involvement, the timing of treatment in relation to disease onset, and severity of disease. Untreated disease is fatal in 5–80 % of patients, depending on the above factors; previously, healthy people with minimal disease have the best outcome, and otherwise ill or immunocompromised patients with extensive pneumonia the worst outcome. Prompt-specific therapy reduces the fatality rate by two- to sixfold. The duration of therapy, depending on the agent used and the presence of immunosuppression, ranges from 3 to 21 days; patients with endocarditis or cavitating pneumonia may require longer courses of therapy. Although antibiotic-resistance genes have been identified in *Legionella* bacteria, including β-lactamases and aminoglycoside kinases (Fu and Neu 1979; Mercuri et al. 2001; Suter et al. 1997; Thompson et al. 1998), clinically relevant resistance has fortunately not emerged.

Epidemiology and Clinical Aspects of Pontiac Fever

Pontiac fever is a self-limited, nonpneumonic illness of short duration (Edelstein 2007; Glick et al. 1978; Goldberg et al. 1989; Luttichau et al. 1998; Mangione et al. 1985; Tossa et al. 2006). Its incubation period is usually 4 h to 6 days (median of 2 days), but it can be as long as 9 days. The attack rate is quite high, with >80 % of those exposed becoming ill. The sources of the *Legionella*-contaminated aerosols for Pontiac fever are similar to those for Legionnaires' disease, with a variety of aerosol-generating devices implicated as well as potting soil (Castor et al. 2005; Cramp et al. 2010; Euser et al. 2010; Jones et al. 2003; Nicolay et al. 2010). No host risk factors have been identified for Pontiac fever (Edelstein and Cianciotto 2010). Most reported cases of Pontiac fever have been linked to outbreaks, although there have been cases occurring in a non-epidemic setting (Bauer et al. 2008). Fever, headache, myalgia, and asthenia are the main symptoms of Pontiac fever. Less common symptoms are cough, dyspnea, anorexia, arthralgia, and abdominal discomfort. Most patients are not ill enough to seek medical attention. Recovery usually occurs without any specific treatment, 3–5 days after disease onset. The basis for Pontiac fever remains obscure (Edelstein 2007). The short incubation period, short duration of illness, and full recovery without antibiotics cause most to conclude that the disease is not the result of a *Legionella* infection. Also, the percentage of persons in outbreaks that have elevated titers of anti-*Legionella* antibodies is quite variable, ranging from 30 % to 85 %. Thus, the prevailing hypotheses to explain this disease include the inhalation of

a bacterial (endo)toxin, an allergic reaction to inhaled live or dead bacteria, or inhalation of amoebae that are also present in the contaminated water (Edelstein 2007). Interestingly, several outbreaks have consisted of both Legionnaires' disease and Pontiac fever; however, it is unclear why some people developed pneumonia whereas others the nonpneumonic form of disease (Benin et al. 2002a; Euser et al. 2010).

Laboratory Diagnosis of *Legionella* Infections

Rapid diagnosis of infection due to *Legionella* spp., in particular *L. pneumophila*, is important for both patient management and effective public health action. The methods currently available for diagnosis of Legionnaires' disease are culture, urinary antigen detection, direct fluorescent antibody testing, and detection of nucleic acids or of specific antibodies in serum samples. However, presently, none of the diagnostic tests available offer the desired quality with respect to sensitivity and specificity.

Culture of *Legionella* spp.

Culture is still the "gold standard" among all diagnostic methods for *Legionella* infections. The medium used, supplemented charcoal yeast extract medium (BCYE), is easily prepared by any large clinical microbiology laboratory and can be made in a selective form. Use of selective media and specimen decontamination with acid are obligatory for optimal culture yield from normally nonsterile tissues and fluids. To obtain optimal yield, specimens with and without acid pretreatment are plated on three different media (all commercially available): BCYE (nonselective), BMPA (selective, also called "CAP" or "PAC"), and MYEA (selective, also called "PAV" or "VAP") (Edelstein 1985a, b; Vickers et al. 1987). Use of two different selective media is required as some *Legionella* spp., and some strains of *L. pneumophila* serogroup 1, will not grow on BMPA, which is the most selective medium. Use of multiple media also increases the chances of detecting very small numbers of *Legionella* spp. bacteria present in the specimen. Specimen dilution before plating is also important, as *Legionella* spp. growth may be inhibited by certain cations, other bacteria, and by tissue factors. The organism has been successfully isolated from sputum, transtracheal aspirates, endotracheal suction specimens, blood, lung biopsy, pleural fluid, bronchial lavage, pericardial fluid, peritoneal fluid, wounds, bowel abscesses, prosthetic heart valves, brain abscesses, myocardium, kidney, liver, vascular grafts, and respiratory sinuses. Cultures generally remain positive for several days after the initiation of antimicrobial therapy and may remain positive for weeks or months from pulmonary abscesses. Broad-spectrum antimicrobial therapy decreases culture yield.

The sensitivity of culture for the diagnosis of Legionnaires' disease has been estimated to be in the range of 11–65 % by retrospective studies performed in different reference laboratories (Den Boer and Yzerman 2004; Hayden et al. 2001; Lindsay et al. 2004). *Legionella* colonies usually form within 3–5 days, which are relatively easy to identify due to their specific colony morphology. To ascertain that it is *Legionella* suspected colonies are subcultured on BCYE agar and on cysteine-free BCYE agar, as *Legionella* have a growth requirement for L-cysteine. So far, a positive culture is the only method that allows the comparison of patient and environmental *Legionella* strains, thus confirming or excluding a given environmental reservoir as the source of infection.

Detection of *Legionella* Antigen in Urine

The most widely used method for laboratory diagnosis of Legionnaires' disease is the urinary antigen test. This ELISA test is based on the identification of a lipopolysaccharide component of *L. pneumophila* that is heat stable, resistant to enzymatic cleavage, and about 10kDa of molecular weight. This soluble antigen appears very early after infection, about 2–3 days after the first clinical symptoms, and can be excreted for a long time. In average, this antigen is present for several days up to 2 month and has been detected in one patient for nearly 1 year (Kohler et al. 1984). Compared to culture, urinary antigen tests are much faster, easy to perform, cheaper, and more sensitive. The specificity of these assays that were mainly evaluated by testing urine samples from patients with urinary tract infections or pneumonia caused by other pathogens has been reported to be more than 99 % (Den Boer and Yzerman 2004; Domínguez et al. 1998; Helbig et al. 2001b, 2003b). In contrast, the sensitivity of the assay varies between 56 % and 99 % according to the study (Birtles et al. 1990; Domínguez et al. 1998, 2001; Helbig et al. 2001b, 2003b; Kazandjian et al. 1997; Plouffe et al. 1995; Ruf et al. 1990; Yzerman et al. 2002). There are several commercial enzyme immunoassay kits available (e.g., Binax, Biotest, Bartels); the best-studied one is made by the Binax Company. However, all available urinary antigen tests have the disadvantage that they lack sensitivity for serogroups other than *L. pneumophila* serogroup 1 (Olsen et al. 2009). Recently, a new kit, Xpect^{TM} *Legionella* test (Oxoid), was introduced, which was designed for the direct, qualitative detection of *L. pneumophila* serogroup 1 and 6 antigens in human urine samples. However, a recent study reported a sensitivity of 79 % for the Binax EIA test, and only 32 % for the Xpect kit. Furthermore, none of the 10 *L. pneumophila* serogroup six samples tested were positive by the Xpect test, which claimed to recognize also serogroup 6 (Svarrer et al. 2012). The sensitivity of the urinary antigen-based tests was reported to correlate highly with the severity of the illness (Blázquez et al. 2005; Lück et al. 2006; Yzerman et al. 2002) and the presence of underlying diseases (Sopena et al. 2002).

Besides these tests, a rapid immunochromatographic (ICT) test (BinaxNOW) has been on the markets since several years. It detects urinary antigen very rapidly, and no lab equipment is required. The sensitivities and specificities were estimated to be 89 % and 100 %, respectively, for the Oxoid Xpect *Legionella* test

kit and 86 % and 100 %, respectively, for the BinaxNOW test (Diederen et al. 2009). Similarly, Higa and colleagues reported a sensitivity and specificity of ICT using respiratory samples of 1.0 and 0.99, respectively (Higa et al. 2008). In contrast, a recent study reported a sensitivity of only 47 % for the BinaxNOW test (Svarrer et al. 2012). Recently, other immunochromatographic assays were released, like the SAS *Legionella* test, the Rapid U test, or the SD Bioline assay.

However, despite the great advantages of the urinary antigen test like easy, rapid, and highly specific, one should keep in mind that a negative urinary antigen test never excludes a *Legionella* infection.

Direct Fluorescence Antibody Testing

Legionella antibodies can be detected in clinical samples by direct florescence antibody (DEA) testing using commercially available monoclonal antibodies specific for the species *L. pneumophila*. However, no monoclonal antibodies are available for *Legionella* species other than *L. pneumophila* (Edelstein et al. 1985). Results can be obtained in 2–4 h. The principal drawback of this method is its low sensitivity reported to be between 25 % and 70 % (Hayden et al. 2001; Lindsay et al. 2004). The specificity which is 60–70 % is due to cross-reactions with certain Gram-negative bacteria like *Pseudomonas aeruginosa*, *P. fluorescens*, *Stenotrophomonas maltophilia*, *Bordetella pertussis*, *Bacteroides fragilis*, and *Francisella tularensis* (Jarraud and Etienne 2012). Therefore, a negative result does not rule out disease, and a positive result is almost always diagnostic of it (Edelstein et al. 1980). The protein antigen detected by this test is not degraded after fixation with formalin. Thus, this test allows the detection of the etiologic agent in formalin-fixed lung tissue, which is not possible with the other methods available (Lück 2008).

Serology

The first test that identified antibodies directed against *L. pneumophila* used indirect immunofluorescent microscopy (IFA) and was set up by the CDC (Centers for Disease Control and Prevention) during the Philadelphia outbreak in 1976 (McDade et al. 1977). The vast majority of laboratories use the IFA technique to determine antibody concentrations. Only measurement of antibody to *L. pneumophila* serogroup 1 by IFA is well standardized and is the "gold standard" test used to diagnose Legionnaires' disease by serologic means.

There are two widely used reference methods of antigen preparation for the IFA test: heat fixation of plate-grown bacteria and formalin fixation of chicken embryo yolk sac-grown bacteria (Edelstein 1997). The latter method may be more specific, although large head-to-head comparative studies have not been performed (Harrison and Taylor 1982). Many commercial laboratories sell kits containing formalin-fixed plate-grown bacteria, but these kits are not known to provide the same results as either of the reference methods. Use and results interpretation of these commercial IFA kits may not give results similar to those obtained using the reference methods. The specificity of the IFA test in a hospitalized population is not well known; this probably approximates 90 % for a fourfold titer rise, although in an epidemic situation in nonhospitalized patients, the specificity is close to 100 %. Cross-reactions for the IFA test have been reported with *Mycobacteria*, *Leptospira*, *Chlamydia*, *Mycoplasma*, *Citrobacter*, *Campylobacter*, *Coxiella burnetii*, *Pseudomonas*, and *Bacteroides fragilis* (Bornstein et al. 1984; Boswell et al. 1996; Collins et al. 1984; Gray et al. 1991; Klein 1980). Cross-reactions have been also frequently observed among the different *Legionella* species and serogroups, which makes diagnostic sometimes difficult (Wilkinson et al. 1983). One of the most frequent cross-reactions occurs between *L. pneumophila* serogroups 1 and 6.

About three-quarters of patients with culture-proven Legionnaires' disease caused by *L. pneumophila* serogroup 1 develop a fourfold rise in IFA titer from 1 to 2 weeks after onset of illness. The mean time required for demonstration of seroconversion is about 2 weeks; however, up to 25 % of seroconversions are missed unless serum is collected up to 8 weeks after onset of illness. However, in certain cases, despite the diagnosis of legionellosis was confirmed, seroconversion was never observed. Furthermore, the possibility of cross-reactions and the fact that serologic testing is retrospective in nature (and does not influence choice of therapy), the other major drawback of diagnosing *Legionella* infections using serologic means is that the test may be negative because the serotype of the infecting organism is not tested for. Thus, serologic testing in the diagnosis of this disease is much more helpful to epidemiologists than to clinicians caring for individual patients.

Detection of Nucleic Acids of *Legionella*

The detection of DNA of *Legionella* by PCR in respiratory samples was reported first in 1992 (Jaulhac et al. 1992). By investigating the performance of PCR in bronchoalveolar lavage fluid specimens, the investigators established the principle that PCR is suitable for detection of Legionella DNA in clinical samples. Meanwhile PCR methods have been developed for testing for the presence of Legionella in many different samples like from bronchoalveolar lavage fluid, throat swaps, blood, peripheral leukocytes, serum, and urine (Helbig et al. 1999; Jaulhac et al. 1992; Jonas et al. 1995; Maiwald et al. 1995; Matsiota-Bernard et al. 1994; Murdoch and Chambers 2000; Ramirez et al. 1996; Weir et al. 1998). The majority of the PCR assays target the 5S and 16S rDNA genes or the intergenic region of the 23S-5S rDNA genes to detect specifically the genus Legionella, and the gene mip (macrophage infectivity potentiator) to detect specifically the species *L. pneumophila*. Recently, a specific real-time PCR for simultaneous detection and identification of *L. pneumophila* serogroup 1 in water and clinical samples was developed (Merault et al. 2011). As *L. pneumophila* is associated with 90 % of human disease, and within the 15 serogroups (Sg), *L. pneumophila* Sg1 causes more

than 84 % of Legionnaires' disease worldwide (Yu et al. 2002), rapid and specific identification of *L. pneumophila* Sg1 is important for the evaluation of the contamination of collective water systems and the risk posed. This PCR targets wzm, a gene present in the *L. pneumophila* serogroup 1 lipopolysaccharide gene cluster (Merault et al. 2011).

PCR, Real-Time PCR, and Multiplex PCR

The sensibility of these tests varies depending on the study from 11 % to 100 %, but the majority of the studies report specificity close to 100 %. However, the performance of the PCR tests depends largely on the kind of sample tested (respiratory sample, urine, serum, etc.) and when the study was reported. Clearly, the improvements of the DNA extraction techniques and the development of real-time PCR methods led to a considerable increase in the sensibility of detection. In particular, the high sensitivity for detection of *Legionella* DNA in respiratory samples demonstrated by several studies suggests that PCR may exceed culture in its ability to detect *Legionella* in respiratory samples (Cloud et al. 2000; Den Boer and Yzerman 2004; Hayden et al. 2001; Koide et al. 2004; Rantakokko-Jalava and Jalava 2001; Reischl et al. 2002). All studies using real-time PCR for detection of *Legionella* in pulmonary samples report 100 % specificity (Ballard et al. 2000; Hayden et al. 2001; Herpers et al. 2003), and for classical PCR, the specificity lies between 93 % and 100 %. The specificity of the PCR on serum is also close to 100 %, but it is with 80–100 % a little less on urine samples. However, the sensibility is relatively weak as it varies between 29 % and 100 %. This weak sensibility seems to be due to the fact that bacteremia occurs only rarely during disease and even when a bacteremia occurs only little amounts of DNA can be found in the blood (Matsiota-Bernard et al. 2000). Similarly, the sensibility for PCR on urine samples is quite variable, as values from 7 % to 86 % have been reported (Matsiota-Bernard et al. 2000; Murdoch et al. 1996). Taken together, if legionellosis is suspected, it seems to be important to take samples of different origin (urine, serum, etc.) and to repeat these sampling for several days to improve the sensibility of the test results (Lindsay et al. 2004). Furthermore, although culture is still the golden standard, PCR and real-time PCR should be considered a useful diagnostic method for Legionnaires' disease (Zarogoulidis et al. 2011).

Recently, multiplex real-time PCR was evaluated as a method for rapid differential detection of five bacterial causes of community-acquired pneumonia (CAP) (*Streptococcus pneumoniae*, *Burkholderia pseudomallei*, and atypical bacterial pathogens, namely, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila*) in blood and respiratory samples of CAP patients attending a hospital in Malaysia (Mustafa et al. 2011). This study showed that multiplex real-time PCR is a useful tool for identifying CAP causative agents. By supplementing traditional diagnostic methods with real-time PCR, a higher microbial detection rate was achieved for both typical and atypical pneumonia (Mustafa et al. 2011). Cho and colleagues reported a comparison of sputum and nasopharyngeal swab specimens for molecular diagnosis of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila* using multiplex PCR using the PneumoBacter assay. To determine the diagnostic performance of this assay, sputum samples were also tested using BD ProbeTec ET Atypical Pneumonia Assay (APA; Becton Dickinson). Sputum testing appeared more sensitive than nasopharyngeal swab specimens testing ($P = 0.002$) for *L. pneumophila* diagnosis, but PneumoBacter and APA tests agreed 100 % (Cho et al. 2012). Although culture is still the golden standard, PCR and real-time PCR should be considered a useful diagnostic method for Legionnaires' disease (Zarogoulidis et al. 2011).

Pathogenesis, Virulence Factors, and Immune Responses

Overview of *L. pneumophila* Pathogenesis

Given that Legionnaires' disease is most associated with infection by *L. pneumophila*, the vast majority of studies on *Legionella* pathogenesis have focused on that species and, in particular, strains belonging to its first serogroup. Therefore, the following description is an account of *L. pneumophila* (serogroup 1) pathogenesis. Observations that have been made concerning other *Legionella* species will be highlighted toward the end of this chapter.

As discussed above, *L. pneumophila* infects humans following the introduction of contaminated water into the lower respiratory tract. The nature of the infectious particle that triggers disease is still unknown, with the possibilities being planktonic (free, extracellular) bacteria, legionellae contained within amoebae or amoebal cysts, and biofilm-containing legionellae. Within the lung, *L. pneumophila* invades and proliferates within the resident macrophages that line the alveoli (Cianciotto et al. 1989a; Horwitz 1992; Swanson and Hammer 2000; Winn 1988). Bacterial growth results in the lysis of the macrophage hosts and subsequent rounds of intracellular infection. The release of tissue-destructive substances from the bacteria and the dead and dying host cells lead to local tissue destruction (Cianciotto 2001; Newton et al. 2010; Winn 1988). Chemokines and cytokines released by the infected macrophages trigger the infiltration of polymorphonuclear leukocytes, additional macrophages, and erythrocytes, and capillary leakage results in local edema (Akamine et al. 2005; Archer and Roy 2006; Blanchard et al. 1987; Case et al. 2009; Coers et al. 2007b; Matsunaga et al. 2001, 2002; McHugh et al. 2000; Molofsky et al. 2006; Neumeister et al. 1998b; Park and Skerrett 1996; Shin et al. 2008; Winn 1988; Zamboni et al. 2006). When the host's innate immune response and/or adaptive, cell-mediated immune response (see below) is functioning normally, further amplification of the *L. pneumophila* infection is usually limited. However, when host defenses are impaired, as described above as host risk factors, bacterial proliferation and lung pathology are further pronounced, and extrapulmonary dissemination may occur.

Ultimately, *L. pneumophila* infection has the potential to be fatal, particularly if the infected individual is not quickly treated with the proper antibiotics. Animal models have been an invaluable tool for determining the basic course of an *L. pneumophila* infection, the role of particular bacterial factors in pathogenesis, and aspects of host defense. Legionnaires' disease is most often reproduced by infection of guinea pigs or susceptible inbred (e.g., A/J) mice, using the aerosol or intratracheal routes of inoculation (Baskerville et al. 1983; Berendt et al. 1980; Brieland et al. 1994; Collins 1986; Davis et al. 1982; Edelstein et al. 1984; Skerrett et al. 1989; Susa et al. 1998; Winn et al. 1982). A number of inbred mice are not susceptible to *L. pneumophila*, and thus crosses between them and A/J mice have been used to identify a mammalian gene (i.e., *Naip5/Birc1e*) that influences host susceptibility (Beckers et al. 1995; Dietrich et al. 1995; Diez et al. 2003; Fortier et al. 2005; Wright et al. 2003; Yamamoto et al. 1992b; Yoshida et al. 1991).

Much evidence indicates that the capacity of the *L. pneumophila* to grow within alveolar macrophages is central to the pathogenesis of Legionnaires' disease (Cianciotto 2001; Newton et al. 2010). First, the majority of bacteria observed in lung samples from infected humans and animals are associated with these cells (Chandler et al. 1979; Davis et al. 1983; Glavin et al. 1979; Katz et al. 1979; Rodgers et al. 1978; Surgot et al. 1988; Watson and Sun 1981; White et al. 1979). Second, *L. pneumophila* readily replicates within alveolar macrophages and monocytes in vitro (Horwitz and Silverstein 1980; Jacobs et al. 1984; Kishimoto et al. 1979; Nash et al. 1984). Third, the susceptibility of an animal species to infection is correlated with the ability of *L. pneumophila* to grow within macrophages from that species (Yamamoto et al. 1987, 1988; Yoshida and Mizuguchi 1986). Fourth, the resistance of animals to infection requires cytokines that activate macrophages (Brieland et al. 1994; Skerrett and Martin 1994). Fifth, mutants impaired in their ability to infect macrophages exhibit reduced virulence (Cianciotto et al. 1990b; Edelstein et al. 1999; Liles et al. 1999; Marra et al. 1992; Viswanathan et al. 2000). Finally, as noted earlier, therapy requires antibiotics that enter host cells. Despite the early recognition of the *Legionella*-macrophage interaction, it was not immediately obvious how *L. pneumophila*, an organism that neither possesses a mammalian reservoir nor has a "natural" route of infection, evolved the facility to parasitize human phagocytes. As noted above, it is now believed that adaptation to intracellular niches within protozoa engendered in *L. pneumophila* the ability to infect mammalian cells. Hence, a number of virulence factors likely evolved in response to selective pressures within the protozoan environment. Given the central role that macrophage infection has in Legionnaires' disease, many studies of *L. pneumophila* pathogenesis have focused on describing that intracellular infection process and characterizing the bacterial and host factors which are involved in promoting macrophage infection (see below). This work has been facilitated by the use of both explanted monocytes/macrophages from human volunteers and experimental animals (e.g., alveolar- and bone-marrow-derived A/J mouse macrophages) and macrophage cell lines such as the human-derived U937, HL-60, THP-1, and Mono Mac 6 lines and the murine-derived MH-S and J774A.1 and RAW264.7 lines (Cirillo et al. 1994; Kura et al. 1994; Marra et al. 1990; McCoy-Simandle et al. 2011; Neumeister et al. 1997; Pearlman et al. 1988; Susa et al. 1996; Yan and Cirillo 2004).

Without diminishing the significance of growth within macrophages, it is likely that other factors also contribute to the survival and virulence of *L. pneumophila* within humans (Cianciotto 2001). For example, the bacterium may replicate or, at a minimum, must survive within extracellular spaces in the alveoli (Chandler et al. 1979; Rodgers et al. 1978; Surgot et al. 1988; Watson and Sun 1981; White et al. 1979). The fact that many strains of *L. pneumophila* are inherently serum resistant may be particularly relevant for extracellular survival following the onset of the inflammatory response (Caparon and Johnson 1988; Horwitz and Silverstein 1981; Luneberg et al. 1998; Plouffe et al. 1985; Verbrugh et al. 1985). Similarly, the ability of *L. pneumophila* to resist cationic peptides and to secrete enzymes that degrade lung surfactant suggests that the bacterium subverts some of the antimicrobial factors released by lung epithelia (Edelstein 1981; Flieger et al. 2000; Robey et al. 2001; Wadowsky and Yee 1981). Moreover, the presence of legionellae within non-macrophage cells in necropsy material indicates that *L. pneumophila* may also grow within the alveolar epithelium (Maruta et al. 1998; Rodgers 1979; Watson and Sun 1981). In support of this hypothesis, the microbe invades and replicates within alveolar types I and II cells in vitro (Chang et al. 2005; Cianciotto et al. 1995; Edelstein et al. 2003; Gao et al. 1998; Mody et al. 1993; N'Guessan et al. 2007; Newton et al. 2006; Yaradou et al. 2007). The importance of growth and persistence outside of the macrophage host cell is further indicated by several more observations. First, those *L. pneumophila* strain types that represent the most common type of clinical isolate (i.e., MAb-2 positive strains) are not necessarily more effective at intracellular infection of macrophages (Edelstein and Edelstein 1993). Second, various types of *L. pneumophila* mutants that are not defective or only moderately defective for macrophage infection in vitro are impaired or more strikingly impaired in animal models of pneumonia (Allard et al. 2009; Chang et al. 2005; DebRoy et al. 2006b; Edelstein et al. 1999; Liles et al. 1999; Rossier et al. 2004). Third, some of the proteins that are secreted by extracellular *L. pneumophila* such as a zinc metalloprotease can directly mediate lung tissue damage (Conlan et al. 1988; Dowling et al. 1992; Moffat et al. 1994). Thus, another area of *Legionella* research has been the identification and characterization of virulence factors that act, entirely or primarily, outside of the macrophage host (see below).

Cell Biology of *L. pneumophila* Intracellular Infection

While protozoa are the natural hosts of *Legionellae*, the infection of human phagocytic cells is opportunistic. Given the pivotal role that intracellular parasitism plays in the biology of *L. pneumophila*, a first-line approach toward understanding

legionellosis has been to study the cellular basis of macrophage infection (Abu Kwaik 1998; Cianciotto et al. 1989b; Horwitz 1992; Hubber and Roy 2010a; Isberg et al. 2009; Ott 1994; Roy 1999; Shuman et al. 1998; Vogel and Isberg 1999). This effort has been aided enormously by the availability of human macrophage-like cell lines, such as U937, HL-60, Mono Mac, and THP-1 cells (Cirillo et al. 1994; Marra et al. 1990; Neumeister et al. 1997; Pearlman et al. 1988).

Adherence and Entry into Host Cells

Uptake of *L. pneumophila* by phagocytic cells, such as macrophages and amoebae, can occur through conventional phagocytosis or coiling phagocytosis as shown in *Fig. 9.1* (Bozue and Johnson 1996; Elliott and Winn 1986; Hilbi et al. 2001; Horwitz and Maxfield 1984; King et al. 1991; Rechnitzer and Blom 1989). However, coiling phagocytosis may not play an important role in intracellular survival since heat-killed and formalin-killed *L. pneumophila* are also ingested within coiled phagosomes but are targeted to the lysosome (Horwitz and Maxfield 1984). Conventional phagocytosis can occur through a complement-mediated mechanism via complement receptor CR1 and CR3 (Payne and Horwitz 1987). Complement component CR3 fixes primarily to the major outer membrane protein MOMP, and the MOMP-CR3 interaction seems to be sufficient to mediate *L. pneumophila* uptake into macrophages (Bellinger-Kawahara and Horwitz 1990). Several bacterial factors have been implicated in non-complement-mediated adherence of *L. pneumophila* to phagocytic cells like type IV pili (Stone and Abu Kwaik 1998), Hsp60 (Garduno et al. 1998b), and RtxA, but the receptors possibly involved have not been elucidated (Cirillo et al. 2000). Furthermore, LaiA and SidE (Chang et al. 2005), two secreted factors of *L. pneumophila*, and EnhC and LpnE, two bacterial proteins containing multiple tetratricopeptide repeats (TPR), are required for efficient host entry (Cirillo et al. 2000; Newton et al. 2006).

Intracellular Replication

Following ingestion by phagocytes *L. pneumophila* inhibits phagosome-lysosome maturation and is instead found within a single-membrane vacuole with numerous small vesicles on the cytoplasmic face. During the first hour following uptake, mitochondria cluster around the *Legionella*-containing vacuole (LCV), and by 4 h, vesicles derived from rough endoplasmic reticulum (ER) collect near the LCV membrane (*Fig. 9.2*). Formalin-killed bacteria do not form such vacuoles, indicating that bacterial factors are responsible for this process (Horwitz 1983). Fluorescence microscopy studies in which markers of secretory vesicles of ER membrane (p58, Sec22b, calnexin) and ER luminal proteins (IgG-binding protein BiP, calreticulin, glucose-6-phosphate, etc.) have been labeled allowed understanding the origin and the kinetics of recruitment of the vesicles surrounding the LCV (Derré and Isberg 2004; Kagan and Roy 2002; Kagan et al. 2004; Swanson and Isberg 1995). Maturation of the LCV occurs in two phases: shortly after uptake, the LCV interacts and fuses with secretory vesicles transiting between ER and Golgi; in a second phase, LCV fuses with ER membranes, resulting in ER content being delivered to the lumen of the LCV (Hilbi and Haas 2012; Robinson and Roy 2006). After the formation of this ER-surrounded LCV, bacterial replication is initiated with generation times of approximately 2 h (*Fig. 9.3*). During the late replicative phase (~18 h postinfection), the phagosome appears to merge with lysosomes as it becomes more acidic and acquires lysosomal markers like LAMP-1. Fusion with the lysosomal compartment seems to lead a nutrient-rich environment that was shown to promote rather than inhibit *L. pneumophila* replication (Sturgill-Koszycki and Swanson 2000). Recently, intact LCVs have been purified from *L. pneumophila*-infected *D. discoideum* amoebae and analyzed by proteomics (Shevchuk et al. 2009; Urwyler et al. 2009). In one study, *D. discoideum* producing calnexin-GFP (an ER and LCV marker) was infected with red fluorescent *L. pneumophila*, and after homogenization of infected amoebae, fluorescent LCVs

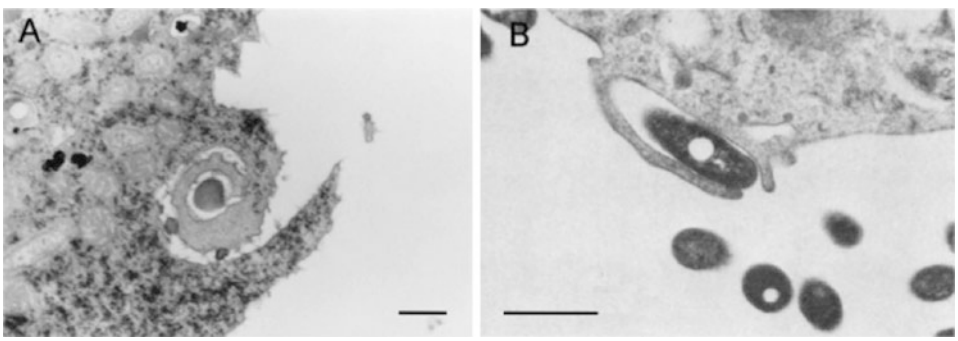

Fig. 9.1

Uptake of L. pneumophila. **(a)** ***Uptake of L. pneumophila by A. castellanii*** **through coiling phagocytosis at 30 min of co-incubation, scale bar 1μm (Bozue and Johnson 1996). (b)** ***Uptake of L. pneumophila by*** **guinea pig alveolar macrophages through conventional phagocytosis, scale bar 1 μm (Elliott and Winn 1986)**

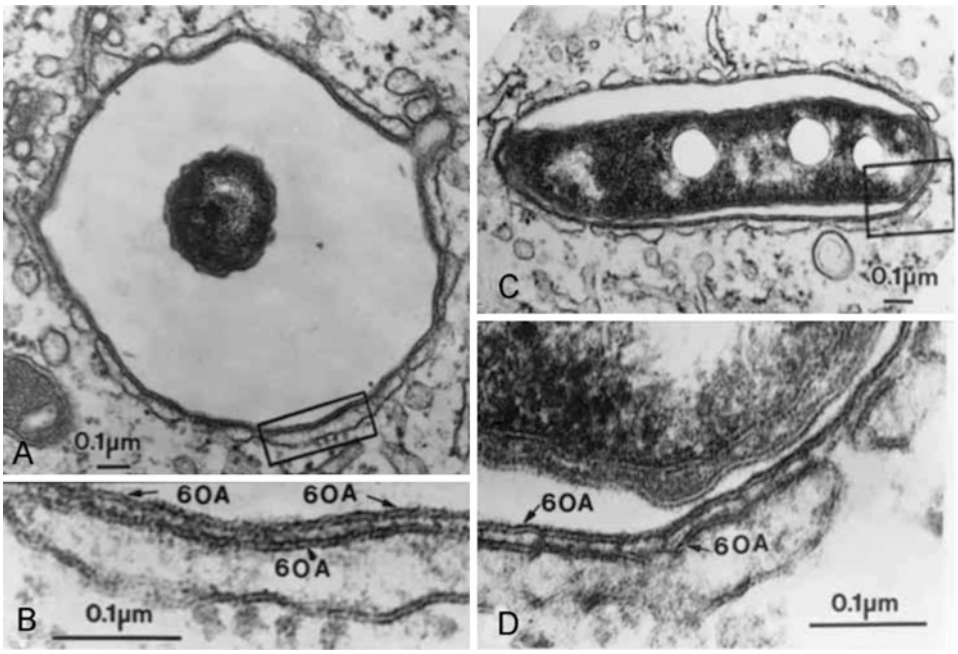

Fig. 9.2
L. pneumophila **recruits ER-derived vesicles. Transverse section through a phagosome containing** ***L. pneumophila*** **in U937 cells after 15 min of infection. Vesicles of ER, both studded with ribosomes and without, are attached to the phagosome. The region indicated is shown at higher magnification in (b). The ER membranes are 60 Å thick. (c) Longitudinal section through a phagosome containing** ***L. pneumophila*** **following 2 h of infection. The area** ***boxed*** **in (c) is shown at a higher magnification in (d) (Tilney et al. 2001)**

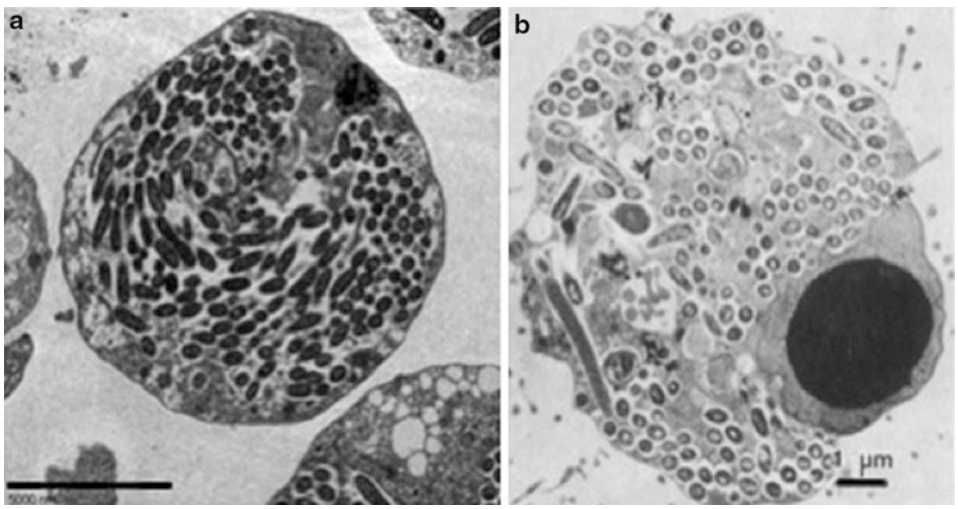

Fig. 9.3
Intracellular replication of ***L. pneumophila*****.** ***L. pneumophila*** **replicating in (a)** ***Hartmannella*****, scale bar 0.5 μm (Source: Photo: Holland/Özel, Robert Koch-Institut), and (b) guinea pig macrophages, Scale bar 1μm (Elliott and Winn 1986)**

were isolated by a straightforward two-step protocol (Urwyler et al. 2010). To this end, immunomagnetic separation was applied using a primary antibody against an *L. pneumophila* "effector protein" (see below) specifically decorating the LCV membrane, and a secondary antibody coupled to magnetic beads, followed by density gradient centrifugation. The proteome of purified LCVs was analyzed by tandem mass spectrometry and revealed more than 560 host proteins (Urwyler et al. 2009). These included several small GTPases of the secretory (Arf1, Rab1, Rab8) or endosomal (Rab7, Rab14) vesicle

trafficking pathway, indicating that LCVs communicate not only with the early and late secretory pathways but also with the early and late endosomal pathways.

It has been shown that the LCV may resemble nascent autophagosomes (Swanson and Isberg 1995). Autophagy is an evolutionarily conserved degradative pathway that captures and transfers a variety of microbes to lysosomes. Biogenesis of *L. pneumophila*-containing vacuoles and autophagosomes shares several features, including ER-derived membranes; contributions by the host GTPases Rab1, Arf1, and Sar1; and a final destiny in lysosomes (Joshi and Swanson 2011). However, there are some differences between LCVs and autophagosomes: (1) autophagosomes lack ribosomes and are probably derived from ribosome-free region of the ER; (2) within 15 min following the infection, a reduction of the membrane thickness of the LCV is observed, and it becomes more similar to that of the ER, a change not observed in autophagy, while the recruitment of rough ER and mitochondria to LCV is achieved within 15 min following uptake, induction of autophagy takes place in 1 h; and (3) a number of *ATG* genes required for autophagy are not required for intracellular replication of *L. pneumophila* (Amer et al. 2005; Amer and Swanson 2005; Dubuisson and Swanson 2006; Otto et al. 2004; Swanson and Molofsky 2005; Tilney et al. 2001). It seems that some bacterial pathogens have evolved mechanisms to evade autophagic recognition or even co-opt the autophagy machinery for their own benefit as a replicative niche (Mostowy and Cossart 2012). *L. pneumophila* might be one of these pathogens.

Exit from the Host Cell and Transmission

Following replication, depletion of nutrients leads to the transition of *L. pneumophila* from a replicative form to a virulent form. This transition is accompanied by phenotypic changes: while replicative *L. pneumophila* is nonmotile, noncytotoxic, sodium resistant, and nonflagellated, bacteria in post-replicative, virulent phase are motile, cytotoxic, and flagellated. This phenotypic switch is observed in both broth cultures and intracellular bacteria. It was postulated that the exit of *L. pneumophila* from the host cell occurs in two stages: firstly, through disruption of the phagosomal membrane and exit into the cytoplasm (Molmeret et al. 2004, 2010) and, secondly, through the lysis of the plasma membrane of the host cell and the exit of bacteria (Molmeret and Abu Kwaik 2002). Both stages could be possibly mediated by a pore-forming activity (Molmeret et al. 2004). However, it has also been proposed that disruption of the host cell can occur through an apoptosis-mediated process. Surprisingly, despite the activation of caspase-3 by *L. pneumophila* during early stages of infection, macrophages are not immediately destroyed, and apoptosis is triggered only in late stages of the infection (Gao and Abu Kwaik 1999a, b; Molmeret et al. 2004; Santic et al. 2007). Two Dot/Icm effectors, LepA and LepB, were implicated in the egress of *L. pneumophila* from protozoa, but not mammalian cells, through a non-lytic process (Chen et al. 2004). These two effectors were initially identified based on their weak homology to eukaryotic SNAREs (Chen et al. 2004), but how LepA and LepB promote egress is unknown. LepB is a Rab1-GAP involved in replicative vacuole biogenesis (Ingmundson et al. 2007). However, LepB may contain other functional domains that contribute to egress (Shin and Roy 2008). In contrast to the series of events just outlined, exposure of a macrophage to large numbers of attached (extracellular) *L. pneumophila* results in immediate pore formation in the host cell plasma membrane and rapid necrotic cell death (Husmann and Johnson 1994; Kirby et al. 1998; Zuckman et al. 1999).

Secreted Virulence Factors of *L. pneumophila*

L. pneumophila secretes a plethora of factors that promote intracellular infection and virulence. These factors include both proteins and non-proteinaceous molecules. Arguably, it is the secretion of myriad proteins that most dramatically promotes *L. pneumophila* pathogenesis. In *L. pneumophila*, as in other Gram-negative bacteria, the secretion of proteins is a complex process that requires transport across the inner membrane, periplasm, and finally the outer membrane. Gram-negative organisms have six, and possibly eight, systems that can mediate protein export from within the bacterial cell to the extracellular milieu and/or into target host cells (i.e., type I, II, III, IV, and so on) (Desvaux et al. 2009). A great deal of research by different laboratories has shown that type IV and type II secretions are functional and critical for *L. pneumophila* (Cianciotto 2009; Hubber and Roy 2010b). The two next sections will focus on type IV and type II secretions, highlighting the mechanisms of secretion, the nature of the secreted proteins, and the role of these secretion events in the bacterium's interactions with its hosts. This will be followed by a discussion of a putative type I secretion system, the Tat system, and other secreted factors, including proteins that appear not to be dependent on type II or type IV secretion, siderophore, pigment, and a quorum-sensing molecule. Genome analysis has suggested that a type V protein secretion system exists in *L. pneumophila*; that is, strain Paris is predicted to encode an autotransporter (Bruggemann et al. 2006). However, more work is needed in order to confirm that this gene functions in protein secretion.

Type IV Protein Secretion

Type IV secretion systems (T4SS) are devices present in a wide range of bacteria, including bacterial pathogens, that deliver macromolecular (proteins and single-strand DNA) across kingdom barriers, as well as between bacteria and into the surroundings. Their components are ancestrally related to the *tra-/trb*-encoded conjugation machinery required for transfer of plasmids between bacteria (Christie et al. 2005; Christie and Vogel 2000). The T4SS are divided into two subgroups, type IVA and type IVB, accordingly to sequence similarity (Juhas et al. 2008). *L. pneumophila* and *Coxiella burnetii* are the only two bacterial species known to date to utilize type IVB secretion system for pathogenesis (Nagai and Kubori 2011; Segal et al. 2005).

The type IVB secretion system in *Legionella* is the *icm* (intracellular multiplication; Marra et al. 1992) and/or *dot* (defective organelle trafficking; Berger and Isberg 1993) system. It is required for intracellular growth in human macrophages as well as in amoebae and for intracellular trafficking. The *dot/icm* type IV secretion system of *L. pneumophila* is probably its most important secretion system as it is involved in many different stages of the intracellular life cycle. It is critical for the ability of *L. pneumophila* to inhibit phagosome-endolysosomal fusions and to establish its unique replicative niche (Andrews et al. 1998; Berger and Isberg 1993; Berger et al. 1994; Brand et al. 1994; Marra et al. 1992; Purcell and Shuman 1998; Sadosky et al. 1993; Segal et al. 1998; Segal and Shuman 1997; Vogel et al. 1998). Several *dot*/*icm* loci have also been shown to be essential for virulence in a guinea pig model of disease (Edelstein et al. 1999; Marra et al. 1992). Interestingly, the Dot/Icm secretion system is also structurally and functionally homologous to bacterial conjugation systems, and it was indeed demonstrated to mediate plasmid DNA transfer between two *L. pneumophila* strains and to *E. coli*, which indicates that the Dot/Icm system retains an ancestral function and mediates transfer of macromolecules between cells (Segal et al. 1998; Segal and Shuman 1998; Vogel et al. 1998). This macromolecular complex is encoded by 25 genes located on two genomic regions: region I contains seven genes (*icmV, W, X, dotA, B, C, D*) and region II is composed of 18 genes (*icmT, S, R, Q, P, O, N, M, L, K, E, G, C, D, J, B, F, H*) as shown in ❯ *Fig. 9.4a*. The *dot* and *icm* gene products are thought to assemble into a multiprotein apparatus that function as a type IV secretion system (T4SS) (❯ *Fig. 9.4b*) (Segal et al. 1998; Segal and Shuman 1999; Vogel et al. 1998). The *L. pneumophila* Dot/Icm system is conserved and present in the same chromosomal location in all strains sequenced to date. When analyzed in six *L. pneumophila* strains (Paris, Lens, Philadelphia, Corby, Lorraine, HL 0604 1035), a very high nucleotide conservation of 98–100 % among orthologs was observed except for *dotA*, *icmX*, and for *icmC* of strain Corby that is shorter and more divergent (84 % nucleotide identity) as compared to *icmC* of strain Paris. These results indicate that strong negative selection acts on these genes (❯ *Table 9.3*) (Gomez Valero et al. 2011). In contrast, between

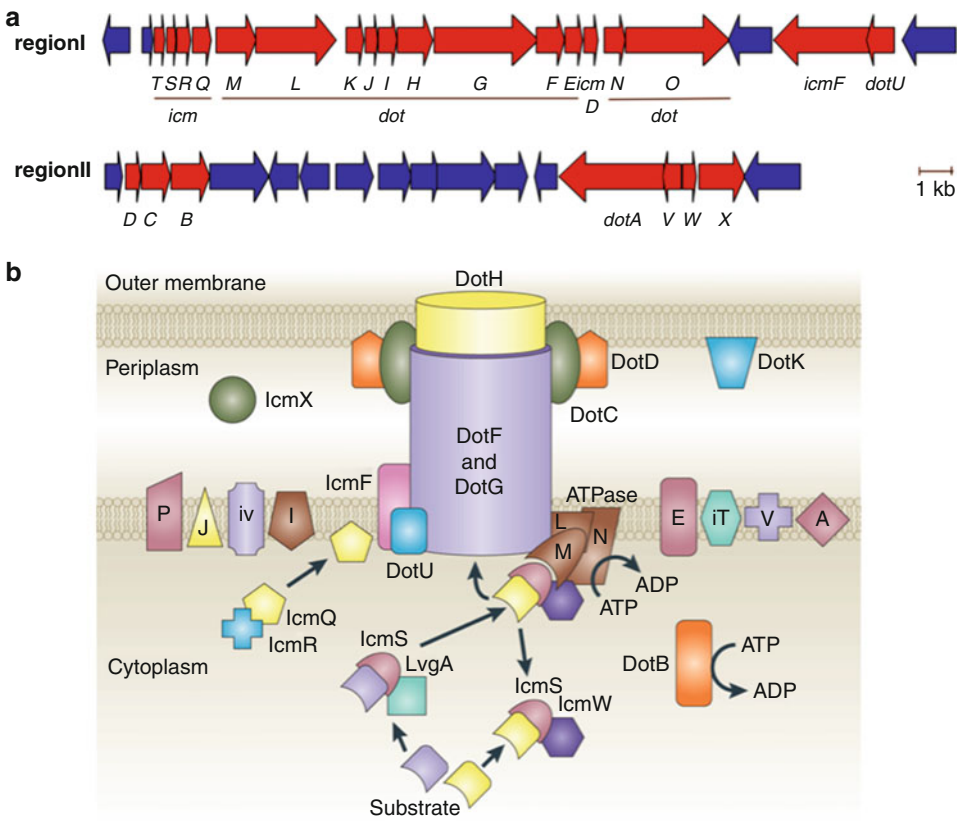

Fig. 9.4

The Dot/Icm type IVB secretion system of *L. pneumophila*. (a) Schematic representation of the genomic regions encoding the *dot/icm* type IV secretion system. This macromolecular complex is encoded by 25 genes located on two different genomic regions (Adapted from Gomez Valero et al. 2011). (b) The presumed locations and topological relationships of the various Dot/Icm components based on a study of the stability of individual proteins in the presence of defined deletion mutations (Buscher et al. 2005). Individual letters represent Dot protein names, whereas letters preceded by an "i" indicate Icm protein names (Isberg et al. 2009)

Table 9.3

Percentage of nucleotide identity of orthologous *dot/icm* genes with respect to the *L. pneumophila* Philadelphia sequence (Gomez Valero et al. 2011)

Components of the Dot/Icm secretion system of the 6 *L. pneumophila* strains and comparison to *L. longbeachae* NSW150														
Gene name	Length (nts)	Philadelphia	Paris	Identity	Lens	Identity	Lorraine	Identity	HL06041035	Identity	Corby	Identity	*L. long*	Identity
icmT	261	*lpg0441*	*lpp0507*	99.65	*lpl0483*	99.14	*lpo0507*	100	*lpv0541*	96	*lpc2902*	99.22	*llo2795*	75.20
icmS	345	*lpg0442*	*lpp0508*	98.54	*lpl0484*	98.83	*lpo0508*	99.12	*lpv0542*	94.44	*lpc2901*	98.25	*llo2794*	76.91
icmR	363	*lpg0443*	*lpp0509*	96.94	*lpl0485*	98.33	*lpo0509*	97.78	*lpv0543*	97.5	*lpc2900*	96.94		
icmQ	576	*lpg0444*	*lpp0510*	97	*lpl0486*	99	*lpo0510*	98	*lpv0544*	98	*lpc2899*	98	*llo2792*	70.72
icmP/dotM	1131	*lpg0445*	*lpp0511*	98	*lpl0487*	99	*lpo0511*	98	*lpv0545*	98	*lpc2898*	99	*llo2791*	74.51
icmO/dotL	2352	*lpg0446*	*lpp0512*	98.42	*lpl0488*	97.90	*lpo0512*	98.13	*lpv0546*	98.34	*lpc2897*	98.3	*llo2790*	77.66
IcmN/DotK	570	*lpg0447*	*lpp0513*	99.29	*lpl0489*	98.59	*lpo0513*	98.94	*lpv0547*	99.65	*lpc2896*	99.65	*llo2789*	67.26
icmM/dotJ	285	*lpg0448*	*lpp0514*	97.87	*lpl0490*	97.87	*lpo0514*	97.87	*lpv0548*	99.29	*lpc2895*	98.58	*llo2788*	61.7
icmL/dotI	639	*lpg0449*	*lpp0515*	99.84	*lpl0491*	99.37	*lpo0515*	99.37	*lpv0549*	99.84	*lpc2894*	99.53	*llo2787*	78.56
icmK/dotH	1083	*lpg0450*	*lpp0516*	94.81	*lpl0492*	94.26	*lpo0516*	95.19	*lpv0550*	94.44	*lpc2893*	94.72	*llo2786*	71.23
icmE/dotG	3147	*lpg0451*	*lpp0517*	93.70	*lpl0493*	94.02	*lpo0517*	94.02	*lpv0551*	93.99	*lpc2892*	94.27	*llo2785*	69.08
icmG/dotF	810	*lpg0452*	*lpp0518*	98	*lpl0494*	97	*lpo0518*	98	*lpv0552*	98	*lpc2891*	97	*llo2784*	55.75
icmC/dotE	585	*lpg0453*	*lpp0519*	99.66	*lpl0495*	99.14	*lpo0519*	99.66	*lpv0553*	99.31	*lpc2890*	54	*llo2783*	69.08
icmD/DotP	399	*lpg0454*	*lpp0520*	97	*lpl0496*	98	*lpo520*	97	*lpv0554*	98	*lpc2889*	97	*llo2782*	77.30
icmJ/dotN	627	*lpg0455*	*lpp0521*	99.04	*lpl0497*	98	*lpo0521*	99	*Lpv0555*	99	*lpc2888*	98	*llo2781*	79.38
IcmB/DotO	3030	*lpg0456*	*lpp0522*	98.08	*lpl0498*	98.25	*lpo0522*	98.32	*lpv0556*	98.18	*lpc2887*	97.62	*llo2780*	76.45
IcmF	2922	*lpg0458*	*lpp0524*	98.22	*lpl0500*	98.49	*lpo0524*	98.32	*lpv0558*	98.49	*lpc2885*	98.15	*llo3075*	69.48
IcmH/DotU	786	*lpg0459*	*lpp0525*	99.36	*lpl0501*	99.49	*lpo0525*	99.66	*lpv0559*	98.98	*lpc2884*	98.98	*llo3074*	68.84
dotD	492	*Lpg2674*	*lpp2728*	98	*lpl2601*	98	*lpo2953*	98	*lpv3018*	98	*lpc0463*	99	*llo0369*	76.49
dotC	912	*Lpg2675*	*lpp2729*	98.68	*lpl2602*	98.46	*lpo2954*	98.79	*lpv3019*	98.57	*lpc0462*	99.89	*llo0368*	74.81
dotB	1134	*Lpg2676*	*lpp2730*	99	*lpl2603*	98	*lpo2955*	98	*lpv3020*	98	*lpc0461*	99	*llo0367*	76.04
dotA	3108	*Lpg2686*	*lpp2740*	83.25	*lpl2613*	96.77	*lpo2967*	82.97	*lpv3032*	83.63	*lpc0450*	85.85	*llo0364*	51.39
icmV	456	*Lpg2687*	*lpp2741*	91	*lpl2614*	91	*lpo2968*	91	*lpv3033*	92	*lpc0449*	92	*llo0363*	64.25
icmW	456	*Lpg2688*	*lpp2742*	94.14	*lpl2615*	97.57	*lpo2969*	95.14	*lpv3034*	95.36	*lpc0448*	95.14	*llo0362*	79.25
icmX	1419	*Lpg2689*	*lpp2743*	84.25	*lpp2616*	85.19	*lpo2970*	85.61	*lpv3035*	85.61	*lpc0447*	84.14	*llo361*	46.90

different *Legionella* species, the nucleotide sequence similarity is less pronounced (46–79 %) (Gomez-Valero et al. 2011; Morozova et al. 2004). The global organization of the Dot/Icm system in different *Legionella* species is conserved; however, it has been shown that in region II in *L. micdadei*, the gene *icmR* is replaced by two genes, *migA* and *migB*, which do not show any homology to *icmR*. The same gene is replaced in *L. longbeachae* by a gene called *ligB* (Feldman and Segal 2004). Further analyses of the region carrying the *icmR* from 29 *Legionella* species revealed the presence of a large hypervariable gene family named functional homologues of *icmR* (*fir*) gene, located at the *icmR* genomic position (Feldman et al. 2005). All *fir* genes were found, together with their corresponding *icmQ* genes, to function similarly during infection. In addition, all FIR proteins were found to interact with their corresponding IcmQ proteins. Their interaction depends on a variable region located between two conserved domains of IcmQ that probably coevolved with the corresponding FIR protein. A FIR-IcmQ pair was also found in *Coxiella burnetii*, the only known non-*Legionella* bacterium that contains a Dot/Icm system, indicating the significance of this protein pair for the function of this type IV secretion system (Feldman et al. 2005). The only other difference observed is present in the DotG/IcmE protein of *L. longbeachae* (1,525 aa) that is 477 amino acids larger than that of *L. pneumophila* (1,048 aa) (Cazalet et al. 2010). DotG of *L. pneumophila* is part of the core transmembrane complex of the secretion system and is composed of three domains: a transmembrane N-terminal domain, a central region composed of 42 repeats of 10 amino acids, and a C-terminal region homologous to VirB10. In contrast, the central region of *L. longbeachae* DotG is composed of approximately 90 repeats. Among the many VirB10 homologues present in bacteria, the *C. burnetii* DotG and the *Helicobacter pylori* Cag7 are the only ones, which also have multiple repeats of 10 amino acids (Segal et al. 2005).

Given the central role of the Dot/Icm system in *Legionella* pathogenesis, many recent studies have aimed at identifying and characterizing its substrates. The first characterized effector was RalF, required for localization of the host protein ARF-1, a key regulator of vesicle trafficking from the endoplasmic reticulum to the phagosomes (Nagai et al. 2002). RalF is conserved in all strains sequenced to date, like LidA, another substrate involved in recruitment of vesicles during vacuole biogenesis and in maintaining integrity of the Dot/Icm complex (Conover et al. 2003; Derre and Isberg 2005). LidA binds to GDI-free Rab1 and thus combined with SidM to intercept host cell vesicles of the Rab1-regulated early secretory pathway (Machner and Isberg 2006). Similarly, the two Dot/Icm effectors LepA and LepB are conserved in all *L. pneumophila* strains investigated. These effectors show weak homology to SNAREs and were shown to be functionally involved in the release of *Legionella* from the vacuole after intracellular multiplication during amoeba infection (Chen et al. 2004). A number of candidate effector proteins named SidA–G were identified in the Philadelphia 1 strain by a two-hybrid screen with IcmG/DotF as bait followed by a screen of proteins transferred interbacterially with a Cre-/loxP-based protein translocation assay (Luo and Isberg 2004). All Sid proteins except SidD contain a coiled-coil domain, a protein motif known to be involved in protein-protein interactions. Most of these effectors have no discernible defects in inhibition of phagolysosome maturation or intracellular growth. Construction of single deletion strains for *sidA*, *sidD*, *sidF*, or *sidG* in strain Philadelphia did not result in attenuated virulence, pointing to functional redundancy, which probably extends individual substrate families. A quadruple mutant strain lacking *sidB* and its three paralogs (*sdbABC*) showed defects in intracellular growth, but this result was not significantly different from a mutant with a single *sdbA* deletion (Luo and Isberg 2004). One SidE paralog, designated LaiA (=SdeA), was identified in an independent study and characterized as a virulence factor: the integrin-like protein is involved in adhesion to and invasion of human lung alveolar epithelial cells (Chang et al. 2005). Another family of effectors, named PieA-PieE, has been identified on a plasticity zone of the *L. pneumophila* genome, indicating that they might have been acquired by horizontal gene transfer (Ninio et al. 2009). One of these proteins, PieA, is recruited in a Dot/Icm-dependent manner to the *L. pneumophila* vacuole and binds to the cytoplasmic face of the vacuole as a result of *L. pneumophila*-induced modifications to this vacuole. These findings demonstrated the first time that the association of an effector with host vacuoles can be spatially controlled through activities mediated by other effector proteins. Six of these genes were also identified independently and called *lirA-F* (Zusman et al. 2008). Recently, whole-genome alignment of strains 130b, Lens, Philadelphia 1, Corby, and Paris revealed that this region is part of a larger genomic region that displays considerable divergence among the five sequenced genomes. In strain 130b, this region is constituted of 96 kb, suggesting that the two regions initially described (Ninio et al. 2009; Zusman et al. 2008) constitute the inner core of a much larger 80- to 100-kb region of high genomic plasticity that represents a strain-specific variable effector region (Schroeder et al. 2010).

In more recent years, several other proteins have been shown to be Dot/Icm effectors by either bioinformatics approaches, the adenylate cyclase assay approach, or the β-lactamase reporter system (Burstein et al. 2009; de Felipe et al. 2005, 2008; Zhu et al. 2011). Currently, 278 proteins of *L. pneumophila* have been described as being translocated by the Dot/Icm T4SS system, a number not equaled by any other bacterial secretion system. Selected substrates and their distribution are listed in *Table 9.4*. Analysis of the distribution of the 278 Dot/Icm substrates identified in *L. pneumophila* strain Philadelphia in six other *L. pneumophila* and five *L. longbeachae* genomes sequenced showed that they are highly conserved among different *L. pneumophila* strains, as over 80 % of the substrates are present in all six *L. pneumophila* strains (Gomez Valero et al. 2011). In contrast, the search for homologues of these *L. pneumophila* Dot/Icm substrates in *L. longbeachae* revealed pronounced differences are present as only 98 of the 278 *L. pneumophila* Dot/Icm substrates have homologues in the *L. longbeachae* genomes (Gomez Valero et al. 2011). Many of them are eukaryotic-like proteins and eukaryotic domain proteins, involved in the virulence of the bacterium described

Table 9.4

Distribution of selected Dot/Icm substrates of strain *L. pneumophila* Philadelphia in five sequenced *L. pneumophila* strains

L. pneumophila							
Philadelphia	Paris	Lens	Corby	Alcoy	130b$^+$	Name	Product
lpg0038	*lpp0037*	*lpl0038*	*lpc0039*	*lpa0049*	*lpw00381*	*ankQ/legA10*	Ankyrin repeat
lpg0096	*lpp0110*	*lpl0096*	*lpc0115*	*lpa0145*	*lpw00961*	*ceg4*	Unknown
lpg0103	*lpp0117*	*lpl0103*	*lpc0122*	*lpa0152*	*lpw01031*	*vipF*	N-terminal acetyltransferase, GNAT
lpg0135	*lpp0150*	*lpl0135*	*lpc0156*	*lpa0204*	*lpw01361*	*sdhB*	Unknown
lpg0171	*lpp0233*	*lpl0234*	–	–	*lpw02651*	*legU1*	F-box motif
lpg0234	*lpp0304*	*lpl0288*	*lpc0309*	*lpa0419*	*lpw03221*	*sidE/laiD*	Unknown
lpg0257	*lpp0327*	*lpl0310*	*lpc0334*	*lpa0450*	*lpw03461*	*sdeA*	Multidrug resistance protein
lpg0275	*lpp0349*	*lpl0327*	*lpc0351/ 3529*	*lpa0477*	*lpw03641*	*sdbA*	Unknown
lpg0276	*lpp0350*	*lpl0328*	*lpc0353*	*lpa0479*	*lpw03651*	*legG2*	Ras guanine nucleotide exchange factor
lpg0376	*lpp0443*	*lpl0419*	*lpc2967*	*lpa0597*	*lpw04591*	*sdhA*	GRIP, coiled-coil
lpg0390	*lpp0457*	*lpl0433*	*lpc2954*	*lpa0613*	*lpw04721*	*vipA*	Unknown
lpg0401	*lpp0468*	*lpl0444*	*lpc2942*	*lpa0629*	*lpw04831*	*legA7/ceg11*	Unknown
lpg0402	–	–	–	–	–	*ankY/legA9*	Ankyrin, STPK
lpg0403	*lpp0469*	*lpl0445*	*lpc2941*	*lpa0630*	*lpw04841*	*ankG/ankZ/ legA7*	Ankyrin
lpg0422	*lpp0489*	*lpl0465*	*lpc2921*	*lpa0657*	*lpw05041*	*legY*	Putative glucan 1,4-alpha-glucosidase
lpg0436	*lpp0503*	*lpl0479*	*lpc2906*	*lpa0673*	*lpw05181*	*ankJ/legA11*	Ankyrin
lpg0437	*lpp0504*	*lpl0480*	*lpc2905*	*lpa0674*	*lpw05191*	*ceg14*	Unknown
lpg0439	*lpp0505*	*lpl0481*	*lpc2904*	*lpa0678*	*lpw05201*	*ceg15*	Unknown
lpg0483	*lpp0547*	*lpl0523*	*lpc2861*	*lpa0739*	*lpw05631*	*ankC/legA12*	Ankyrin
lpg0515	*lpp0578*	*lpl0554*	*lpc2829*	*lpa0776*	*lpw05951*	*legD2*	Phytanoyl-CoA dioxygenase domain
lpg0621	*lpp0675*	*lpl0658*	*lpc2673*	*lpa0975*	*lpw06951*	*sidA*	Unknown
lpg0642	*lpp0696/97*	*lpl0679*	*lpc2651*	*lpa1005*	*lpw07161*	*wipB*	Unknown
lpg0695	*lpp0750*	*lpl0732*	*lpc2599*	*lpa1082*	*lpw07721*	*ankN/ankX legA8*	Ankyrin
lpg0940	*lpp1002*	*lpl0971*	*lpc2349*	*lpa1415*	*lpw10251*	*lidA*	Unknown
lpg0944	*lpp1006*	–	*lpc2345*	*lpa1421*	–	*ravJ*	Unknown
lpg0945	*lpp1007*	*lpl1579*	*lpc2344*	*lpa1423*	*lpw10311*	*legL1*	LLR
lpg0968	*lpp1030*	*lpl0997*	*lpc2319*	*lpa1460*	*lpw10541*	*sidK*	Unknown
lpg1227	*lpp1235*	*lpl1235*	*lpc0696*	*lpa1899*	*lpw12861*	*vpdB*	Unknown
lpg1312	–	–	–	–	*lpw13261*	*legC1*	Unknown
lpg1328	*lpp1283*	*lpl1282*	*lpc0743*	*lpa1958*	–	*legT*	Thaumatin domain
lpg1355	*lpp1309*	–	–	–	–	*sidG*	Coiled-coil
lpg1426	*lpp1381*	*lpl1377*	*lpc0842*	*lpa2090*	*lpw14431*	*vpdC*	Patatin domain
lpg1483	*lpp1439*	*lpl1545*	*lpc0898*	*lpa2161*	*lpw15031*	*legK1*	STPK
lpg1488	*lpp1444*	*lpl1540*	*lpc0903**	*lpa2168*	*lpw15081*	*lgt3/legc5*	Coiled-coil
lpg1588	*lpp1546*	*lpl1437*	*lpc1013*	*lpa2305*	*lpw16131*	*legC6*	Coiled-coil
lpg1602	*lpp1567*	*lpl1423/ 26**	*lpc1028*	*lpa2318*	*lpw16241*	*legL2*	LRR
lpg1642	*lpp1612a/b*	*lpl1384*	*lpc1071*	*lpa2371*	*lpw16681*	*sidB*	Putative hydrolase
lpg1660	*lpp1631*	*lpl1625*	*lpc1090*	*lpa2398*	*lpw16861*	*legL3*	LRR
lpg1661	*lpp1632*	*lpl1626*	*lpc1091*	*lpa2399*	*lpw16871*	–	Putative N-acetyltransferase
lpg1687	*lpp1656*	*lpl1650*	*lpc1118*	*lpa2437*	*lpw17121*	*mavA*	Unknown
lpg1701	*lpp1666*	*lpl1660*	*lpc1130*	*lpa2455*	*lpw17231*	*ppeA/legC3*	Coiled-coil
lpg1702	*lpp1667*	*lpl1661*	*lpc1131*	*lpa2456*	*lpw17241*	*ppeB*	Unknown

Table 9.4 (continued)

L. pneumophila							
Philadelphia	Paris	Lens	Corby	Alcoy	130b$^+$	Name	Product
lpg1718	*lpp1683*	*lpl1682*	*lpc1152*	*lpa2484*	*lpw17411*	*ankI/legAS4*	Ankyrin
lpg1884	*lpp1848*	*lpl1845*	*lpc1331*	*lpa2714*	*lpw19161*	*ylfB/legC2*	Coiled-coil
lpg1890	–	*lpl1852*	*lpc1338*	*lpa2726*	*lpw19231*	*legLC8*	LRR, coiled-coil
lpg1948	–	–	–	–	–	*legLC4*	LRR, coiled-coil
lpg1949	*lpp1931*	*lpl1918*	*lpc1422*	*lpa2837*	*lpw19961*	*lem17*	Unknown
lpg1950	*lpp1932*	*lpl1919*	*lpc1423*	*lpa2838*	*lpw19971*	*ralF*	Sec7 domain
lpg1953	*lpp1935*	*lpl1922*	*lpc1426*	*lpa2842*	*lpw20041*	*legC4*	Coiled-coil
lpg1958	*lpp1940*	–	–	–	–	*legL5*	LRR
lpg1960	*lpp1942*	*lpl1934**	*lpc1437*	*lpa2859*	*lpw20111*	*lirA*	Unknown
lpg1962	*lpp1946*	*lpl1936*	*lpc1440*	*lpa2861*	*lpw20131*	*lirB*	Rotamase
lpg1963	–	–	*lpc1441/42*	*lpa2863*	–	*pieA/lirC*	Unknown
lpg1964	–	–	–	–	–	*pieB/lirD*	Unknown
lpg1965	–	–	*lpc1443/45*	*lpa2865*	*lpw20141*	*pieC/lirE*	Unknown
lpg1966	*lpp1947*	–	*lpc1446*	*lpa2867*	*lpw20151*	*pieD/lirF*	Unknown
lpg1969	*lpp1952*	*lpl1941*	*lpc1452*	*lpa2874*	*lpw20201*	*pieE*	Unknown
lpg1972	*lpp1955*	*lpl1950*	*lpc1459*	*lpa2884*	*lpw20291*	*pieF*	Unknown
lpg1976	*lpp1959*	*lpl1953*	*lpc1462*	*lpa2889(2)*	*lpw20351*	*pieG/legG1*	Regulator of chromosome condensation
lpg1978	*lpp1961*	*lpl1955*	*lpc1464*	*lpa2892*	*lpw20371*	*setA*	Putative glycosyltransferase
lpg2137	*lpp2076*	*lpl2066*	*lpc1586*	*lpa3060*	*lpw23101*	*legK2*	STPK
lpg2144	*lpp2082*	*lpl2072*	*lpc1593*	*lpa3071*	*lpw23181*	*ankB/legAU13/ ceg27*	Ankyrin, F-box
lpg2153	*lpp2092*	*lpl2081*	*lpc1602*	*lpa3083*	*lpw23271*	*sdeC*	Unknown
lpg2154	*lpp2093*	*lpl2082*	*lpc1603*	*lpa3086*	*lpw23281*	*sdeC*	Unknown
lpg2155	*lpp2094*	*lpl2083*	*lpc1604*	*lpa3087*	*lpw23291*	*sidJ*	Unknown
lpg2156	*lpp2095*	*lpl2084*	*lpc1605*	*lpa3088*	*lpw23301*	*sdeB*	Unknown
lpg2157	*lpp2096*	*lpl2085*	*lpc1618*	*lpa3037*	*lpw23331*	*sdeC*	Unknown
lpg2176	*lpp2128*	*lpl2102*	*lpc1635*	*lpa3118*	*lpw23561*	*legS2*	Sphingosine-1-phosphate lyase
lpg2215	*lpp2166*	*lpl2140*	*lpc1680*	*lpa3179*	*lpw24011*	*legA2*	Ankyrin
lpg2222	*lpp2174*	*lpl2147*	*lpc1689*	*lpa3191*	*lpw24081*	*lpnE*	Putative beta-lactamase (sel1 domain)
lpg2224	–	–	–	–	–	*ppgA*	Regulator of chromosome condensation
lpg2298	*lpp2246*	*lpl2217*	*lpc1763*	*lpa3296*	*lpw24841*	*ylfA/legC7*	Coiled-coil
lpg2300	*lpp2248*	*lpl2219*	*lpc1765*	*lpa3298*	*lpw24871*	*ankH/legA3, ankW*	Ankyrin, NfkappaB inhibitor
lpg2322	*lpp2270*	*lpl2242*	*lpc1789*	*lpa3328*	*lpw25121*	*ankK/legA5*	Ankyrin
lpg2391	*lpp2458*	*lpl2315*	*lpc2086*	*lpa3485*	*lpw26021*	*sdbC*	Unknown
lpg2392	*lpp2459*	*lpl2316*	*lpc2085*	*lpa3486*	*lpw26041*	*legL6*	LRR
lpg2400	–	*lpl2323*	–	–	*lpw26121*	*legL6*	LRR
lpg2410	*lpp2479*	*lpl2334*	*lpc2065*	*lpa3513*	*lpw26261*	*vpdA*	Patatin domain
lpg2416	–	*lpl2339*	*lpc2057*	*lpa3527*	*lpw26351*	*legA1*	Unknown
lpg2452	*lpp2517*	*lpl2370*	*lpc2026*	*lpa3574*	*lpw26701*	*ankF/legA14/ceg31*	Ankyrin
lpg2456	*lpp2522*	*lpl2375*	*lpc2020*	*lpa3583*	*lpw26751*	*ankD/legA15*	Ankyrin
lpg2464	–	*lpl2384*	–	–	*lpw26851*	*sidM/drrA*	Unknown
lpg2465	–	*lpl2385*	–	–	*lpw26861*	*sidD*	Unknown
lpg2490	*lpp2555*	*lpl2411*	*lpc1987*	*lpa3628*	*lpw27131*	*lepB*	Coiled-coil, Rab1 GAP
lpg2482	*lpp2546*	*lpl2402*	*lpc1996*	*lpa3615*	*lpw27041*	*sdbB*	Unknown
lpg2498	*lpp2566*	*lpl2420*	*lpc1975*	*lpa3646*	*lpw27241*	*mavJ*	Unknown

Table 9.4 (continued)

L. pneumophila							
Philadelphia	Paris	Lens	Corby	Alcoy	130b^{+}	Name	Product
lpg2504	*lpp2572*	*lpl2426*	*lpc1967*	*lpa3658*	*lpw27301*	*sidI/ceg32*	Unknown
lpg2508	*lpp2576*	*lpl2430*	*lpc1962/63**	*lpa3666*	*lpw27341*	*sdjA*	Unknown
lpg2509	*lpp2577*	*lpl2431*	*lpc1961*	*lpa3667*	*lpw27351*	*sdeD*	Unknown
lpg2510	*lpp2578*	*lpl2432*	*lpc1960*	*lpa3668*	–	*sdcA*	Unknown
lpg2511	*lpp2579*	*lpl2433*	*lpc1959*	*lpa3669*	*lpw27371*	*sidC*	PI(4)P binding domain
lpg2556	*lpp2626*	*lpl2481*	*lpc1906*	*lpa3745*	*lpw27911*	*legK3*	STPK
lpg2577	*lpp2629*	*lpl2499*	*lpc0570*	*lpa3768*	*lpw28241*	*mavM*	Unknown
lpg2584	*lpp2637*	*lpl2507*	*lpc0561*	*lpa3779*	*lpw28321*	*sidF*	Unknown
lpg2588	*lpp2641*	*lpl2511*	*lpc0557*	*lpa3784*	*lpw28361*	*legS1*	Unknown
lpg2694	*lpp2748*	*lpl2621*	*lpc0442*	*lpa3931*	*lpw29481*	*legD1*	phyhd1 protein
lpg2718	*lpp2775*	*lpl2646*	*lpc0415*	*lpa3966*	*lpw29771*	*wipA*	Unknown
lpg2720	*lpp2777*	*lpl2648*	*lpc0413*	*lpa3968*	*lpw29791*	*legN*	cAMP-binding protein
lpg2793	*lpp2839*	*lpl2708*	*lpc3079*	*lpa4063*	*lpw30471*	*lepA*	Effector protein A
lpg2829	*lpp2883/86**	–	–	–	*lpw30861*	*sidH*	Unknown
lpg2830	*lpp2887*	–	–	–	*lpw30881*	*lubX/legU2*	U-box motif
lpg2831	*lpp2888*	–	–	–	*lpw30891*	*VipD*	Patatin-like phospholipase
lpg2862	–	–	–	–	–	*Lgt2/legC8*	Coiled-coil
lpg2999	*lpp3071*	*lpl2927*	*lpc3315*	*lpa4395*	*lpw32851*	*legP*	Astacin protease

The selection of the substrates in this table is based on Isberg et al. (2009), de Felipe et al. (2008), Ninio et al. (2007), Zhu et al. (2011), and Gomez Valero et al. (2011) (de Felipe et al. 2008; de Felipe et al. 2005; Gomez Valero et al. 2011; Isberg et al. 2009; Ninio and Roy 2007; Zhu et al. 2011); *pseudogene, $^{+}$130b is not a finished sequence and not manually curated. Thus, absence of a substrate can also be due to gaps in the sequence

afterward (Bruggemann et al. 2006; Cazalet et al. 2004; de Felipe et al. 2005; Franco et al. 2009; Nora et al. 2009).

The first *L. pneumophila* protein encoding a eukaryotic domain was identified before genome sequencing. The gene *ralF* encodes a protein with a Sec-7 domain. These domains are found in eukaryotes as components of Arf-specific guanine nucleotide exchange factors (GEFs). GEFs catalyze the nucleotide exchange of Arfs, thereby converting them from an inactive state (GDP-bound) to the active one (GTP-bound). To promote the fusion to ER membranes, *L. pneumophila* recruits host factors to the surface of the LCVs like Arf-1 and Rab-1, important cell signaling proteins involved in the regulation of the ER-Golgi traffic. This is in part achieved by RalF. Following secretion by T4SS, RalF recruits Arf-1 and then functions like an Arf-1 specific GEF (Nagai et al. 2002). Until recently, the translocation signal for the type IV secretion effectors was not known. Nagai and colleagues, who investigated the mechanism of translocation of RalF (Nagai et al. 2002), identified a 20-amino-acid C-terminal region of the RalF protein as necessary and sufficient for translocation. In particular, a hydrophobic residue at the C-terminal −3 position is critical for secretion of RalF, as a substitution to hydrophilic residues resulted in a severe defect in translocation (Nagai et al. 2005). Comparison with other Dot/Icm substrates identified in most of them a hydrophobic residue or a proline residue at the −3 or −4 position, supporting the idea that these residues are critical for secretion by the type IV system. Additional features have been suggested like the enrichment in alanine, glycine, serine, and threonine at positions −8 to −2, and polar amino acids at positions −13 to +1 (Kubori et al. 2008; Nagai et al. 2005), as well as a region of 6–8 amino acids rich in glutamates, called the E Block motif (Huang et al. 2011).

Interestingly, many effector proteins show no sequence similarity to proteins present in other microorganisms, which indicates the uniqueness of the mechanism by which *L. pneumophila* subverts eukaryotic host cell functions. Another study identified candidate effector proteins capable of altering endosomal trafficking by screening a *L. pneumophila* genomic library for genes that induce a VPS (vacuole protein sorting)-negative phenotype in yeast (Shohdy et al. 2005). In *Saccharomyces cerevisiae*, VPS pathway components control several distinct vesicle trafficking pathways. The identified effectors were designated Vip (VPS inhibitor protein). Three Vip proteins (VipA, VipD, and VipF) are translocated from *L. pneumophila* into host macrophages via the Dot/Icm apparatus. The *L. pneumophila* genome encodes in addition three paralogs of VipD (VpdA, VpdB, VpdC), and VpdA and VpdB have also been shown to be translocated by the Dot/Icm T4SS (Shohdy et al. 2005). As VipD is predicted to contain a phospholipase A domain with homology to the type III-secreted protein ExoU from *Pseudomonas aeruginosa* which is a potent toxin for mammalian cells, VanRheenen and colleagues tested the toxicity of VipD for *S. cerevisiae*. VipD was shown to be mildly toxic when overproduced in eukaryotic cells (VanRheenen et al. 2006).

Recently, VipA was shown to constitute a novel type of actin nucleator that may contribute to the intracellular lifestyle of *Legionella* by altering cytoskeleton dynamics to target host cell pathways. VipA binds actin in vitro and directly polymerizes microfilaments without the requirement of additional proteins, displaying properties distinct from other bacterial actin nucleators (Franco et al. 2012; Franco and Shuman 2012). Intracellularly, neither the strains bearing deletions of individual genes nor the strain bearing the quadruple mutation was significantly impaired for growth in macrophages (VanRheenen et al. 2006). Thus, the different studies that aimed at characterizing the Dot/Icm substrates point to a probably high functional redundancy of this protein family in *Legionella*.

Another screen in a different yeast genetic system identified *Legionella* proteins that conferred a conditional growth defect when overproduced by yeast cultured in the presence of galactose. This screen led to the identification of a new Dot/Icm substrate that was called YlfA, for yeast lethal factor A. The YlfA protein could be observed on the ER-derived replicative vacuole and on punctuate structures throughout the host cell at late stages of the infectious cycle. However, the precise function of YlfA is still under investigation (Campodonico et al. 2005). A systematic analysis of 127 characterized and putative effector proteins for disruption of vesicle trafficking in yeast revealed the disruption of vesicle trafficking by Ceg9, Ceg19, and the novel effector SetA (Heidtman et al. 2009). SetA localized in LAMP-1-positive compartments when expressed in mammalian cells and was proposed to interact with the ubiquitination machinery at late endosomal compartments and to exhibit glycosyltransferase activity (see also below) (Heidtman et al. 2009).

Many of the secreted Dot/Icm substrates mediate posttranslational modifications (PTMs) of host proteins, to promote survival and replication of *L. pneumophila* inside eukaryotic cells. One of the conserved pathways hijacked by *L. pneumophila* is the host ubiquitination system. Ubiquitination is one of the best-known PTMs exploited by pathogenic bacteria to interfere with host signaling pathways. Several T3SS and T4SS translocated effectors from bacterial pathogens have been shown to exploit the ubiquitin-proteasome system of the host to their advantage (Rytkonen and Holden 2007). These secreted effectors may function as E3 ubiquitin ligases or deubiquitinating enzymes, leading to their proteasome-dependent degradation (Rohde et al. 2007; Zhang et al. 2006), or they possess deubiquitination (DUB) activity (Le Negrate et al. 2008). *L. pneumophila* interferes with the host ubiquitination system by translocating different proteins. One is LubX (for *Legionella U-box*), a protein containing two U-box domains (U-box1 and U-box2) (Kubori et al. 2008). LubX functions as an ubiquitin ligase in conjunction with host UbcH5a or UbcH5c E2 enzymes and mediates polyubiquitination of cellular Clk1 (Cdc2-like kinase 1). The U-box1 domain seems to be critical for E2 binding and the subsequent ubiquitin ligation, whereas the U-box2 domain interacts with the substrate. Interestingly, LubX has a second target within host cells, the *L. pneumophila* effector protein SidH. LubX directly binds and polyubiquitinates SidH in vitro and mediates its proteasomal degradation in infected cells (*Fig. 9.5*). LubX is the first example of a bacterial "meta-effector" that regulates in space and time the expression level of another effector (Kubori et al. 2010).

Other *L. pneumophila* proteins that interfere with the host ubiquitination pathway are three F-box motifs containing proteins (Cazalet et al. 2004; de Felipe et al. 2005; Gomez Valero et al. 2011). In eukaryotes, F-box proteins have been shown to function as E3 ubiquitin ligases within the modular SCF (*Skp1-Cullin-F-box protein*) multiprotein complex. Indeed, soon after infection of eukaryotic cells with *L. pneumophila*, ubiquitinated proteins accumulate on the LCV containing wild-type bacteria, but not on vacuoles containing mutants with a nonfunctional Dot/Icm system (Dorer et al. 2006). The addition of a proteasomal inhibitor during host cell infection led to reduced intracellular replication of *L. pneumophila*, suggesting that interference with the host ubiquitination pathway is important for the intracellular survival of *L. pneumophila* and dependent on secreted Dot/Icm substrates (Dorer et al. 2006). Among the three F-box-containing proteins encoded by *L. pneumophila* strain Philadelphia and Paris, AnkB/LegAU13/Ceg27 (Lpg2144/Lpp2082) is characterized best. Besides its F-box domain, this protein possesses a C-terminal ankyrin (Ank) domain (Cazalet et al. 2004) and a CAAX motif (where C represents cysteine and A an aliphatic amino acid) (Ivanov et al. 2010; Price et al. 2010b). Deletion of this conserved F-box protein-encoding gene (*ankB/lpg2144)* in strain AA100/130B resulted in a mutant exhibiting a severe replication defect within eukaryotic cells (Al-Khodor et al. 2008). Strain Paris missing AnkB/Lpp2082 is slightly attenuated in infection of protozoan cells, but it is outcompeted during competitive infection of lungs of A/J mice (Lomma et al. 2010). AnkB is a Dot/Icm-translocated effector that is involved in the recruitment of polyubiquitinated proteins around the LCV and interacts with Skp1 during infection (Lomma et al. 2010; Price et al. 2009). Silencing of Skp1 by RNAi blocked intracellular replication of *L. pneumophila*, indicating that Skp1 recruitment by *L. pneumophila* is essential for hijacking the host ubiquitination machinery, which is leading to an advantage in intracellular replication (Price et al. 2009) (*Fig. 9.5*). A yeast two-hybrid screen and co-immunoprecipitation analysis identified Parvin-β (ParvB) as one target of the *L. pneumophila* F-box protein AnkB/Lpp2082 encoded by strain Paris (Lomma et al. 2010). Parvins are known to have important roles in focal adhesion, cell spreading, and motility (Sepulveda and Wu 2006). ParvB is endogenously ubiquitinated and co-immunoprecipitates in vivo with AnkB/Lpp2082 (Lomma et al. 2010). Surprisingly, expression of AnkB/Lpp2082 led to a decrease of ubiquitination of ParvB. Thus, *L. pneumophila* seems to modulate ubiquitination of ParvB by competing with the eukaryotic E3 ligase for the specific protein-protein interaction site of ParvB, in order to promote caspase-3 activation and apoptosis (Lomma et al. 2010) (*Fig. 9.5*). Another appealing hypothesis is that AnkB/Lpp2082 targets the eukaryotic ubiquitin ligase that ubiquitinates ParvB, which would also lead to a decrease in ubiquitinated ParvB as observed during *L. pneumophila* infection (*Fig. 9.5*).

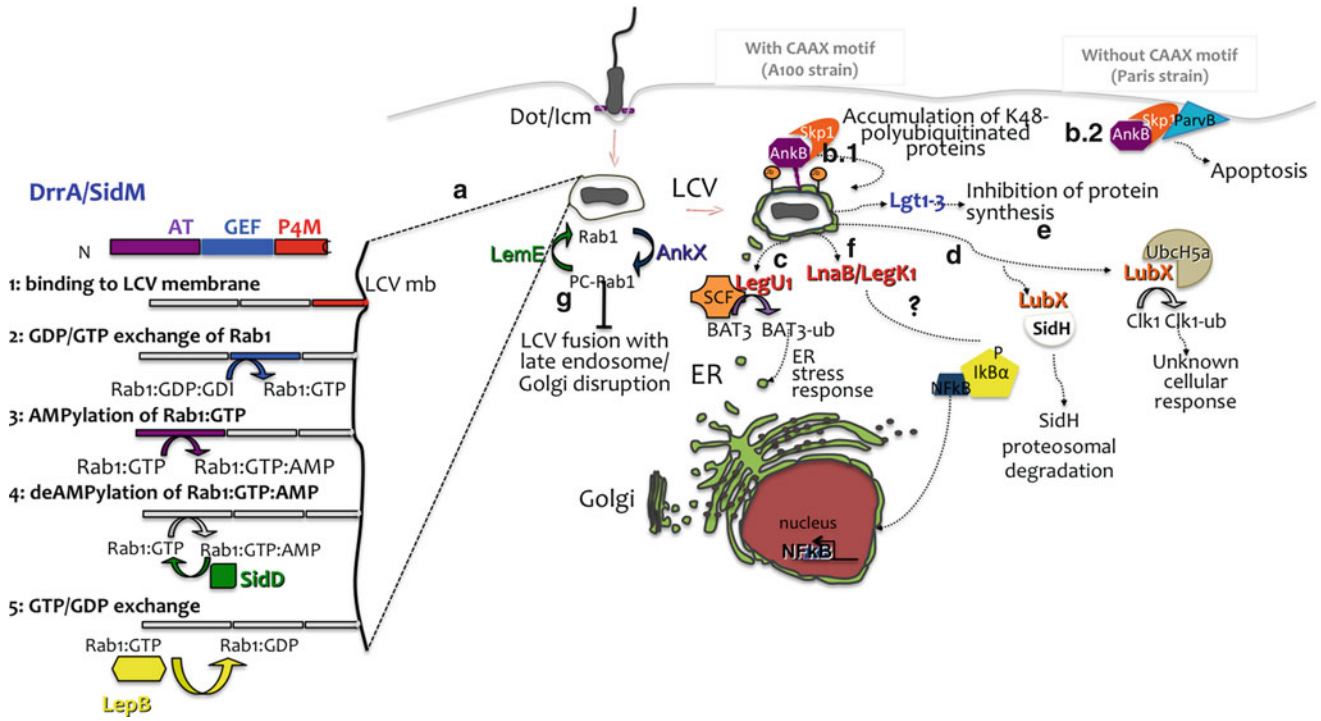

Fig. 9.5

Posttranslational modifications induced by *L. pneumophila*. (*a*) *L. pneumophila* exploits AMPylation and deAMPylation. DrrA/SidM possesses three domains: an AMP-transfer domain (AT) in its N-terminal region, a nucleotide exchange factor (*GEF*) domain in the central part, and a phosphatidylinositol-4-phosphate (PI-4P) binding domain (P4M) in its C-terminal part. After association of DrrA/SidM with the membrane of the LCV (LCV mb) via P4M (*1*), it recruits Rab1 *via* the GEF domain and catalyzes the GDP-GTP exchange (*2*). Rab1 is then adenylylated by the AT domain (*3*), leading to inhibition of GAP-catalyzed Rab1 deactivation. In step (*4*), SidD deAMPylates Rab1 and enables LepB to bind Rab1 and promotes its GTP-GDP exchange (*5*). (*b–d*) *Legionella* modulates the ubiquitin-signaling pathway. (*b.1*) AnkB of strain AA100 and Lp01 ubiquitinates host proteins and exploits the host prenylation machinery to anchor at the cytosolic face of LCV. (*b.2*) AnkB of *L. pneumophila* strain Paris that lacks the C-terminal CAAX farnesylation motif binds Skp1 and modulates the ubiquitination level of ParvB, a host protein present in focal adhesions and in lamellipodia. (*c*) The LegU1 effector interacts with a functional SCF complex through its F-box domain and specifically targets the host protein BAT3, a key regulator of the ER stress response. (*d*) The effector LubX contains two U-box domains that target cellular Clk1 during infection and promote bacterial replication. Furthermore, LubX functions as an E3 ubiquitin ligase that hijacks the host proteasome to specifically target the bacterial effector protein SidH for degradation to temporally coordinate its function. (*e*) Effector glycosyltransferases in *Legionella*. Lgt1, Lgt2, and Lgt3 act as glycosyltransferases that specifically target eEF1A and thereby inhibit the eukaryotic protein translation machinery. (*f*) Control of host cell phosphorylation. LegK1 and LnaB target the NF-kB pathway by phosphorylation activity. (*g*) *Legionella* exploits the cell phosphocholination (PC)/de-PC pathway to modify Rab1 (Adapted from Rolando and Buchrieser 2012)

Another F-box protein of *L. pneumophila* protein is LegU1/Lpg0171, which is also a translocated Dot/Icm type IV substrate and can be integrated into a functional SCF complex that confers E3 ubiquitin ligase activity. LegU1 specifically targets the host chaperon protein BAT3, a key regulator of the endoplasmic reticulum (ER) stress response (*Fig. 9.5*). LegU1 associates with BAT3 and mediates its polyubiquitination in vitro (Ensminger and Isberg 2010). Moreover, another translocated *L. pneumophila* protein, Lpg2160, plays a role in this complex, by binding both the SCF complex and BAT3, suggesting that this multicomplex formation leads to BAT3 ubiquitination, probably to modulate the ER response during infection (Ensminger and Isberg 2010).

Furthermore, *L. pneumophila* is able to block DALIS (*d*endritic cell *a*ggresome-*l*ike *i*nduced *s*tructure) formation in infected macrophages and dendritic cells (DC) (Ivanov and Roy 2009). DALIS formation in macrophages in response to *L. pneumophila* infection occurs downstream of TLR2 activation, but intracellular *L. pneumophila* blocks this activation in a Dot/Icm-dependent manner to maintain an LCV decorated with ubiquitinated proteins (Ivanov and Roy 2009). Interestingly, *L. pneumophila* is the only pathogen known to disrupt DALIS formation. A hypothesis is that the ability of *L. pneumophila* to disrupt DALIS might result in premature or inefficient antigen presentation (Ivanov and Roy 2009). Thus, the exploitation of ubiquitin signaling is a remarkable example of how *L. pneumophila* exploits conserved eukaryotic pathways and regulations to proliferate.

In addition to hijacking the host ubiquitination machinery, *L. pneumophila* exploits the host prenylation apparatus (Ivanov and Roy 2009; Price et al. 2010b). Prenylation (farnesylation or

geranylgeranylation) is a PTM of eukaryotic proteins that covalently links a lipid moiety at a CAAX tetrapeptide motif in the C-terminal region of proteins (Wright and Philips 2006). It renders proteins hydrophobic to target them to membranes by facilitating their anchoring to the lipid bilayer of membranes or their association with other hydrophobic proteins (Wright and Philips 2006). Interestingly, in silico analysis of the proteins predicted in the *L. pneumophila* genome identified several proteins encoding a CAAX motif (Ivanov et al. 2010; Price et al. 2010c). One of the secreted effectors of *L. pneumophila*, AnkB, of strains *L. pneumophila* AA100 and Philadelphia Lp01 contains such a CAAX motif. Interestingly, this protein can be lipidated at its CAAX motif by the host farnesylation machinery. This is thought to facilitate its anchoring to the membrane of the LCV in vivo (Ivanov et al. 2010; Price et al. 2010c) (*Fig. 9.5*). Lipidation of *L. pneumophila* effectors by the host is important for intracellular replication, as perturbation of host prenyltransferases during infection adversely affected the remodeling of the LCV (Ivanov et al. 2010). Thus, *L. pneumophila* utilizes the host prenylation machinery to facilitate targeting of effector proteins to membrane-bound organelles during intracellular infection (Ivanov et al. 2010; Price et al. 2010b; Price et al. 2010c). Most interestingly, in some *L. pneumophila* strains like strain Paris, AnkB does not contain this CAAX motif, which suggests that other CAAX motif proteins might take over this function. It was further proposed that AnkB functions as platform for the docking of polyubiquitinated proteins to the LCV to enable intravacuolar proliferation in macrophages and amoeba (Al-Quadan et al. 2011; Price et al. 2010b) and that AnkB helps *L. pneumophila* to exploit the eukaryotic proteasomal degradation pathway of K48-linked polyubiquitinated proteins to generate amino acids for its own replication (Al-Quadan et al. 2012; Price et al. 2011). This is an appealing idea, but as the AnkB homologue of a considerable number of *L. pneumophila* strains does not contain the CAAX motif, AnkB might not be the key effector of *L. pneumophila* that is generating nutrients for intracellular growth. However, AnkB is a versatile protein as it can take advantage of two distinct posttranslational mechanisms, one targeting host proteins and leading to modulation of their ubiquitination status and another one where the bacterial effector itself is modified by host proteins to help its intracellular proliferation.

One of the best characterized and most common PMTs is protein phosphorylation. Nearly all cellular processes are regulated by reversible phosphorylation; hence, many pathogens and also *L. pneumophila* interfere with the host phosphorylation machinery to target major signaling pathways to promote their own survival. *L. pneumophila* encodes three eukaryotic serine/threonine protein kinases, and one, LegK1, directly phosphorylates the NF-κB inhibitor IκBα, leading to a robust NF-κB activation, independently of the IKK (Iκβ kinase) complex (Ge et al. 2009). NF-κB is a master transcriptional regulator of the mammalian innate immune response, leading to activation of proinflammatory cytokines, chemokines, and cell survival genes (Karin and Lin 2002). The activity of LegK1 appears to be specific toward the Iκβ (inhibitor of κB) family proteins by mimicking the host IKK. However, the authors observed the phosphorylation activity in vitro, but a deletion of *legK1* in *L. pneumophila* strain Philadelphia had no notable effect on intracellular replication (Ge et al. 2009). Many studies investigated NF-κB activation by *L. pneumophila*. Activation occurs through both a TLR-dependent pathway, shortly after contact, as a robust and a persistent Dot/Icm-dependent pathway (Bartfeld et al. 2009). Even though LegK1 activates the NF-κB pathway, the redundancy of effectors in *Legionella* and the fact that a *legK1* deletion mutant does not impact the intracellular replication suggest that several additional proteins might contribute to the NF-κB response. Indeed, another Dot/Icm substrate, LnaB, has been shown to activate the NF-κB pathway. LnaB is a protein that has no sequence similarity to any known protein; however, it strongly activates NF-κB (Losick et al. 2010) (*Fig. 9.5*). LegK2, another serine-protein kinase encoded by *L. pneumophila*, does not seem to act in the NF-κB pathway, but it plays a key role in virulence during amoeba infection, and it exhibits protein kinase activity in vitro. It was shown that LegK2 is able to phosphorylate the general eukaryotic protein kinase substrate myelin basic protein (MBP), but its cellular target is still unknown (Hervet et al. 2011). Thus, *L. pneumophila* is exploiting the host phosphorylation system to perturb key signaling pathways, like the NF-κB pathway, in order to modify and then to block the cellular response against the bacteria (Haenssler and Isberg 2011).

The glycosyltransferases Lgt1, Lgt2, and Lgt3 of *L. pneumophila* are Dot/Icm-secreted substrates and are able to glycosylate host proteins (Belyi et al. 2011). Glycosyltransferases catalyze the transfer of a sugar residue from an activated sugar donor to various acceptor molecules, which may be proteins, lipids, saccharides, or metabolites. These protein modifications are emerging as regulators of central processes like cell signaling or gene transcription (Lairson et al. 2008). Lgt1 is a glucosyltransferase with sugar-specific enzymatic activity (only UDP-glucose). Its specific cellular target is the host elongation factor 1A (eEF1A) that Lgt1 modifies by mono-O-glucosylation at Ser53 of eEF1A (Belyi et al. 2003; Belyi et al. 2006). As eEF1A represents one of the key players in ribosome-dependent synthesis due to its GTPase activity is necessary for recruitment of aminoacylated tRNA to the A-site of ribosomes charged with translated mRNA, Lgt1 blocks protein synthesis and causes death of target cells (Belyi et al. 2006). The two other glycosyltransferases encoded by *L. pneumophila*, Lgt2 and Lgt3, also target eEF1A at Ser53 and kill infected cells (Aktories 2011; Belyi et al. 2008) (*Fig. 9.5*). By comparison of uncharged tRNA with two distinct aminoacyl-tRNAs (His-tRNA(His) and Phe-tRNA(Phe)), it could be shown that aminoacylation is crucial for Lgt-catalyzed glucosylation. Aminoacyl-tRNA had no effect on the enzymatic properties of the Lgt proteins and did not enhance the glucosylation rate of eEF1A truncation mutants, consisting of the GTPase domain only or of a 5-kDa peptide covering Ser-53 of eEF1A. Furthermore, binding of aminoacyl-tRNA to eEF1A was not altered by glucosylation. The authors thus suggest that the ternary complex, consisting of eEF1A, aminoacyl-tRNA, and

GTP, is the bona fide substrate for Lgt proteins (Tzivelekidis et al. 2011). Inhibition of protein synthesis of the host cell seems to be very important for intracellular replication of *L. pneumophila*, as not only Lgt1-3 target protein synthesis but also two other effectors of the Dot/Icm secretion system, SidI and SidL. SidI exhibits toxicity for eukaryotic cells, but it has no glycosyltransferase activity. Direct binding in vitro and in vivo between SidI and eEF1A and eEF1Bγ and a role in protein synthesis inhibition have been clearly determined, but no specific enzymatic activity could be defined (Shen et al. 2009). SidL is toxic for mammalian cells and able to inhibit protein translation in vitro via an unknown mechanism, and the concerted action of all five effectors targeting protein synthesis (Lgt1, Lgt2, Lgt3, SidI, SidL) is critical for the induction of the Dot/Icm-dependent transcriptional response of host cells (Fontana et al. 2011; Massis and Zamboni 2011). During infection, these five effectors induce a global decrease of host translation, thereby preventing synthesis of the transcription factor NF-κB inhibitor IκB. An additional putative glycosyltransferase produced by *L. pneumophila*, SetA (subversion of eukaryotic vesicle trafficking A), has been identified by a large-scale screening of candidate Dot/Icm effectors that modulate host vesicle trafficking pathways (Heidtman et al. 2009). SetA possesses a functional glycosyltransferase domain containing the conserved DxD motif that essential for the activity of bacterial glycosylating enzymes (Heidtman et al. 2009). SetA has a multidomain protein with an N-terminal glucosyltransferase domain and a C-terminal phosphatidylinositol 3-phosphate binding domain. Thus, the catalytic activity is located at the N-terminus of SetA, and the C-terminus (amino acids 401–644) is essential for guidance of SetA to vesicular compartments of host cells. Both the localization and the glucosyltransferase domains of SetA are crucial for cellular functions (Jank et al. 2012).

Phosphoinositide (PI) lipids are phosphorylated products of phosphatidylinositol (PtdIns) that play a key role in the regulation of eukaryotic signal transduction, cytoskeleton architecture, membrane dynamics, and vesicle trafficking pathways (Di Paolo and De Camilli 2006). Accordingly, many bacterial pathogens subvert PI metabolism to promote cell infection and intracellular replication (Weber et al. 2009b). *L. pneumophila* replicates more efficiently in absence of PI 3-kinases (PI3Ks) (Weber et al. 2006) or PI 5-phosphatases (Dd5P4/OCRL) (Weber et al. 2009a). LCVs are decorated with PtdIns (Abu Kwaik et al. 1993) *P*, and a number of *L. pneumophila* effector proteins bind non-covalently to PIs (Hilbi et al. 2011). The Dot/Icm substrate SidC and its paralog SdcA selectively bind to PtdIns(4)*P* via a novel "P4C" domain (Ragaz et al. 2008; Weber et al. 2006), and LidA preferentially binds monophosphorylated PIs (Brombacher et al. 2009). Moreover, a screen for *L. pneumophila* PI-binding proteins using different PIs coupled to agarose beads revealed that the Dot/Icm-translocated effector DrrA/SidM specifically binds to PtdIns(4)*P* via the "P4M" domain and competes with SidC for PI binding (Brombacher et al. 2009). The P4C and P4M PtdIns(4)*P* binding domains are neither related to one another nor to eukaryotic PI-binding folds. Together, these results indicate that *L. pneumophila* modulates host cell PI metabolism in a Dot/Icm-dependent manner and exploits monophosphorylated PIs to anchor Dot/Icm-translocated effector proteins to the LCV membrane (Hilbi et al. 2011).

AMPylation or adenylylation consists of adding covalently an adenosine monophosphate (AMP) moiety to a threonine, tyrosine residues, or possibly, serine residue of a protein by using ATP (Ribet and Cossart 2011). Via the Dot/Icm-secreted effector DrrA/SidM, *L. pneumophila* modulates AMPylation of Rab1b, a small GTPase involved in intracellular vesicular transport (Müller et al. 2010). Interestingly, the catalytic domain of DrrA/SidM is distinct from the Fic domains observed in other bacterial proteins able to AMPylate host proteins (Roy and Mukherjee 2009). DrrA/SidM is a protein with three functional domains that have been defined biochemically and resolved structurally (Müller et al. 2010; Schoebel et al. 2009; Suh et al. 2010; Zhu et al. 2010). In the carboxy-terminal region, it carries a phosphatidylinositol-4-phosphate (PI-4P) binding domain (P4M), responsible for anchoring to the LCV shortly after Dot/Icm translocation (Brombacher et al. 2009) that targets DrrA/SidM to the plasma membrane (Murata et al. 2006). Then DrrA/SidM recruits the vesicular trafficking regulator Rab1 through its guanine nucleotide exchange factor (GEF) domain that is localized in the central region of the protein (*Fig. 9.5*). Rab1 belongs to the Rab proteins, involved in organizing membranes for the formation of vesicular carriers. They interconvert between an active, GTP-bound and an inactive GDP-bound form. GTP activation occurs via enzymes known as GEFs that exchange the bound GDP nucleotide for a GTP, whereas GTPase-activating proteins (GAP) help Rabs to hydrolyze Rab-bound GTP to produce GDP. Moreover, inactive Rabs are maintained in the cytosol by binding to GDP-dissociation inhibitor (GDI). Hence, specialized proteins known as GDI-displacement factors (GDFs) are required to displace GDI from Rabs before Rab activation by GEFs (Sprang 1997). DrrA/SidM functions as both a GDF and a GEF (Ingmundson et al. 2007; Machner and Isberg 2007). It releases Rab1 from GDI, loads it with GTP, and due to its own LCV localization, recruits it to this organelle (*Fig. 9.5*). However, DrrA/SidM is a catalytically highly efficient GEF, and this activity seems sufficient for effective GDI displacement from the Rab1/GDP/GDI complex (Schoebel et al. 2009; Suh et al. 2010; Zhu et al. 2010). Thus, when Rab1/GTP is localized on the LCV, it is AMPylated by the N-terminal domain of DrrA/SidM. This domain has adenylylation activity (AT) toward Rab1, resulting in posttranslational modification of the GTPase on Tyr77 with an adenosine monophosphate (AMP) moiety (Müller et al. 2010) (*Fig. 9.5*). Interestingly, Rab1 AMPylation blocks its binding to another *L. pneumophila* effector, LepB, which possesses specific GAP activity toward Rab1/GTP (Ingmundson et al. 2007) and loosens this activity toward Rab1/GTP/AMP. Whereas DrrA/SidM and Rab1 appear on the LCV already shortly after infection, the recruitment of LepB is delayed (Ingmundson et al. 2007). During the progression of infection, DrrA/SidM and Rab1 are removed from the LCV, whereas LepB increases. Thus, DrrA mediates the recruitment and activation

of Rab1 on the plasma membrane-derived vacuole that harbors *Legionella*. However, the molecular details of how ER-derived vesicles fuse with the LCV remain unknown. A possible role for Rab1 (Arasaki and Roy 2010) was reported. Furthermore, membrane fusion between the LCV and ER-derived vesicles involves interactions between the v-SNARE Sec22b on the ER-derived vesicles and a plasma membrane t-SNARE complex containing host syntaxins (Arasaki and Roy 2010). Recently, it was shown that the DrrA protein promotes also the tethering of ER-derived vesicles with the plasma membrane-derived organelle, which leads to membrane fusion through Sec22b interactions with PM-localized syntaxins (Arasaki et al. 2012).

AMPylation is a reversible process and may be modulated by proteins exhibiting deAMPylation activity. Indeed, *L. pneumophila* is able to reverse AMPylation. It was the first bacterial pathogen that was reported to mediate deAMPylation of a host protein (Neunuebel et al. 2011; Tan and Luo 2011). During infection, Rab1 can be removed from the LCV only when it is in a GDP-bound form. The *L. pneumophila* LepB protein was shown to be a Rab1 GTPase-activating protein (GAP) capable of inactivating Rab1 (Ingmundson et al. 2007). However, LepB can catalyze its GDP binding only on deAMPylated Rab1. This deAMPylation activity is mediated by another effector of the Dot/Icm secretion system, by SidD (Neunuebel et al. 2011; Tan and Luo 2011). SidD mediates the removal of the AMP moiety from modified Rab1 and allows in this way, via the LepB-GAP activity, the displacement of Rab1 from the LCV (*Fig. 9.5*). Importantly, SidD is not present in all *L. pneumophila* strains. This effector is missing in strain Paris, suggesting that another, not yet identified, secreted effector catalyzes Rab1/GTP deAMPylation. Rigden and colleagues showed that the deAMPylase SidD contains a metal-dependent protein phosphatase (PPM) fold catalytic domain (Rigden 2011).

As mentioned above, AMPylation activity is known to be mediated by proteins containing FIC domains from the Fido domain superfamily (Kinch et al. 2009). Interestingly, *L. pneumophila* contains a FIC domain protein, AnkX, but it has no AMPylation activity. Instead it prevents microtubule-dependent vesicular transport to disrupt LCV fusion with late endosomes (Pan et al. 2008) and mediates a novel PTM on Rab1, phosphocholination (Mukherjee et al. 2011). Using mass spectrometry, Mukherjee and colleagues identified a covalent addition of a phosphocholine group on Rab1 during *L. pneumophila* infection (Mukherjee et al. 2011). The serine preceding the tyrosine that is the site of AMP addition was phosphocholinated. Golgi disruption mediated by AnkX is dependent on its phosphocholine transferase activity (Mukherjee et al. 2011). Similar to the capacity to reverse AMPylation, *L. pneumophila* has also evolved the capacity to reverse phosphocholination. This activity is mediated by the Dot/Icm effector Lem3 that regulates AnkX activity (Tan and Luo 2011). Lem3 (*lpg0696*) possesses a biochemical activity allowing it to remove the phosphocholine moiety from Rab1 (*Fig. 9.5*). Importantly phosphocholination interferes with the GTPase activity of Rab1, through blocking GTP loading and LepB-induced GTPase activity, suggesting that this enzymatic activity may account for the inhibition of the eukaryotic secretory pathway by AnkX (Tan and Luo 2011).

Interestingly, many genes coding for Dot/Icm substrates form clusters: *ankX* and *lem3* are closely linked genes, as well as *lubX* and *sidH* (Kubori and Nagai 2011) and *sidM/DrrA* and *sidD* (Neunuebel et al. 2011; Tan and Luo 2011). This observation emphasizes the spatiotemporal regulation of Dot/Icm effectors, each of which is playing a subtle role during infection, and all together modulate multiple cellular pathways at the same time to orchestrate *Legionella* survival.

Type II Protein Secretion

Type II protein secretion (T2S) systems are common, although not universal, among the various types of Gram-negative bacteria (Cianciotto 2005; Evans et al. 2008). T2S is a multistage process (Filloux 2004; Johnson et al. 2006). The proteins that are to be secreted are first translocated across the inner membrane. In the majority of cases, unfolded protein substrates transit across the inner membrane via the Sec pathway. However, sometimes, folded substrates are transported across the inner membrane by the twin-arginine translocation (Tat) (Lee et al. 2006; Ochsner et al. 2002; Sargent et al. 2006). Upon delivery into the periplasm, the unfolded substrates assume their tertiary conformation and in some instances oligomerize. In the final step of secretion, protein substrates are translocated across the outer membrane by a complex of proteins that is specifically dedicated to T2S, namely, the T2S apparatus. The T2S apparatus has 12 "core" proteins, that is, a cytosolic ATPase (T2S E), three inner membrane proteins that make a platform for T2S E (T2S F, L, M), major and minor pseudopilins which create a pilus-like structure that spans the periplasmic space (T2S G, H, I, J, K), an inner membrane peptidase that cleaves pseudopilins before their placement into the apparatus (T2S O), an outer membrane "secretin" that oligomerizes to create the secretion pore (T2S D), and finally a protein that appears to link inner and outer membrane components (T2S C) (Filloux 2004; Forest 2008; Johnson et al. 2006; Korotkov et al. 2011; Yanez et al. 2008). The current model for T2S is that substrates destined for secretion are somehow recognized by the apparatus and then, using energy generated at the inner membrane, the pseudopilus behaves like a piston to push the proteins through the secretin pore. The characteristic that defines a protein as a substrate for T2S is still not known but likely involves the protein's tertiary structure and initial interactions with T2S C and D (Korotkov et al. 2011).

The first indication that *L. pneumophila* has a T2S system was the discovery of the *pilD* gene, encoding the pseudopilin peptidase (T2S O) (Liles et al. 1998). Mutation of *pilD* in serogroup 1 strain 130b altered secretion, as shown by the loss of proteins in mutant culture supernatants (Liles et al. 1999). Study of serogroup 1 strain Philadelphia 1 revealed the presence of *lspFGHIJK*, which encodes the T2S F, G, H, I, J, and K proteins (Hales and Shuman 1999a). In further studies of strain 130b, genes encoding homologues of T2S D and E (*lspDE*), C (*lspC*), and L and M (*lspLM*) were reported, with mutation of *lspDE*

affirming the role of the genes in secretion (Rossier and Cianciotto 2001; Rossier et al. 2004). That *L. pneumophila* has a complete set of T2S-specific genes was confirmed when the genomes of serogroup 1 strains Alcoy, 130b, Corby, Lens, Paris, and Philadelphia 1 were sequenced (Cazalet et al. 2004; Chien et al. 2004; D'Auria et al. 2010; Glockner et al. 2008; Schroeder et al. 2010). Southern blots and PCR analysis have determined the presence of T2S genes in many more strains of *L. pneumophila* (Costa et al. 2011; Huang et al. 2006; Rossier et al. 2004). Additional analysis has confirmed that *L. pneumophila* contains genes encoding the Sec and Tat systems (De Buck et al. 2004; Geukens et al. 2006; Lammertyn and Anne 2004; Rossier and Cianciotto 2005) (see below for further discussion of Tat). T2S-specific mutants of *L. pneumophila* replicate normally in bacteriologic media at 37°C (Rossier and Cianciotto 2001; Rossier et al. 2004), indicating that T2S is not required for optimal extracellular growth at 37°C.

Initially, 12 exoenzymes were shown to be dependent upon the T2S system of strain 130b (Banerji et al. 2005; Cianciotto 2005; Rossier et al. 2004). This conclusion was based upon the loss of activities from the culture supernatants of *lsp* mutants grown in BYE broth at 37 °C (Aragon et al. 2000; Liles et al. 1999; Rossier and Cianciotto 2001; Rossier et al. 2004). The activities identified were the tartrate-sensitive and tartrate-resistant acid phosphatases; Mip-dependent and Mip-independent phospholipases C; phospholipase A; lysophospholipase A; glycerophospholipid cholesterol acyltransferase (GCAT); mono-, di-, and triacylglycerol lipases; ribonuclease; and protease (Aragon et al. 2000, 2001, 2002; Banerji et al. 2005; DebRoy et al. 2006a; Flieger et al. 2001, 2002; Hales and Shuman 1999a; Liles et al. 1999; Rossier and Cianciotto 2001; Rossier et al. 2004). Recent analysis of strain Paris has identified a starch/glycogen-degrading activity that is T2S dependent (Herrmann et al. 2011), and a similar result has been obtained for strain 130b (J. Schmitt and N. Cianciotto, unpublished results). In some cases, the structural genes (proteins) encoding the T2S-dependent secreted activities have been identified. These include *map* (Map) for the tartrate-sensitive acid phosphatase (Aragon et al. 2001), *plcA* (PlcA) for phospholipase C activity (Aragon et al. 2002; Rossier and Cianciotto 2005), *plaA* (PlaA) for the lysophospholipase A (Flieger et al. 2002), *plaC* (PlaC) for GCAT (Banerji et al. 2005), *lipA* (LipA) and *lipB* (LipB) for mono- and triacylglycerol lipases (Aragon et al. 2002), *gamA* (GamA) for the starch hydrolase (Herrmann et al. 2011), and *proA/msp* (ProA/Msp) for a metalloprotease (Hales and Shuman 1999a; Liles et al. 1999). The analysis of supernatants from *proA* mutants demonstrates that some secreted proteins are cleaved and perhaps activated by the T2S-dependent protease (Banerji et al. 2005; Flieger et al. 2002). Interestingly, one of the first secreted substrates to be described, the Map acid phosphatase, shows striking sequence similarity to eukaryotic enzymes (Aragon et al. 2001), suggesting that *L. pneumophila* has usurped the strategies of its host cells over the course of its evolution as an intracellular parasite. Many more examples of *Legionella* eukaryotic-like proteins have emerged from the study of the *L. pneumophila* type IV secretion system as well as the further study of T2S.

In order to define additional T2S-dependent proteins secreted by *L. pneumophila*, strain 130b and an *lsp* mutant were grown in broth at 37 °C, and then supernatants were compared by two-dimensional PAGE. Mass spectrometry (MS) was then utilized to identify the secreted proteins that were present for wild type but absent for the T2S mutant (DebRoy et al. 2006b). Three of the identified proteins had been previously defined as T2S substrates, that is, ProA, Map, and PlaA. A fourth (SrnA) proved to encode the previously found ribonuclease activity (Rossier et al. 2009). Others were predicted, based upon their sequence, to be "new" enzymes, and subsequent cloning and mutational analyses confirmed that CelA encodes an endoglucanase (cellulase); ChiA, a chitinase; and LapA and LapB, two distinct aminopeptidases (DebRoy et al. 2006b; Pearce and Cianciotto 2009; Rossier et al. 2008, 2009). Others showed sequence similarity to eukaryotic proteins, with one having collagen-like repeats, and the other relatedness to astacin-like proteinases (DebRoy et al. 2006b). Subsequent studies showed that the protein with collagen-like repeats (Lcl) has heparin-binding activity and may promote adherence events (Duncan et al. 2011; Vandersmissen et al. 2010). The astacin-like proteinase (LegP) was later found to be translocated by Dot/Icm type IV secretion when the legionellae were growing within macrophages (de Felipe et al. 2008), raising the possibility that some proteins may be secreted or influenced by multiple pathways, with the environmental conditions potentially dictating which secretion pathway(s) is most critical. Other type II-dependent proteins showed weak similarity to bacterial amidases and cysteine proteases, but their true activities are yet to be confirmed (DebRoy et al. 2006b). Interestingly, five others had no similarity to any known protein in the database and thus might encode novel activities (DebRoy et al. 2006b). Results similar to these obtained from using strain 130b have now been reported after proteomic analysis of Philadelphia 1 (Galka et al. 2008). The results of the proteomics combined with assessments of secreted enzymatic activities indicate that the number of proteins secreted by *L. pneumophila* T2S is at least 25. That the T2S output is >25 is supported by several arguments; for example, (1) low-level expression or degradation is very likely to have impaired detection of some secreted proteins, (2) the comparisons between wild type and mutant utilized bacterial cultures grown under a single condition, (3) mutations eliminating specific genes did not always completely abolish the corresponding enzymatic activity in supernatants, and (4) in silico analysis of *L. pneumophila* genomes reveals 60 proteins that contain a signal sequence and are predicted to be extracellular by at least one bioinformatics program (DebRoy et al. 2006b). Finally, recent studies have shown that *Legionella* T2S is required for the secretion of a surfactant that mediates bacterial translocation (i.e., sliding) over surfaces (Stewart et al. 2009, 2011). However, in this case, it appears that T2S has an indirect role, promoting the release of surfactant through a TolC-containing efflux pump (see below) (Stewart et al. 2011).

Although T2S mutants of *L. pneumophila* replicate normally at 30–37 °C, they are defective for growth in media at 25 °C, 17 °C, and 12 °C (Söderberg et al. 2004). In experiments that

mimic aquatic habitats, T2S mutants show reduced survival in tap water incubated at 25, 17, 12, and 4 °C (Söderberg et al. 2008). T2S mutants grow better at low temperatures when plated next to wild type or wild-type supernatants, suggesting that a secreted factor promotes low-temperature growth (Söderberg et al. 2004, 2008). Supporting this idea, when wild-type *L. pneumophila* is grown at 17 °C or 12 °C, new proteins appear in culture supernatants, including a Sec-dependent protein that is predicted to have PPIase activity (Söderberg and Cianciotto 2008). In another type of study, transcriptional profiling revealed that a number of genes encoding T2S-dependent exoproteins are hyperexpressed when *L. pneumophila* is grown in a biofilm at 20 °C (Hindre et al. 2008). Finally, the T2S-dependent surfactant of *L. pneumophila* is antagonistic toward other species of *Legionella* (Stewart et al. 2011). Overall, these various data implicate T2S as a key factor in the planktonic persistence of *L. pneumophila* in the environment and as such identify T2S as an important factor in disease transmission.

L. pneumophila T2S mutants are highly impaired for intracellular growth in amoebae, including *Hartmannella vermiformis* and *Acanthamoeba castellanii* (Hales and Shuman 1999a; Liles et al. 1999; Polesky et al. 2001; Rossier and Cianciotto 2001; Rossier et al. 2004). Indeed, T2S mutants of strain 130b and Philadelphia 1 show very little, if any, evidence of growth in amoebae. The reduced infectivity of the T2S mutants is complemented (i.e., reversed) when an intact copy of the T2S gene is introduced, confirming that T2S is required for infection. Although the initial assessments of amoebal infection were done at 35–37 °C, more recent studies have shown that the T2S mutant defect is also manifest when amoebae are cultured at 22–25 °C (Söderberg et al. 2008). Additional assays have determined that the T2S mutants are not impaired for entry into the amoebal hosts (Söderberg et al. 2008), indicating that T2S is promoting bacterial resistance to intracellular killing and/or facilitating bacterial replication itself. Among T2S effectors, the ProA protease and SrnA ribonuclease are necessary for optimal infection of *H. vermiformis* (Rossier et al. 2008, 2009). This implies that the infection defects of T2S mutants are due to the loss of secreted effectors vs. being simply due to changes in the bacterial cell envelope. Double mutants lacking both ProA and SrnA show a defect that is greater than the corresponding single mutants, implying that the role of T2S in amoebal infection is due to the combined effect of multiple secreted proteins (Rossier et al. 2009). The protease and ribonuclease may be facilitating growth by helping legionellae to generate amino acids, nucleotides, or phosphate for nutrient acquisition. Alternatively, ProA and SrnA might be degrading amoebal proteins and RNA that can influence *Legionella* growth. Interestingly, ProA exhibits differential importance among the amoebae tested, being important for infection of hartmannellae but not acanthamoebae (Rossier et al. 2008). These data were the first to demonstrate that some secreted factors have evolved to target certain protozoan hosts. A similar result occurs for SrnA (J. Schmitt & N. Cianciotto, unpublished results) as well as some type IV-secreted proteins (O'Connor et al. 2011). Given the key role of protozoa in *L. pneumophila* survival in water, these data further establish T2S as a major factor in *Legionella* persistence in the environment. Because infected amoebae might be part of the infective dose that initiates lung infection (Brieland et al. 1996; Cirillo et al. 1999), these data also signal the relevance of T2S for disease.

Importantly, T2S mutants of *L. pneumophila* are also very defective in an animal model of Legionnaires' disease (McCoy-Simandle et al. 2011; Rossier et al. 2004). Whereas the parental wild-type strain increases at least tenfold in the lungs of A/J mice, a T2S mutant exhibits no increase in number and is cleared much more rapidly. An examination of sera obtained from animals infected with the wild-type strain further revealed that T2S-dependent proteins are expressed in vivo (Rossier et al. 2004). Thus, T2S is an important contributor to *L. pneumophila* virulence. Among all of the effectors tested thus far, the chitinase stands out as being necessary for bacterial survival in the lungs (DebRoy et al. 2006b; Rossier et al. 2008, 2009). ChiA mutants are impaired fourfold when tested in the mouse model, and immunoblot analysis showed that ChiA is one of the T2S-dependent proteins that is expressed in vivo (DebRoy et al. 2006b). Since the *chiA* mutant grows normally in macrophages in vitro and since its reduced survival in the lung was only manifest in the later stages of infection, ChiA likely promotes persistence vs. initial replication. Since mammals do not have chitin, these data lead to the hypothesis that there is a chitin-like factor in the lung whose degradation aids bacterial persistence. Alternately, ChiA could be a bifunctional enzyme that has another substrate. That a protein having chitinase activity can promote the survival of a pathogen in a mammalian host had not been previously seen. Thus, factors that are traditionally viewed as only being important in the environment may actually have direct relevance to disease. Although *proA* mutants have not been shown to clearly exhibit reduced growth or survival in the lungs of experimental animals, ProA is believed to contribute to disease by promoting the destruction of lung tissue (Baskerville et al. 1986; Blander et al. 1990; Conlan et al. 1986, 1988; DebRoy et al. 2006b; Moffat et al. 1994; Williams et al. 1987). It can also degrade transferrin and therefore may contribute to iron acquisition (James et al. 1997).

Finally, *L. pneumophila* T2S mutants are impaired for intracellular infection of macrophages and lung epithelial cells (Liles et al. 1999; McCoy-Simandle et al. 2011; Polesky et al. 2001; Rossier et al. 2004). Thus, T2S also promotes infection by facilitating bacterial growth in resident lung cells. Besides the *chiA* mutant, *L. pneumophila map*, *plcA*, *plaA*, *plaC*, *lipA*, *lipB*, *proA/msp*, *lapA*, *lapB*, *srnA*, *celA*, and *gamA* mutants that lack particular T2S effectors have been tested for alterations in infection of macrophages. However, all grow normally, indicating that the proteins encoded by these genes are not required for macrophage infection (Aragon et al. 2001; Aragon et al. 2002; Banerji et al. 2005; DebRoy et al. 2006b; Flieger et al. 2002; Herrmann et al. 2011; Pearce and Cianciotto 2009; Rossier et al. 2008, 2009). These data indicate that the T2S system secretes a yet-to-be-defined factor that is necessary for macrophage infection. On the other hand, there might be redundancy

in the effectors such that one secreted factor can compensate for the loss of another. Because T2S mutant numbers do not increase in the lungs, whereas they do, although not optimally, in macrophages and epithelial cells in vitro, it was hypothesized that *L. pneumophila* T2S also promotes processes that are relevant to disease. Following infection of macrophages, T2S mutants (but not a complemented mutant) elicit significantly higher levels of cytokines and chemokines (McCoy-Simandle et al. 2011). A similar result was obtained with infected lung epithelial cell lines and the lungs of infected A/J mice. Infection with a mutant specifically lacking the T2S-dependent ProA protease (but not a complemented *proA* mutant) results in a partial elevation of cytokine levels (McCoy-Simandle et al. 2011). These data indicate that the T2S system of *L. pneumophila* dampens the cytokine/chemokine output of infected host cells. Based on quantitative RT-PCR analysis of infected host cells, a T2S mutant, but not the *proA* mutant, produced significantly higher levels of cytokine transcripts, implying that some T2S-dependent effector dampens signal transduction and transcription, whereas others, such as ProA, act at a posttranscriptional step in cytokine expression (McCoy-Simandle et al. 2011).

In summary, the role of T2S in Legionnaires' disease is a combination of at least seven factors, that is, (1) extracellular survival in environmental water samples (which is the source of infection), (2) growth in amoebae (which is the main replicative niche for *L. pneumophila* in water and which may be part of the infective particle), (3) intracellular infection of lung macrophages (which are the primary host cell in the lung), (4) intracellular infection of lung epithelial cells (which are an alternative host cell in vivo), (5) dampening of the chemokine and cytokine output of infected macrophages and epithelial cells (which is predicted to dampen the inflammatory cell infiltrate into the lung, allowing for prolonged bacterial growth), (6) the elaboration of ChiA (which appears to promote intrapulmonary persistence independent of macrophage infection), and (7) the secretion of ProA (which degrades both host cytokines and lung tissue). Further research will undoubtedly reveal even more roles for this multifactorial secretion system.

Type I Protein Secretion

All sequenced strains of *L. pneumophila* encode the outer membrane protein TolC and putative inner membrane translocase proteins that would together constitute a type I secretion system (Ferhat et al. 2009; Jacobi and Heuner 2003; Newton et al. 2010; Stewart et al. 2011). Well studied in many other bacteria, TolC is best known as the outer membrane component of multidrug efflux pumps (Blair and Piddock 2009; Koronakis et al. 2004; Nikaido and Takatsuka 2009; Zgurskaya 2009). Indeed, *L. pneumophila tolC* mutants exhibit increased sensitivity to various drugs (Ferhat et al. 2009; Stewart et al. 2011). More interestingly, in *L. pneumophila*, TolC mediates the secretion of a surfactant that allows the bacterium to move across surfaces, that is, sliding motility (Stewart et al. 2011). The surfactant also has antimicrobial activity against other *Legionella* species. Structurally, the surfactant appears to be a lipoprotein or lipopeptide, providing the first experimental indication that *L. pneumophila* encodes a functional type I secretion system (Stewart et al. 2011). Equally important, *L. pneumophila tolC* mutants are impaired for in vitro intracellular infection, suggesting that TolC and one or more of its substrates are required for *Legionella* pathogenesis (Ferhat et al. 2009).

The *rtxA* gene of *L. pneumophila* is predicted to encode a very large (approximately 7,000-aa) protein that has sequence similarity to Rtx ("repeats-in-toxin") toxins that are found in other bacterial pathogens and secreted in those cases via a type I system (Cirillo et al. 2001; D'Auria et al. 2008). A *L. pneumophila* mutant lacking *rtxA* is impaired for entry and intracellular growth within macrophages, epithelial cells, and amoebae (Cirillo et al. 2001, 2002). The mutant also shows impaired growth within the lungs of infected mice (Cirillo et al. 2001), indicating that RtxA is a virulence factor for *L. pneumophila*. The *rtxA* gene is well conserved among clinical isolates of *L. pneumophila* (Huang et al. 2006). RtxA has not been studied biochemically, and its location in *L. pneumophila* has not been determined. But it is reasonable to suspect that the protein is either secreted outside of the bacterial cell via the type I system or present in the outer membrane.

Tat-Dependent Secretion, Other Secreted Proteins, and Outer Membrane Vesicles

In Gram-negative bacteria, the twin-arginine translocation (Tat) pathway mediates translocation of proteins across the inner membrane to the periplasm (De Buck et al. 2008b). The majority of Tat substrates remain cell associated, residing in the periplasm or inner membrane (De Buck et al. 2004, 2007; Rossier and Cianciotto 2005). However, as noted above, some Tat substrates are further secreted out of the cell via the T2S system. In the case of *L. pneumophila*, a T2S effector that has been shown to be Tat dependent is PlcA phospholipase C (Rossier and Cianciotto 2005). Bioinformatics and subsequent proteomic analysis has determined that additional proteins are secreted into culture supernatants in a Tat-dependent manner, including a 3′, 5′-cyclic nucleotide phosphodiesterase (De Buck et al. 2008a; Rossier and Cianciotto 2005). More work is needed in order to determine whether these other proteins are delivered into the extracellular space via T2S or an alternative secretion system. Phenotypic analysis of *tat* mutants has determined that an intact Tat pathway is required for a variety of other aspects of *L. pneumophila*, including virulence-associated traits (De Buck et al. 2005; Rossier and Cianciotto 2005). When tested for infection of macrophages, *tat* mutants show an approximate 15-fold reduction in growth. Double mutants lacking Tat and T2S are even more defective, indicating that Tat has an intracellular role that is independent of T2S. The mutants are also impaired for cytochrome *c* oxidase, growth in amoebae cultured in presence of an iron chelator, extracellular growth on low-iron bacteriologic

media, and biofilm formation in plastic microtiter plates. All these mutant phenotypes are reversed by reintroduction of intact *tat*. Thus, the Tat pathway of *L. pneumophila* has a role in secretion of exoenzymes, formation of a respiratory complex, growth in low-iron conditions, and intracellular infection.

There are several *L. pneumophila* proteins that are secreted into culture supernatants by an unknown mechanism. Lpg1905 is an ecto-nucleoside triphosphate diphosphohydrolase with ATPase and ADPase activities (Sansom et al. 2007; Vivian et al. 2010). The release of Lpg1905 in supernatants was shown to not be dependent upon a type II or Dot/Icm type IV secretion system. Importantly, this protein is required for intracellular infection of macrophages and alveolar epithelial cells (Sansom et al. 2007; Vivian et al. 2010). PlaD is a secreted phospholipase A that does not contain a signal sequence typical of a T2S substrate (Banerji et al. 2008). Taken together, these data further suggest that more than two protein secretion systems are operative in *L. pneumophila*.

In addition to secreting various sorts of proteins, *L. pneumophila* releases outer membrane vesicles (OMVs) when it grows within liquid culture (Fernandez-Moreira et al. 2006; Flesher et al. 1979; Galka et al. 2008). *L. pneumophila* shares this trait with many other types of Gram-negative bacteria (Ellis and Kuehn 2010). OMVs of *L. pneumophila* contain LPS and as many as 74 different proteins, including 33 proteins that do not appear to be also secreted via type II or Dot/Icm type IV secretion (Fernandez-Moreira et al. 2006; Galka et al. 2008). The OMVs can also associate with the plasma membrane of human epithelial cell (Galka et al. 2008), suggesting that they may represent an alternate means of delivering toxins and other effectors into host cells. It has also been posited that OMVs produced by intracellular legionellae can fuse with the phagosomal membrane and alter trafficking events (Fernandez-Moreira et al. 2006).

Non-protein Secretion: Siderophore, Pigment, and Quorum-Sensing Molecules

A critical non-protein molecule that is secreted by *L. pneumophila* is the siderophore legiobactin. The ability of *L. pneumophila* to replicate in the mammalian host is highly dependent on iron (Cianciotto 2007). For example, iron supplementation increases the susceptibility of animals and macrophages to infection, and legionellae grown under iron-limiting conditions exhibit reduced virulence (Gebran et al. 1994; James et al. 1995). Furthermore, human macrophages treated with iron chelators do not support *Legionella* growth, and some host cytokines inhibit intracellular growth by limiting iron (Byrd and Horwitz 2000; Viswanathan et al. 2000). The first genetic data on the role of iron in *Legionella* was the identification of the gene for the transcriptional repressor Fur (Hickey and Cianciotto 1994). The importance of *L. pneumophila* iron acquisition became evident from the identification of iron- and Fur-regulated genes that are required for optimal intracellular infection (Hickey and Cianciotto 1997). The principal means of *L. pneumophila* Fe^{3+} assimilation is now known to be secreted legiobactin. When *L. pneumophila* is grown in a low-iron, chemically defined medium, it secretes this low-molecular-weight siderophore that is most readily detected by the chrome azurol S assay (Allard et al. 2006; Liles et al. 2000). Legiobactin is also recognized by its ability to stimulate the growth of iron-starved legionellae (Allard et al. 2006, 2009). Legiobactin contains 13 aliphatic carbons (three carbonyls) and no aromatic carbons, and spectra further indicate that it is a polycarboxylate (Allard et al. 2009). Two linked genes, *lbtA* and *lbtB*, are required for the expression of legiobactin; that is, supernatants from mutants inactivated for *lbtA* or *lbtB* lack CAS reactivity and show an inability to stimulate the growth of iron-starved legionellae (Allard et al. 2006, 2009). Cytoplasmic LbtA is homologous to siderophore synthetases and is undoubtedly involved in the synthesis of legiobactin. LbtB, a member of the major facilitator superfamily (MFS) of transporters, is akin to inner membrane siderophore exporters and most likely promotes the transport of legiobactin across the inner membrane prior to its final export. A third gene that has been implicated in the secretion or maturation of legiobactin is *cyc4*, which encodes a periplasmic *c*-type cytochrome (Yip et al. 2011). Importantly, *lbtA* mutants, but not their complemented derivatives, are defective for infection of the murine lung, demonstrating a role for legiobactin in *L. pneumophila* virulence (Allard et al. 2009). Because legiobactin mutants are not impaired for intracellular infection in vitro, the pathogenic role of legiobactin may derive from extra-macrophage events. Given the in vivo relevance of legiobactin, recent studies have characterized its outer membrane receptor, LbtU (Chatfield et al. 2011). Interestingly, LbtU has a 16-stranded transmembrane β-barrel, multiple extracellular domains, and short periplasmic tails. The sequence and structure of LbtU are distinct from known siderophore receptors, which generally have a 22-stranded β-barrel and an N-terminus that binds TonB in order to transduce energy from the inner membrane. This observation coupled with the fact that *L. pneumophila* does not encode TonB suggests that *L. pneumophila* uses a mode of siderophore uptake that is mechanistically distinct from existing paradigms (Chatfield et al. 2011). In further support of this hypothesis, it has recently been shown that the inner membrane protein (LbtC) required for utilization of legiobactin is unlike known permeases in the MFS. In addition to its ferric iron-legiobactin pathway, *L. pneumophila* can utilize ferrous iron through the action of an inner membrane Fe^{2+} transporter known as FeoB. Mutants lacking *feoB* are impaired for both intracellular infection and intrapulmonary growth (Robey and Cianciotto 2002), demonstrating the importance of ferrous iron transport.

For a long time, it has been known that *L. pneumophila* secretes a brown pigment when it is grown in bacteriologic media (Baine and Rasheed 1979; Baine et al. 1978; Feeley et al. 1978; Orrison et al. 1981; Pine et al. 1979; Ristroph et al. 1981; Vickers and Yu 1984; Warren and Miller 1979). Early reports showed that the production of the pigment is dependent upon L-tyrosine in the growth medium and most apparent in bacteria experiencing slowed growth

(Baine and Rasheed 1979; Baine et al. 1978; Berg et al. 1985). It was later established that the pigment results from the spontaneous and oxidative polymerization of homogentisic acid (HGA) which is secreted into the supernatant (Steinert et al. 2001). HGA, in turn, is made through the action of Lly, a *p*-hydroxyphenylpyruvate dioxygenase (Gillespie et al. 2002; Steinert et al. 2001; Wintermeyer et al. 1991, 1994). Thus, the pigment is a pyomelanin or HGA-melanin, a type of molecule that is produced by a variety of other environmental bacteria as well as some pathogens (Plonka and Grabacka 2006; Turick et al. 2002; Weiner 1997). Based upon the behavior of *lly* mutants, it is believed that the pigment is not a required for intracellular infection (Steinert et al. 1995; Wintermeyer et al. 1994). However, a recent study has determined that the pyomelanin has intrinsic ferric reductase activity, converting Fe^{3+} to Fe^{2+} (Chatfield and Cianciotto 2007). Compatible with the nature of HGA-melanin, the secreted ferric reductase activity was positively influenced by the amount of tyrosine in growth media, resistant to protease, acid precipitable, and heterogeneous in size. Thus, *L. pneumophila* secretes a ferric reductase activity that likely facilitates the acquisition of ferrous iron. Since the virulence of *L. pneumophila* is dependent upon its capacity to acquire ferrous iron, it is possible that secreted pyomelanin has a nutritional role within the infected lung. The mechanism of pyomelanin secretion is unknown.

L. pneumophila secretes a quorum-sensing molecule known as LAI-1 (*Legionella* autoinducer-1) (Spirig et al. 2008). LAI-1 is further identified as 3-hydroxy-pentadecan-4-one. Signaling through this quorum-sensing system promotes a number of processes pertinent to intracellular infection and virulence, including phagocytosis, formation of the replicative phagosome, and intracellular replication (Tiaden et al. 2007). This is explained at least in part by the fact that the LAI-1 system modulates the expression of Dot/Icm type IV effectors (Tiaden et al. 2008). However, *Legionella* 3-hydroxy-pentadecan-4-one influences a variety of other processes including motility, the formation of extracellular filaments, and the regulation of a wide array of genes that goes well beyond type IV secretion (Tiaden et al. 2010a, b). The production of LAI-1 is controlled by RpoS and LetA (Newton et al. 2010; Tiaden et al. 2007, 2010a); however, the mechanism of LAI-1 secretion has not been defined.

Other Surface Structures, Outer Membrane Proteins, and LPS

In addition to its secretion systems and vast array of secreted factors, *L. pneumophila* expresses several surface structures that are important in infection. The first prominent surface feature of *L. pneumophila* is its flagella (Chandler et al. 1980; Rodgers et al. 1980). The gene encoding the flagellin subunit has been cloned, sequenced, and found to be regulated by temperature, growth phase, amino acids, viscosity, and osmolarity (Albert-Weissenberger et al. 2010; Hammer and Swanson 1999; Heuner et al. 1995, 1999; Ott et al. 1991). Although *Legionella* flagella are not required for intracellular replication per se, they promote bacterial entry into host cells, presumably by hastening the approach of legionellae to the host cell (Dietrich et al. 2001; Merriam et al. 1997; Pruckler et al. 1995). *L. pneumophila* flagellin that is released into the cytosol of infected macrophages is recognized by Naip5/Birc1e which helps trigger the innate immune response (Molofsky et al. 2006; Ren et al. 2006; Whitfield et al. 2010). A second prominent surface feature of *L. pneumophila* is its pili. The bacterium has at least two types of pili, including temperature-regulated, bundle-forming type IV pili that mediate twitching motility (Chandler et al. 1980; Coil and Anne 2009, 2010; Liles et al. 1998; Rodgers et al. 1979, 1980; Stone and Abu Kwaik 1998). Although type IV pili modestly facilitate bacterial attachment to host cells in vitro (Stone and Abu Kwaik 1998), they appear not to be required for lung infection in the A/J mouse model (Rossier et al. 2004).

Besides its multiprotein structures, the surface of *L. pneumophila* contains a variety of outer membrane proteins that play different roles in disease. The first to mention is the genus-wide, peptidoglycan-linked, outer membrane porin (Gabay et al. 1985; Hoffman et al. 1992a, b). This protein, which is also known as the major outer membrane protein (MOMP), binds complement components C3 and C1q and thus can mediate phagocytosis by macrophages (Bellinger-Kawahara and Horwitz 1990; Mintz et al. 1995). A cloned copy of a MOMP gene can confer increased adherence upon *E. coli* (High et al. 1993; Krinos et al. 1999). Since MOMP mutants cannot be made, the importance of this surface protein in lung infection is presumed but not proven. The second surface protein that has received attention is a 19-kDa outer membrane lipoprotein. The gene encoding this genus-wide antigen, known as Pal, has been cloned and sequenced (Engleberg et al. 1991; Hindahl and Iglewski 1987; Kim et al. 2003; Ludwig et al. 1991). Although the inability to mutate the gene has precluded an assessment of the importance of the protein in an infection model, Pal does trigger cytokine production by binding to TLR2 on macrophages (Shim et al. 2009). A third surface-exposed protein is the 60-kDa heat shock protein known as Hsp60 or HtpB (Garduno et al. 1998a; Hindahl and Iglewski 1987; Hoffman et al. 1990; Sampson et al. 1990). Upon intracellular infection, HtpB appears, based upon studies done using HeLa cells, to promote epithelial cell invasion (Abu Kwaik et al. 1993; Fernandez et al. 1996; Garduno et al. 1998b). When introduced into a host cytoplasm using protein-coated beads or ectopic expression, HtpB alters mitochondrial trafficking and microfilament organization (Chong et al. 2009). Within host cells, HtpB may also function to ensure a supply of polyamines, which are required for intracellular growth of *L. pneumophila* (Nasrallah et al. 2011). Finally, purified HtpB elicits proinflammatory cytokine (IL-1) expression by macrophages (Retzlaff et al. 1994, 1996). A fourth protein associated with the outer membrane is the PlaB (lyso) phospholipase A (Bender et al. 2009; Lang and Flieger 2011). PlaB has contact-dependent hemolytic activity and is important in a guinea pig model of disease (Schunder et al. 2010).

Arguably, the most studied surface protein is the now-crystallized 24-kDa Mip, a genus-wide protein that exists as

a homodimer and possesses peptidyl-proline isomerase (PPIase) activity (Cianciotto et al. 1990a; Engleberg et al. 1989; Fischer et al. 1992; Helbig et al. 1995b, 2001a; Ludwig et al. 1994; Riboldi-Tunnicliffe et al. 2001; Riffard et al. 1996; Schmidt et al. 1994). The protein is notable for sharing sequence similarity with eukaryotic proteins belonging to the FK506-binding protein family (Cianciotto and Fields 1992; Fischer et al. 1992). The first virulence factor to be defined in *Legionella*, Mip, is required for the early (postentry) stages of intracellular infection of macrophages, protozoa, and lung epithelia and for virulence following intratracheal inoculation of guinea pigs (Cianciotto et al. 1989b, 1990b, 1995; Cianciotto and Fields 1992; Helbig et al. 2001a, 2003a; Kohler et al. 2000; Susa et al. 1996; Wieland et al. 2002; Wintermeyer et al. 1995). The target of Mip action within the infected host cell is still unknown; however, it has recently been reported that Mip is required for efficient secretion of a novel T2S-dependent phospholipase C-like enzyme (DebRoy et al. 2006a), suggesting that Mip may facilitate expression of multiple other infectivity determinants. More recent studies indicate that Mip promotes pathogenesis by binding collagen IV and enabling *L. pneumophila* to transmigrate through a lung epithelial barrier (Unal et al. 2011; Wagner et al. 2007).

Because *L. pneumophila* is Gram-negative, another one of its critical surface molecules is lipopolysaccharide. *L. pneumophila* LPS is the serogroup-specific O antigen (Ciesielski et al. 1986; Conlan and Ashworth 1986; Knirel et al. 1997; Nolte et al. 1986; Otten et al. 1986; Zahringer et al. 1995). A particular LPS epitope of serogroup 1 that is recognized by the typing monoclonal antibodies MAb 2 and MAb3/1 is more frequently expressed on clinical vs. environmental isolates (Dournon et al. 1988; Helbig et al. 1995a). However, the loss of the epitope itself does not diminish the ability of *L. pneumophila* to infect macrophages (Mintz and Zou 1992; Zou et al. 1999), indicating that the increased prevalence of MAb2-positive strains is due to other factors, including physiochemical surface properties (Gosselin et al. 2011). On the other hand, other sorts of antigenic changes in *Legionella* LPS expression do result in reductions in serum resistance, intracellular growth, and virulence (Luneberg et al. 1998; Rogers et al. 1992). Additionally, recent studies indicate that LPS is shed during intracellular growth and delivered into the cytoplasm of the host cell where it may play a role in bacterial evasion of lysosomes (Reichardt et al. 2010; Seeger et al. 2010). The lipid A component of *L. pneumophila* LPS has relatively weak endotoxin activity, and this appears to be due to its unusual long-chain and branched fatty acids as well as its low affinity for the CD14 receptor on macrophages (Highsmith et al. 1978; Moll et al. 1992; Neumeister et al. 1998a; Schramek et al. 1982; Wong et al. 1979; Zahringer et al. 1995). A more significant role for lipid A in *L. pneumophila* pathogenesis was documented when a gene (*rcp*) involved in the palmitoylation of lipid A was inactivated and there was a simultaneous reduction in resistance to cationic antimicrobial peptides, intracellular infectivity, and virulence in an animal model of disease (Robey et al. 2001). As is the case for the LPS of other pathogens, the LPS of *Legionella* is recognized by the host innate immune system. However, unlike most LPS molecules, which are recognized by the host TLR4 receptor, the LPS of *L. pneumophila* is recognized by TLR2 (Braedel-Ruoff et al. 2005; Girard et al. 2003). *L. pneumophila* LPS is also bound by lung surfactant proteins that can suppress the growth of the bacterium (Sawada et al. 2010).

Periplasmic and Cytoplasmic Virulence Factors

Several important infectivity determinants have been localized to the *L. pneumophila* periplasm or cytoplasm. The first group of these is proteins involved in detoxification and stress responses. A copper-zinc superoxide dismutase (SOD) resides in the periplasm, affording resistance to toxic superoxide anions (by converting them to H_2O_2) and promoting survival during stationary phase (John and Steinman 1996). Mutational analysis indicates, however, that the enzyme is not required for infection of macrophages. A second SOD, which bears iron as its cofactor and exists within the *Legionella* cytoplasm, is essential for bacterial viability, and thus its role in pathogenesis cannot be assessed by the genetic approach (Sadosky et al. 1994). *L. pneumophila* has two catalase-peroxidase enzymes that convert H_2O_2 to water and oxygen. The KatA catalase-peroxidase is located in the periplasm, induced during stationary phase, and required for optimal intracellular infection of macrophages (Amemura-Maekawa et al. 1999; Bandyopadhyay et al. 2003; Bandyopadhyay and Steinman 2000). The KatB enzyme is cytoplasmic, and mutational analysis has identified a role for the *katB* gene in macrophage infection (Bandyopadhyay et al. 2003; Bandyopadhyay and Steinman 1998). Though not individually required for infection, AhpC1 and AhpC2 are expressed intracellularly and are important detoxifiers of H_2O_2 and organic peroxides (LeBlanc et al. 2006, 2008; Rankin et al. 2002). HtrA, a periplasmic chaperone-protease that facilitates the refolding or degradation of defective outer membrane proteins, is required for *L. pneumophila* infection of macrophages (Pedersen et al. 2001; Wrase et al. 2011). *L. pneumophila* has two periplasmic oxidoreductases (DsbA1, DsbA2) that catalyze the formation of disulphide bonds in extracytoplasmic proteins; important targets for DsbA2 are components of the type IV secretion system (Jameson-Lee et al. 2011). Although not required for intracellular infection, McoL, a multicopper oxidase that is associated with the inner membrane, acts to prevent toxic effects of ferrous iron during aerobic growth (Huston et al. 2008). NudA is a nucleoside diphosphate pyrophosphatase (Nudix hydrolase) that degrades toxic intracellular compounds, and based upon mutational analysis, the *nudA* gene is required for infection of macrophages and the lungs (Edelstein et al. 2005). The inner membrane protein LadC is a putative adenylate cyclase that is required for adherence to macrophages and growth in the lungs (Newton et al. 2008).

Other infectivity determinants are involved in intracellular metabolism and growth. As for respiration considerations, the cytochrome *c* maturation system is critical for both intracellular and intrapulmonary growth, with the *c*1 and *c*5 cytochromes being most important for intracellular infection

(Yip et al. 2011). Finally, as mentioned above, another important aspect of intracellular infection and virulence is iron acquisition. In addition to legiobactin, pyomelanin, and FeoB-mediated ferrous iron transport, factors that are involved in iron acquisition include periplasmic and cytoplasmic ferric reductases, a methyltransferase (*iraA*) and membrane (iron-)peptide transporter (*iraB*), the inner membrane cytochrome *c* biogenesis system (*ccm*), an LbtA-like synthetase (*frgA*), and a hemin-binding protein (*hbp*) (Cianciotto et al. 2005; Hickey and Cianciotto 1997; James et al. 1997; Johnson et al. 1991; Naylor and Cianciotto 2004; O'Connell et al. 1996b; Poch and Johnson 1993; Pope et al. 1996; Robey and Cianciotto 2002; Viswanathan et al. 2000; Viswanathan et al. 2002). Other genes encoding cytosolic proteins that have been shown to be required for optimal intracellular infection of macrophages include *bdhA-patD* (polyhydroxybutyrate metabolism) (Aurass et al. 2009), *oad* (oxaloacetate decarboxylase) (Jain et al. 1996), *asd* (aspartate-β-semialdehyde) (Harb and Abu Kwaik 1998), *pts* (phosphoenolpyruvate phosphotransferase) (Edelstein et al. 1999; Higa and Edelstein 2001), *pmi* (phosphomannose isomerase) (Gao et al. 1997), *prp* (propionate catabolism) (Stone et al. 1999), and *ssrS* (regulatory 6S RNA) (Faucher et al. 2010). Finally, it has recently been determined that cyclic diguanylate signaling proteins modulate intracellular growth of *L. pneumophila* (Levet-Paulo et al. 2011; Levi et al. 2011).

Pathogenesis of Other *Legionella* Species

Besides *L. pneumophila*, 37 of the other *Legionella* species have been associated with human disease (*Table 9.1*). Thus, many of these species have been tested for their ability to grow within macrophages, as an initial attempt to explain their pathogenicity. Twenty-two of these species are known to replicate in one or more types of macrophages, including *L. anisa*, *L. brunensis*, *L. birminghamensis*, *L. bozemanae*, *L. cardiaca*, *L. cherrii*, *L. dumoffii*, *L. feeleii*, *L. gormanii*, *L. hackeliae*, *L. jordanis*, *L. lansingensis*, *L. longbeachae*, *L. maceachernii*, *L. micdadei*, *L. oakridgensis*, *L. parisiensis*, *L. sainthelensi*, *L. santicrucis*, *L. spiritensis*, *L. tusconensis*, and *L. wadsworthii* (Alli et al. 2003; Buse et al. 2011; Doyle et al. 2001; Izu et al. 1999; Levi et al. 1987; Miyamoto et al. 1996; Neumeister et al. 1997; O'Connell et al. 1996a; Pearce and Cianciotto 2012; Pearce et al. 2012; Weinbaum et al. 1984; Whitfield et al. 2010). Thus, the pneumonia caused by these 22 species likely derives from the ability of the legionellae to infect and grow within alveolar macrophages, as is the case for *L. pneumophila*. In contrast to these results, six other species that have been linked to disease, that is, *L. cincinnatiensis*, *L. erythra*, *L. gratiana*, *L. londiniensis*, *L. quinlivanii*, and *L. rubrilucens*, do not show evidence of intracellular replication (Alli et al. 2003; Izu et al. 1999; O'Connell et al. 1996a). Although these data might suggest that some *Legionella* species do not cause disease by being an intracellular parasite, it is also quite possible that the studies on these bacteria were limited by their in vitro infection assay and/or employed a bacterial strain that had been attenuated by laboratory passage. The remaining nine species that have been linked to disease, that is, *L. drancourtii*, *L. drozanskii*, *L. fallonii*, *L. lytica*, *L. nagasakiensis*, *L. nautarum*, *L. rowbothamii*, *L. waltersii*, and *L. worsleiensis*, have not been tested. Five of the 19 *Legionella* species that have not been linked to human disease have been tested for in vitro infection of macrophages. Interestingly, among these, *L. jamestowniensis* and *L. steigerwaltii* show an ability to grow intracellularly (Neumeister et al. 1997; O'Connell et al. 1996a). These data support the hypothesis that these two species, and perhaps other environmental legionellae, have pathogenic potential. A similar hypothesis had initially been put forward for *L. parisiensis* (O'Connell et al. 1996a), and soon thereafter pneumonia caused by *L. parisiensis* was reported (Igel et al. 2004).

A limited number of studies have sought to further define the intracellular infection process of the non-*pneumophila* species. In some cases, the non-*pneumophila* species exhibit traits that are similar to those of *L. pneumophila*. For example, *mip* and *dot/icm* genes promote intracellular infection by *L. longbeachae* and *L. micdadei*, as they do for *L. pneumophila* (Doyle et al. 1998; Feldman and Segal 2004; O'Connell et al. 1995). However, there are instances where their mode of macrophage infection is notably different from that of *L. pneumophila*. Strains of *L. micdadei*, for the most part, do not reside within phagosomes that evade lysosomes and recruit rough endoplasmic reticulum (Gao et al. 1999; Gerhardt et al. 2000; Joshi and Swanson 1999; Rechnitzer and Blom 1989; Weinbaum et al. 1984). *L. parisiensis* and *L. tucsonensis* do not effectively evade LAMP-1 (Whitfield et al. 2010). *L. longbeachae* colocalizes with early endosomal (EEA1) and late endosomal (LAMP-2) markers (Asare and Abu Kwaik 2007). *L. dumoffii* and *L. oakridgensis* multiply free within the cytoplasm of non-macrophage hosts (Maruta et al. 1998; Takekawa et al. 2012). Other sorts of studies have found that secreted activities can vary significantly between the *Legionella* species, including type II protein effectors, type IV protein effectors, siderophore, and surfactant (Cazalet et al. 2010; Kozak et al. 2010; Nagai et al. 2002; Newton et al. 2006; Pearce and Cianciotto 2009; Pearce et al. 2012; Söderberg et al. 2008; Starkenburg et al. 2004; Stewart et al. 2009). Thus, as various legionellae become associated with human disease, it will become increasingly important for researchers to examine them carefully and not assume that they are simple equivalents of *L. pneumophila*.

Immune Response and Host Susceptibility to *Legionella* Infections

Upon inhalation of contaminated aerosols, *L. pneumophila* reaches the lung and triggers an acute inflammation. In line with a higher virulence of protozoa-grown *L. pneumophila*, coinfection of the bacteria with *Hartmannella vermiformis* amoeba significantly enhances intrapulmonary bacterial growth and aggravates inflammation (Brieland et al. 1996, 1997a, b). A prerequisite for *L. pneumophila* to cause an inflammatory disease is the Dot/Icm-dependent ability to resist but not

necessarily replicate in macrophages (Spörri et al. 2006). Growth of *L. pneumophila* is restricted in macrophages from most laboratory mouse strains including BALB/c or C57BL/6, yet macrophages from the A/J mouse strain support replication of the bacteria in vitro (Yamamoto et al. 1988), and A/J mice are a suitable model for Legionnaires' disease (Brieland et al. 1994). In A/J mice, *L. pneumophila* elicits an acute inflammatory reaction and strong innate immune responses (Blanchard et al. 1988a, b; Brieland et al. 1995, 1998; Tateda et al. 2001a, b). Early after infection, inflammatory cytokines such as tumor necrosis factor (TNF)-α (Blanchard et al. 1987, 1988a; Brieland et al. 1995), interferon (IFN)-γ (Blanchard et al. 1988b, 1989), interleukin (IL)-12 (Brieland et al. 1998), and IL-18 (Brieland et al. 2000) are released and restrict the replication of the pathogen. In particular, IFN-γ is crucial to resolve an infection with *L. pneumophila* and also restricts growth of the bacteria in monocytes and alveolar macrophages, thus further contributing to limiting the infection (Bhardwaj et al. 1986; Byrd and Horwitz 1989; Nash et al. 1988).

Inflammatory cytokines act in concert with the activation and recruitment of polymorphonuclear neutrophil granulocytes (PMN) (Blanchard et al. 1988a, 1989; Tateda et al. 2001b). PMN are important innate effector cells that resolve *Legionella* infection and also function as cytokine producers. A/J mice depleted of PMN or natural killer (NK) cells, or mice lacking the type II IFN receptor, are unable to clear *L. pneumophila* due to a lack of the critical cytokine IFN-γ or its receptor (Spörri et al. 2006, 2008). In response to *L. pneumophila* infection, PMN activate caspase-1, leading to the production of mature IL-18. This cytokine then activates NK cells, which in turn produce IFN-γ (Spörri et al. 2008). In a feedback loop, IFN-γ stimulates dendritic (DC) cells, which produce IL-12 to activate NK cells, and thus, DC cells are also essential to control *L. pneumophila* infection (Ang et al. 2010). Interestingly, DCs restrict the intracellular growth of *L. pneumophila*, despite the fact that the bacteria reside in an ER-derived compartment (Neild and Roy 2003).

The innate immune response toward pathogens is directed against specific prokaryotic or viral "pathogen-associated molecular patterns" (PAMPs), which are recognized by eukaryotic "pattern recognition receptors" (PRRs) (Janeway and Medzhitov 2002). The PRRs include transmembrane toll-like receptors (TLRs), and the cytoplasmic nod-like receptors (NLRs) or retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) (Creagh and O'Neill 2006). *Legionella* spp. evolved as parasites of free-living protozoa, and the human host represents a dead end for the "accidental" pathogen. Thus, *L. pneumophila* likely has not been exposed to a rigorous evolutionary selection to avoid recognition by mammalian PRRs, and accordingly, the bacteria trigger activation of all families of PRRs (Massis and Zamboni 2011).

The activation of TLRs proceeds through dimerization and recruitment of adaptor proteins such as MyD88 (myeloid differentiation primary response gene 88) and culminates in the activation of the transcription factor NF-κB and the expression of inflammatory genes. TLR2 recognizes bacterial lipopeptides and lipoproteins. Mice lacking TLR2 are impaired for PMN migration to the site of *L. pneumophila* infection and, consequently, cannot efficiently clear the bacteria (Archer and Roy 2006; Fuse et al. 2007; Hawn et al. 2006). TLR4 responds to bacterial LPS. In contrast to TLR2, the absence of TLR4 does not affect the susceptibility of mice toward *L. pneumophila* (Archer and Roy 2006; Fuse et al. 2007; Lettinga et al. 2002). This lack of response might be due to the structure of *L. pneumophila* LPS, which contains a lipid A moiety with very long acyl chains that triggers TLR4 less potently than enterobacterial LPS. Yet, the situation is more complicated, as TLR4 polymorphisms are associated with resistance to Legionnaires' disease in humans (Hawn et al. 2005). TLR5 senses bacterial flagellin, including the flagellum of *L. pneumophila*. TLR5 plays an important role in *L. pneumophila* infection, as a common dominant *TLR5* polymorphism that abolishes flagellin signaling is associated with an increased susceptibility to Legionnaires' disease (Hawn et al. 2003). A role for TLR5 in Legionnaires' disease is corroborated by mice lacking the gene, which show altered leukocyte recruitment and inflammatory responses upon infection with *L. pneumophila* (Hawn et al. 2007). Taken together, mice lacking individual *TLR* genes are not substantially more susceptible to *L. pneumophila*. In contrast, deletion of MyD88 generates mice that fail to produce cytokines such as NK cell-derived IFN-γ and are highly susceptible to *L. pneumophila* infection (Archer et al. 2009, 2010; Archer and Roy 2006; Hawn et al. 2006; Neild et al. 2005; Spörri et al. 2006).

The NLRs represent a large family of PRRs that promote the expression of inflammatory genes and the activation of caspase-1 in multiprotein complexes termed inflammasomes (Schroeder et al. 2010). Three groups of NLRs can be distinguished: (1) nucleotide-binding oligomerization domain (NOD) proteins, (2) NALP (NRLP) receptors that require the adaptor protein ASC to trigger inflammasomes, and (3) NAIP (IPAF/NLRC) proteins that do not require ASC. The NOD-1 and NOD-2 receptors, which detect bacterial cell wall molecules, modulate the in vivo pulmonary immune response against *L. pneumophila*, albeit in reciprocal ways (Berrington et al. 2010; Shin et al. 2008). NOD-dependent signaling proceeds through the RIP-2 kinase and, in concert with MyD88- and NAIP5/NLRC4-dependent signaling (see below), cooperatively contributes to protection from *L. pneumophila* (Archer et al. 2010). ASC-dependent inflammasome activation leads to the secretion of mature IL-1β and restricts *L. pneumophila* replication in human macrophages (Abdelaziz et al. 2011), but neither activates the NALP3 (NLRP3) inflammasome nor contributes to controlling *L. pneumophila* infection in mice (Case and Roy 2011; Case et al. 2009; Molofsky et al. 2006; Ren et al. 2006; Zamboni et al. 2006). In contrast, ASC-independent recognition of *L. pneumophila* flagellin through the NAIP5 (Birc1e)/ NLRC4 (Ipaf) inflammasome triggers caspase-1 activation, pore formation, and pyroptosis (Amer et al. 2006; Case et al. 2009; Coers et al. 2007a; Derré and Isberg 2004; Fortier et al. 2007; Molofsky et al. 2006; Ren et al. 2006; Silveira and Zamboni 2010; Vinzing et al. 2008; Whitfield et al. 2010; Zamboni et al. 2006). Recognition of flagellin through the NAIP5/NLRC4 inflammasome is arguably the most important mechanism of *L. pneumophila* restriction. The elucidation of this intricate pathway finally provided a molecular and mechanistic rational for the

long-standing fact that A/J mice fail to restrict *L. pneumophila*, a feature that previously was mapped to the *NAIP5* gene (Diez et al. 2003; Wright et al. 2003) within the *Lgn1* locus (Dietrich et al. 1995). In agreement with this concept, macrophages as well as DC restrict *L. pneumophila* replication through a cell death pathway mediated by NAIP5, caspase-1, and also caspase-3 (Nogueira et al. 2009). Moreover, mice lacking NAIP5 fail to activate caspase-1 and restrict flagellated *L. pneumophila* (Lightfield et al. 2008).

Finally, RLRs are nucleic acid-sensing PRRs, which upon activation lead to the production of type I IFNs, such as IFN-α and IFN-β. During *L. pneumophila* infection, type I IFNs are produced dependent on the bacterial Dot/Icm T4SS and the eukaryotic regulators IFN-β promoter stimulator-1 (IPS-1) as well as IFN regulatory factor (IRF-3) (Lippmann et al. 2008; Opitz et al. 2006; Stetson and Medzhitov 2006). A role of RIG-1 for *L. pneumophila* DNA-dependent type I IFN production was debated; yet, recent data suggest that *L. pneumophila* RNA triggers the RIG-I-dependent production of type I IFNs (Monroe et al. 2009). Moreover, in this study, a Dot/Icm-secreted effector protein, SdhA, was identified as a key suppressor of the IFN response to *L. pneumophila*. Similar to IFN-γ, but through a different pathway, the addition of type I IFNs to macrophages abolishes intracellular growth of *L. pneumophila* in vitro (Plumlee et al. 2009; Schiavoni et al. 2004). In contrast, the role of type I IFNs in murine infection is less prominent (Ang et al. 2010; Monroe et al. 2009).

Genomics, Transcriptomics, and Metabolomics

Genetics and Genomics

Since the publication of the first bacterial genome sequence in 1995 (Fleischmann et al. 1995), a tremendous increase in genomic information has substantially altered our view on bacterial pathogenesis and has led to the application of many different genomics and post-genomics approaches in microbial research. In *Legionella* research, the genomics era started only in 2004 with the completion, analysis, and publication of the genome sequence of three clinical *L. pneumophila* isolates (Cazalet et al. 2004; Chien et al. 2004). The sequenced strains are the endemic strain Paris and the epidemic strain Lens, responsible for two major outbreaks in France in 2001 and 2004 (Cazalet et al. 2004), and strain Philadelphia 1, isolated from the first recognized outbreak of the disease in 1976 (Chien et al. 2004). Recently, the genomes of five additional isolates were determined: *L. pneumophila* strain Corby, a virulent strain isolated from a human legionellosis case (Jepras et al. 1985; Steinert et al. 2007); *L. pneumophila* strain Alcoy, a particularly persistent and recurrent strain in the region of Alcoy, Spain, that was isolated during one of the most significant outbreaks between the years 1999 and 2000 in Alcoy (D'Auria et al. 2010); strain *L. pneumophila* 130b, a clinical isolate from the Wadsworth Veterans Administration Hospital, Los Angeles, CA (Edelstein 1986; Engleberg et al. 1984; Schroeder et al. 2010); and two stains isolated in France. *L. pneumophila* strain Lorraine is rarely isolated from the environment, but its prevalence in human disease is increasing considerably in the last years (Ginevra et al. 2008), and *L. pneumophila* strain HL 0604 1035 has been frequently isolated from a hospital water system since over 10 years but never caused disease (Gomez-Valero et al. 2011). Knowledge of these sequences is now the basis for major breakthroughs in understanding the biology of *L. pneumophila*, as it gives new insight into the bacterium's lifestyle and its way of adapting to the host. As described below in detail, the analysis of these genome sequences revealed several specific features of *L. pneumophila*, some of which are undoubtedly related to its intracellular life, and allowed generation of hypothesis on how *L. pneumophila* subverts host functions to its advantage. Furthermore, the availability of the genome sequence of *L. pneumophila* provided the basis for the application of new powerful approaches like bioinformatics analyses, transcriptomics, and proteomics studies to better understand the biology of this organism.

General Features of the *L. pneumophila* Genomes

Legionella pneumophila has a single, circular chromosome of in average 3.4 Mb, with an average GC content of 38 % (Table 9.5). Strains Paris, Lens, and Lorraine each contain a plasmid, 131.9 kb, 59.8 kb, and 150.4 kb in size, respectively. In the other sequenced strains, no plasmid was identified. The genomes contain each ~3,000 genes distributed fairly evenly between the two strands (~57 % on the leading strand) and accounting for ~88 % of the potential coding capacity. No function can be predicted for about 40 % of the *L. pneumophila* genes, and about 20% of the predicted genes are unique to the genus *Legionella*. As seen in Table 9.5, the main features of the sequenced *L. pneumophila* genomes (e.g., genome size, GC content, and coding density) are highly conserved. The core genome of the *L. pneumophila* genomes comprises about 2,200 genes, which represents roughly 80 % of the predicted genes in each genome. Comparative analysis of the genome structure of the *L. pneumophila* genomes showed high colinearity, with only few translocations, duplications, deletions, or inversions. Principally, the genomes contain four large plasticity zones, where the synteny is disrupted: a 260-kb inversion in strain Lens with respect to all other strains, a 130-kb fragment which is partly similar partly different and which is inserted in different genomic location in the different strains, the genomic island-like region carrying the Pie effectors, and the chromosomal region carrying the Lvh T4SS.

Diversity, Mobility, and Plasticity Characterize the *L. pneumophila* Genomes

When determining the non-orthologous genes specific of each completely sequenced *L. pneumophila* genome, about 130 and

Table 9.5

Complete and draft genomes of *L. pneumophila* obtained by classical or new generation sequencing

Legionella pneumophila								
	Paris	Lens	Philadelphia	Corby	Alcoy	130b [c]	Lorraine	HL06041035
Chromosome size (kb)[a]	3,504 (131.9)[b]	3,345 (59.8)[b]	3,397	3,576	3,516	3,490	3,467 (150.4)[b]	3,492
G + C content (%)	38.3 (37.4)	38.4 (38)	38.3	38	38.4	38.2	38.3	38.4
No. of genes[a]	3,123 (142)	2,980 (60)	3,031	3,237	3,197	3,288	3,117	3,132
No. of protein-coding genes[a]	3,078 (140)	2,921 (60)	2,999	3,193	3,097	3,141	3,080	3,079
Coding density (%)	86.9	87.7	88.2	87.2	87.0	87.9	87.2	86.9
No. of 16S/23S/5S	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	ND	3/3/3	3/3/3
No. of transfer RNA	44	43	43	43	43	42	44	43
Plasmids	1	1	0	0	0	0	1	0

ND not determined; *kb* kilo bases

[a]Updated annotation; *CDS* coding sequence

[b]Data from plasmids in parenthesis

[c]The 130-b sequence is a not manually curated sequence

300 strain-specific genes mainly encoded on mobile genetic elements are identified. Thus, the *L. pneumophila* genomes have a highly dynamic accessory genome of up to 300 genes each, mainly formed by mobile genetic elements, genomic islands, and genes of unknown function. One of these mobile genetic elements is the above-mentioned Lvh carrying region. Lvh is a genomic island-like region that encodes a T4ASS implicated in conjugation and in virulence-related phenotypes under conditions mimicking the spread of Legionnaires' disease from environmental niches (Bandyopadhyay et al. 2007; Ridenour et al. 2003). This region can be integrated in the chromosome but can also excise in a site-specific manner and exist as a low copy plasmid (Doleans-Jordheim et al. 2006). It is present in all sequenced *L. pneumophila* strains except Lorraine, Corby, and Alcoy. In *L. pneumophila* Corby, there are instead two similar large genomic islands present called Trb-1 and Trb-2 (Steinert et al. 2007). Both islands encode all genes necessary for a functional T4SS with a *trb*/*tra* gene organization similar to the *tra*/*trb* region of plasmid R751 (IncP) of *Enterobacter aerogenes* (Thorsted et al. 1998) and contain an *oriT*-like site. Besides the *trb*/*tra* genes, the gene content of each island is specific. Trb-1 is integrated in a Pro tRNA gene, while Trb-2 is inserted in a tmRNA. The ladder integration site is identical to that of the pathogenicity island containing the *lvh* region in strain Paris and strain Lens. Both Trb-1 and Trb-2 can exist as an integrated and an excised form (Glockner et al. 2008; Steinert et al. 2007). It is interesting to note that in the *Legionella* strains where the Lvh T4SS is not present, another Tra system, a P-type T4SS that codes for short and rigid pili that allow surface mating for conjugation, is present in the same chromosomal position (Gomez-Valero et al. 2011). Recently, several other regions coding proteins homologous to Tra proteins that might code for a conjugative machinery and/or T4ASS were identified in all *Legionella* plasmids. They are similar to F-type T4SS that allow the synthesis of a long and flexible pilus for conjugation in liquid and solid media (Lawley et al. 2003). However, such a system is also found in a chromosomal localization in the *L. pneumophila* strain Philadelphia (Gomez-Valero et al. 2011). These regions are inserted in a tRNA gene next to an integrase and are bordered by flanking repeats. The presence of these elements is suggesting that these T4SSs are mobile and that their heterogeneous distribution is the result of the lateral movement of these plasmids. Furthermore, these different T4SS or conjugative elements can be found in different plasmids or can be completely or partially present in the chromosome, indicating that these regions might have the capacity to integrate and excise from the *Legionella* genomes as shown for the Lvh carrying region (Doleans-Jordheim et al. 2006; Gomez-Valero et al. 2011). Interestingly, a feature shared by most of the mobile elements (plasmids, *lvh* gene cluster, 65-kb putative pathogenicity island originally identified in strain Philadelphia 1 (Brassinga et al. 2003), the 130-kb fragment encoding several multidrug efflux pumps, and the putative mobile genetic elements coding P-type and F-type T4SSs) is that they encode a paralog of CsrA, a protein described in *L. pneumophila* as a repressor of transmission traits and an activator of replication traits (Fettes et al. 2001; Molofsky and Swanson 2004). It is tempting to assume that the paralogs of CsrA encoded on the mobile elements control their expression and/or also regulate the switch between integrated and circular forms of these regions (Cazalet et al. 2008; Gomez-Valero et al. 2011).

Thus, plasticity and genomic diversity are specific features of the *L. pneumophila* genomes due to the presence of integrative plasmids, putative conjugation elements, and genomic islands. In addition to DNA interchange between different bacterial genera, horizontal gene transfer within the genus *Legionella* and within the species *L. pneumophila* has been reported. Several studies suggested that recombination events may also play a role in the evolution of the species *L. pneumophila* (Bumbaugh et al. 2002; Coscolla et al. 2011; Coscolla and Gonzalez-Candelas 2007; Ko et al. 2003, 2006). Indeed, recently, it was shown that recombination and horizontal gene transfer are frequent in

L. pneumophila (Gomez-Valero et al. 2011). In particular, the analyses of the distribution of nucleotide polymorphisms suggested that large chromosomal fragments of over 200kbs can be exchanged between *L. pneumophila* strains contributing to the genome dynamics in the natural population (Gomez-Valero et al. 2011). *L. pneumophila* has all necessary features for incorporating foreign DNA, as these bacteria are naturally competent and possess an intact recombination machinery (Mintz 1999; Sexton and Vogel 2004; Stone and Kwaik 1999). Taken together, the *L. pneumophila* genomes are highly dynamic, a feature allowing different clones to evolve into predominant disease clones and others to replace them subsequently within relatively short periods of time but also allowing *L. pneumophila* to adapt to the many diverse conditions and environments it encounters.

Coevolution with Protozoa Is Reflected in the *L. pneumophila* Genome Sequence

Analysis and comparison of the first sequenced genomes revealed an intriguing feature of *Legionella*, which is the presence of an extended array of eukaryotic-like proteins (Cazalet et al. 2004). A systematic search in the genome sequence identified about 100 genes having their best hit against eukaryotic genomes including both housekeeping genes and genes that may play a role in the virulence of *L. pneumophila* (Cazalet et al. 2004). Examples for eukaryotic-like proteins are two secreted apyrases, a sphingosine-phosphate lyase and sphingosine kinase, eukaryotic-like glucoamylase, cytokinin oxidase, zinc metalloprotease, or an RNA binding precursor (Bruggemann et al. 2006; Cazalet et al. 2004; de Felipe et al. 2005). Further analyses of additional genomes showed that eukaryotic-like proteins are present in all sequenced *L. pneumophila* genomes, and most of them are even highly conserved, suggesting their importance for *L. pneumophila* virulence and survival (Amaro et al. 2012; Cazalet et al. 2004; D'Auria et al. 2010; de Felipe et al. 2005; Gomez Valero et al. 2011; Gomez-Valero et al. 2011; Schroeder et al. 2010). This high conservation was also confirmed by hybridization analyses of over 200 *L. pneumophila* strains (Cazalet et al. 2008). A search against Pfam and Prosite databases identified over 30 genes coding proteins containing motifs that are present mostly in eukaryotes like ankyrin repeats, Sel-1, Sec7, serine threonine kinase domains (STPK), F-box, or U-box motifs. The identification of these so-called eukaryotic-like proteins (Cazalet et al. 2004) or *Legionella* *e*ukaryotic *g*enes (leg) (de Felipe et al. 2005) led to the hypothesis that the eukaryotic proteins of *L. pneumophila* might help to mimic the function of host proteins to manipulate the host physiology and certain cellular functions for the pathogens benefit (Bruggemann et al. 2006; Cazalet et al. 2004). Indeed, many of these eukaryotic-like proteins are substrates of the Dot/Icm T4SS (de Felipe et al. 2008; de Felipe et al. 2005), and for several, it has been shown meanwhile that they are indeed modulating different host cell pathways (Hubber and Roy 2010a; Nora et al. 2009).

A particular example is the large family of ankyrin repeat proteins identified in the *L. pneumophila* genomes. Different studies showed that many of the *L. pneumophila* ankyrin proteins, namely, AnkB, AnkC, AnkF, AnkK, AnkQ, AnkW/AnkH, AnkX/AnkN, AnkY, and AnkZ/AnkG, are substrates of the Dot/Icm secretion system and that AnkB, AnkK, AnkQ, AnkX, and AnkY induce a growth defect in *Saccharomyces cerevisiae* (Al-Khodor et al. 2008; de Felipe et al. 2008; Heidtman et al. 2009; Pan et al. 2008). However, the determination of whether proteins are secreted by the T4SS is not always clear, as, for example, AnkY and AnkZ are not translocated Dot/Icm effectors according to the assay used by de Felipe and colleagues (de Felipe et al. 2008). Recent functional analysis of certain of these ankyrin proteins showed that they are multifunctional and are involved in many cellular pathways. AnkX/AnkN was shown to be an effector of membrane transport that promotes fragmentation of the Golgi apparatus when expressed in mammalian cells (Pan et al. 2008). Golgi fragmentation is presumably the result of inhibition of ER-to-Golgi vesicle transport by interference with microtubule-dependent transport of vesicles (Pan et al. 2008). Furthermore, AnkW, AnkX, AnkY, and AnkZ showed different patterns of subcellular localization in mammalian cells (Pan et al. 2008). AnkB plays an important role in intracellular growth and in exploiting the ubiquitin system and the farnesylation machinery of the host (see above, paragraph type IV secretion) (Al-Khodor et al. 2008; Al-Quadan and Kwaik 2011; Al-Quadan et al. 2012, 2011; Habyarimana et al. 2008; Ivanov et al. 2010; Price et al. 2009, 2010a, b, c, 2011; Price and Kwaik 2010). AnkH and AnkJ show a significant defect in intracellular replication in amoebae, human macrophages, and protozoa (Habyarimana et al. 2009). Furthermore, when expressed in *Saccharomyces cerevisiae*, AnkB, AnkF, AnkQ, AnkX, and AnkY caused severe growth defects, indicating that these proteins impact essential host cell pathways (Heidtman et al. 2009). Thus, ankyrin proteins are translocated effectors of distinct secretion systems that may have many different functions in the eukaryotic hosts by aiding intracellular bacteria to modulate host functions to their advantage.

Another example is the apyrase coding genes. Apyrases or ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases, apyrases) (gene family ENTPD) of the CD39 family are important ectonucleotidases that are characterized by the presence of five "apyrase-conserved regions" (ACR1 to ACR5) and by the ability to hydrolyze nucleotide tri- and diphosphatases to the monophosphate form. Nucleoside monophosphates may then be catalyzed to nucleosides such as adenosine by the action of ecto-5-nucleotidases (e.g., mammalian CD73) (Sansom et al. 2008). While CD39/NTPDase 1 family members are present in all higher eukaryotes, the first prokaryotic ecto-nucleoside triphosphate diphosphohydrolase was identified and characterized in *L. pneumophila* (Cazalet et al. 2004; Sansom et al. 2007). *L. pneumophila* encodes two secreted ecto-NTPDases Lpp1880/Lpg1905 and Lpp1033/Lpg0971. Lpp1880/Lpg1905 shares similarities with human CD39 and other eukaryotic ecto-NTPDases, and it has been shown to play a role during uptake of *L. pneumophila* into the host cell. In humans, CD39 is located on the surface of endothelial cells, and it controls extracellular levels of ATP by converting it in its

diphosphate and monophosphate forms. In this way, it plays a major role in maintaining vascular fluidity by regulating platelet aggregation (Marcus et al. 2005). CD39/NTPDases are found in a wide range of pathogens such as in protozoan parasites, but their role in infection is poorly understood. One of the two predicted ecto-NTPDases in *L. pneumophila* is secreted into the host cell, and its activity is required for successful infection. This defect was not correlated with the ability to recruit the ER or avoiding phagolysosomal fusion but mainly to a less efficient entry (Sansom et al. 2007). Recently, it was shown that the enzyme catalyzed the hydrolysis of ATP and ADP and also of GTP and GDP but had only limited activity against CTP, CDP, UTP, and UDP. Furthermore, mutational analysis revealed that all five apyrase domains are necessary for infection following intratracheal inoculation of A/J mice (Sansom et al. 2008). Interestingly, Lpp1880/Lpg1905 is the first example in which a bacterial ecto-NTPDase is implicated in virulence. In contrast, Lpp1033/Lpg0971, the second ecto-NTPDase encoded by *L. pneumophila*, is not necessary for entry and replication within amoebae, alveolar epithelial cells, and macrophages (Sansom et al. 2007), but contributes to virulence in a mouse lung infection model (Sansom et al. 2008).

Another intriguing protein identified in the *L. pneumophila* genomes (Cazalet et al. 2004) is a eukaryote-like sphingosine-1-phosphate lyase. Up to now, SPL-encoding genes have been found in very few prokaryotes only. In eukaryotes, SPL is an enzyme that uses pyridoxal 5'-phosphate as a cofactor for catalyzing the irreversible cleavage of sphingosine-1-phosphate (S1P). S1P, a sphingolipid, like ceramide or sphingosine, is implicated in various physiological processes like cell survival, apoptosis, proliferation, migration, differentiation, platelet aggregation, angiogenesis, lymphocyte trafficking, and development (Alvarez et al. 2007; Bandhuvula and Saba 2007). Despite the fact that the function of the *L. pneumophila* sphingosine-1-phosphate lyase remains actually unclear, the hypothesis is that it plays a role in autophagy and/or apoptosis. Recently, it has been shown that *L. pneumophila* SPL is translocated into host cells using a C-terminal translocation domain absent in its eukaryotic homologues and that it is able to complement the sphingosine-sensitive phenotype of *Saccharomyces cerevisiae* (Degtyar et al. 2009). Unlike the eukaryotic SPL that localizes to the endoplasmic reticulum, *L. pneumophila* SPL was found to be targeted mainly to host cell mitochondria (Degtyar et al. 2009). Eukaryotic-like proteins are clearly helping *L. pneumophila* to modulate host functions to its advantage. Thus, molecular mimicry seems to be a main virulence strategy of this environmental pathogen.

The Evolution of Virulence in *L. pneumophila*: The Evolution of the Eukaryotic-Like Proteins

Eukaryotic-like proteins have also been identified in other bacterial pathogens like *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Stebbins and Galan 2001; Vance et al. 2004). Furthermore, a particular high number of serine protein kinases has been identified in *Mycobacterium tuberculosis* (Cole et al. 1998), or large families of ankyrin repeat proteins are present in *Coxiella burnetii* (Luhrmann et al. 2010; Pan et al. 2008; Seshadri et al. 2003; Voth et al. 2009), *Rickettsia felis* (Ogata et al. 2005), or *Wolbachia pipientis* (Wu et al. 2004). However, *L. pneumophila* ranks as one of the pathogens that encodes the most and the widest variety of eukaryotic-like proteins or proteins with eukaryotic domains. Thus, the question arises with respect to the origin of these eukaryotic-like proteins. Two hypotheses can be proposed: (1) either they have been acquired by horizontal gene transfer (2) or they evolved by convergent evolution. Most of the *Legionella* eukaryotic-like genes have a GC content, ranging from 32 % to 48 %; thus, it differs significantly from that of the rest of the genome (38 %), supporting the hypothesis that these genes have been acquired through horizontal gene transfer. Another line of evidence supporting this hypothesis is the presence of an 11.3-kb gene cluster in strain Paris containing 11 genes with similarity to eukaryotic genes that is bordered by a Lys-tRNA which might be the integration site as in many genomic and pathogenicity islands (Hacker and Kaper 2000). *ralF* was the first gene suggested to have been acquired by *L. pneumophila* from eukaryotes by HGT, as RalF carries a eukaryotic Sec 7 domain (Nagai et al. 2002). Recently, it has been reported that *Legionella drancourtii*, a relative of *L. pneumophila*, has acquired a sterol reductase gene from the *Acanthamoeba polyphaga Mimivirus* genome, a virus that grows in amoeba (Moliner et al. 2009b). Thus, the acquisition of some of the eukaryotic-like genes of *L. pneumophila* by HGT from protozoa is plausible and has thus been suggested by different groups (Bruggemann et al. 2006; Cazalet et al. 2008; de Felipe et al. 2005; Hubber and Roy 2010a; Moliner et al. 2010; Moliner et al. 2009a). In order to study their evolutionary origin, a systematic phylogenetic analysis has been undertaken (Lurie-Weinberger et al. 2010). It demonstrated that both lateral gene transfer from eukaryotic hosts and bacterial genes that became eukaryotic like by gradual adaptation to the intracellular milieu or gene fragment acquisition contributed to the existing repertoire of ELPs, which comprise over 3 % of the putative proteome of *L. pneumophila* (Lurie-Weinberger et al. 2010). A clear example is the eukaryote-like sphingosine-1-phosphate lyase of *L. pneumophila* described earlier. The phylogenetic analysis shown in ❯ *Fig. 9.6* revealed that it was most likely acquired from a eukaryotic organism early during *Legionella* evolution (Degtyar et al. 2009; Gomez Valero et al. 2011; Nora et al. 2009) as the protein sequence of *L. pneumophila* clearly falls into the eukaryotic clade of SPL sequences. Similarly, as shown in ❯ *Fig. 9.7*, phylogenetic analyses of an atypical member of the arylamine N-acetyltransferase family encoded in the *L. pneumophila* genomes, which allows this bacterium to detoxify aromatic amine chemicals and thus to grow in their presence, indicated that this gene has been acquired by horizontal gene transfer (Kubiak et al. 2012).

Interaction between *L. pneumophila* and amoeba or more generally freshwater protozoa is central to the ecology and the pathogenesis of *L. pneumophila*. Thus, it is very likely that *L. pneumophila* has acquired some of its eukaryotic-like genes from amoeba. Interestingly, *L. pneumophila* is not the only

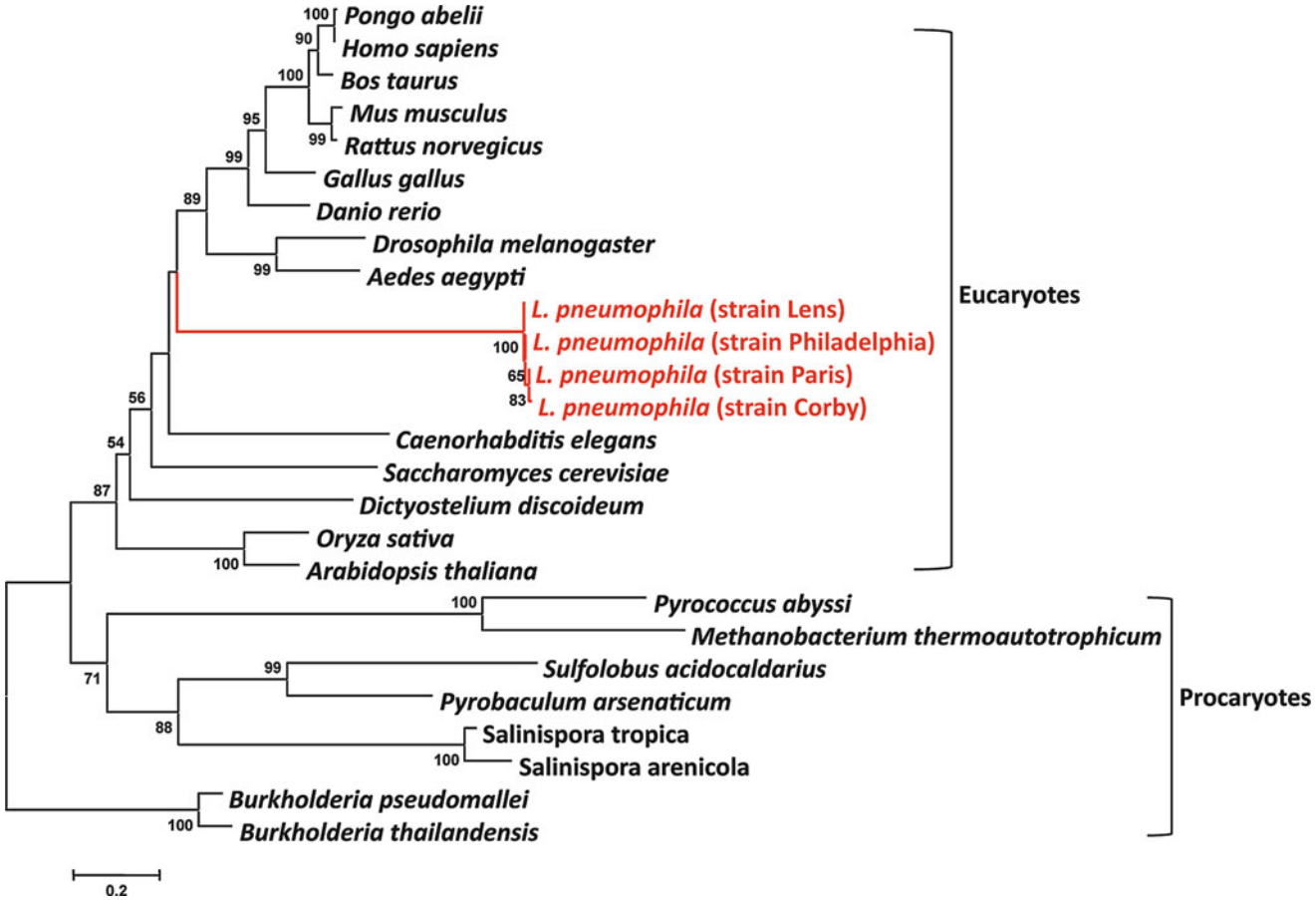

Fig. 9.6

Phylogenetic tree of a multiple sequence comparison of sphingosine-phosphate lyase proteins present in eukaryotic and prokaryotic genomes. Phylogenetic reconstruction was done with MEGA using the neighbor-joining method. *Numbers* indicate bootstrap values after 1,000 bootstrap replicates. The *red lines* indicate the *L. pneumophila* sequences that are embedded in the eukaryotic clade. The *bar at the bottom* represents the estimated evolutionary distance (Gomez Valero et al. 2011; Nora et al. 2009)

prokaryote whose genome shows an enrichment of proteins with eukaryotic domains, but it seems to be a common feature of amoeba-associated bacteria. For example, *Rickettsia bellii* and *Protochlamydia amoebophila*, both bacteria that live inside amoeba, contain many eukaryotic-like genes, again arguing for acquisition of some of these genes from amoeba. Another example is the genome of "*Ca. Amoebophilus asiaticus*," a Gram-negative, obligate intracellular amoeba symbiont whose genome encodes a large number of proteins with eukaryotic domains (Schmitz-Esser et al. 2010). To further investigate the distribution of these protein domains in other bacteria, an enrichment analysis comparing the fraction of all functional protein domains among 514 bacterial proteomes (Schmitz-Esser et al. 2010) has been undertaken. It revealed that the genomes of bacteria for which replication in amoeba has been demonstrated were enriched in protein domains that are predominantly found in eukaryotic proteins. Due to the phylogenetic diversity of these bacteria containing eukaryotic domains and proteins, it is most likely that these traits were acquired independently during evolutionary early interaction with ancient protozoa. Furthermore, comparison of the genome of *R. bellii*, an obligate intracellular pathogen of amoeba, revealed that many *R. bellii* ORFs (about 8 %) exhibit a high level of sequence similarity to homologues found in *L. pneumophila* and the amoeba-endosymbiont *P. amoebophila* (Ogata et al. 2005). This percentage is significantly higher than that seen for alphaproteobacteria that do not live within amoeba like *Mesorhizobium loti* (less than 1 %) and *Pelagibacter ubique* (about 2 %), suggesting that horizontal gene transfers among these different bacterial species may take place within amoeba and that amoebae constitute a gene melting pot, allowing diverse microorganisms to evolve by the same pathway characterized by gene acquisition, and then either adapt to the intra-amoebal lifestyle or create new pathogens (Moliner et al. 2009a, 2010).

Transcriptomics

L. pneumophila has developed a variety of strategies by which it adapts its genetic expression to meet the challenges of the

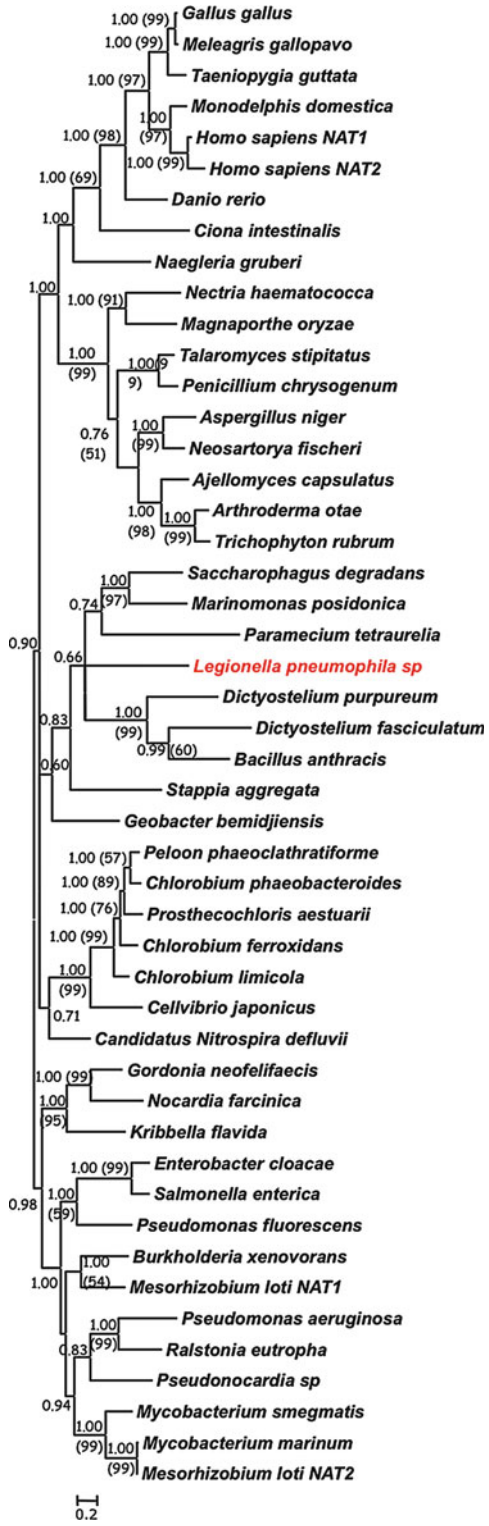

Fig. 9.7

Phylogenetic tree inferred from NAT amino acid sequences. The tree was constructed by Bayesian analysis. *Numbers besides nodes* are posterior probabilities recovered by the Bayesian analysis. *Numbers in parentheses* are bootstrap values based on 1,000 replicates in maximum likelihood analyses. Values below 50 % are not shown. The *scale bar* represents the estimated evolutionary distance (Kubiak et al. 2012)

ever-changing surrounding environment. These include specific sigma factors, two-component systems, quorum-sensing systems, repressors, positive regulators, as well as small regulatory RNAs. Alone or in combination, these mechanisms enable *L. pneumophila* to communicate with its environment, its hosts, and with each other, to adopt specific responses, express specific proteins, or develop specialized structures such as biofilms to ensure survival, colonization of their ecological niches, and dissemination. In the last years, microarray technology has been the method of choice for large-scale gene expression studies. It provides an efficient and rapid method to investigate the entire transcriptome of a cell. In vivo time course transcriptome analysis of *L. pneumophila* upon infection of amoeba and macrophages as well as in vitro analysis of different regulatory networks has been undertaken. Very recently, the advent of new generation sequencing allowed the application of RNA-seq to *L. pneumophila*, leading to new and exciting information about small noncoding RNAs and allowing to establish the precise transcriptional map.

Regulatory Repertoire of *L. pneumophila*

Consistent with the intracellular lifestyle, the regulatory repertoire of *L. pneumophila* is rather small as compared to other free-living bacteria, for example, *Pseudomonas aeruginosa* (Stover et al. 2000). Genome analysis identified in average 90 transcriptional regulators per sequenced genome, which represents only 3.0 % of the predicted genes. *L. pneumophila* encodes six putative sigma factors, the homologues of *rpoD* (the major sigma factor), *rpoH*, *rpoS*, *rpoN*, *fliA*, and the ECF-type sigma factor *rpoE*. With about 14 two-component systems (TCS) encoded in its genome, the number is also lower than that of free-living bacteria like *E. coli* that encodes 35. The most abundant class of regulators identified in the *L. pneumophila* genome belongs to the GGDEF/EAL family (over 20 per strain). Analyses of the regulatory proteins of *L. pneumophila* that contain domains related to cyclic diguanylate synthesis, hydrolysis, and recognition indicated that components of the cyclic diguanylate signaling pathway play an important role in regulating the ability of *L. pneumophila* to grow in host cells (Levi et al. 2011). Furthermore, one of these proteins was recently shown to belong to a new histidine kinase subfamily based on the conservation of an original H box that we named HGN H box and to be the first example of a bifunctional enzyme that modulates synthesis and turnover of c-di-GMP in response to phosphorylation of its receiver domain (Levet-Paulo et al. 2011). Key regulatory networks of *L. pneumophila* known to date include that of the two-component systems PmrA/PmrB (Zusman et al. 2007), CpxR/CpxA (Altman and Segal 2008), and LetA/LetS (Hammer et al. 2002), that of the sigma factor RpoS (σS) (Hovel-Miner et al. 2009) and the RNA-binding protein CsrA (Fettes et al. 2001; Molofsky and Swanson 2003), and probably that under the control of Hfq (McNealy et al. 2005).

Regulation of the Intracellular Life Cycle of *L. pneumophila*

L. pneumophila that reaches the alveolar space of the lungs is engulfed by macrophages. The pathogen inhibits phagosome acidification and fusion with lysosomes. *L. pneumophila*-containing phagosomes are completely isolated from the endosomal pathway and become surrounded by endoplasmic reticulum, and within these protected vacuoles, *L. pneumophila* converts to a replicative form. Once the vacuole is no longer favorable for replication, a regulatory cascade coordinates entry into the stationary phase with expression of traits that promote transmission to a new host cell (for a review, see Molofsky and Swanson 2004). Thus, it has been proposed that *L. pneumophila* cycles between an infectious, non-replicating form thought to promote transmission to a new host and an intracellular, replicative form, which does not express transmission traits (Byrne and Swanson 1998; Rowbotham 1986). This biphasic life cycle can be mimicked in broth culture with exponential (replicative) and postexponential (transmissive) grown bacteria (Molofsky and Swanson 2004). Adaptation of *L. pneumophila* is governed by a complex regulatory system. Current knowledge of these regulatory networks and the transcriptome responses of *L. pneumophila* are mainly based on microarray analysis (Brüggemann et al. 2006; Dalebroux et al. 2009, 2010; Faucher et al. 2011; Hovel-Miner et al. 2009, 2010; Sahr et al. 2009).

Genome-wide analyses of the transcriptional response of *L. pneumophila* grown in broth as well as inside *Acanthamoeba castellanii* showed that in vitro and inside the host cell *L. pneumophila* ensures a precise timing of its life cycle reflected by a major shift in gene expression from replicative to transmissive phase, concerning nearly half of the genes predicted in the genome (Brüggemann et al. 2006). Furthermore, in three different *L. pneumophila* strains – Paris, Philadelphia, and Lens – similar gene expression patterns were found, indicating that the *Legionella* life cycle is based on common regulatory mechanisms (Brüggemann et al. 2006). During the replicative phase, most parts of the carbohydrate, amino acid, fatty acid, and nucleotide uptake and/or metabolism were strongly expressed as well as genes encoding proteins of the respiratory chain, ATP synthesis, and ribosome biogenesis. This suggests the consumption of host nutrients by *L. pneumophila* to gain energy and components for DNA replication, protein biosynthesis, and lipid biosynthesis necessary for efficient multiplication. Traits necessary for bacterial entry and lysosome evasion (EnhC, RalF, LidA, VipA, etc.) or motility – in particular the most abundant flagellar protein FlaA – are not activated. These genes are expressed during transmissive phase together with many other substrates of the Dot/Icm secretion system, proteins related to UV, heat or osmotic stress response, the flagellar regulon, or numerous *Legionella*-specific yet unknown proteins (Brüggemann et al. 2006). Similarly, the analyses of the gene expression profile of *L. pneumophila* during infection of macrophage-like cells derived from the human THP-1 monocyte cell line found that genes involved in the metabolism of amino acids, lipids, carbohydrates, nucleotides, cofactors, and vitamins were induced inside cells; however, the authors report that this induction was independent of the time postinfection (Faucher et al. 2011). Furthermore, many transport systems involved in amino acid and iron uptake and genes involved in catabolism of glycerol were also induced during intracellular growth, suggesting that glycerol could be used as a carbon source. Like during amoeba infection, the genes encoding several translocated effectors were strongly induced (Faucher et al. 2011). Recently, Weissenmayer and colleagues used the RNA-seq technique to study the intracellular response of *L. pneumophila* at different time points. Their results largely confirmed the microarray results but also identified small ncRNAs induced during infection (Weissenmayer et al. 2011). How these different traits are activated and deactivated in *Legionella* is beginning to be examined in molecular detail, and the study of exponential and postexponential phase forms has provided valuable data about the regulatory networks that control life cycle-related phenotypic changes of *L. pneumophila*. Central in the regulation of the biphasic life cycle is the CsrA system of *L. pneumophila* that includes the two-component system LetA/LetS and two small noncoding RNAs (ncRNA) RsmY and RsmZ and in some strains also RsmX (Edwards et al. 2010; Fettes et al. 2001; Hammer et al. 2002; Molofsky and Swanson 2004; Rasis and Segal 2009; Sahr et al. 2009, 2012). Furthermore, the sigma factor RpoS, the RNA chaperone Hfq, the sigma 28 factor FliA, and the Lqs system have been shown to be part of this regulatory network (Bachman and Swanson 2004; Brüggemann et al. 2006; Hovel-Miner et al. 2009; McNealy et al. 2005; Tiaden et al. 2007, 2010b).

The LetA/LetS System and Posttranscriptional Regulation by CsrA

One of the best-studied TCS in *Legionella* is the LetA/LetS system. LetA/LetS has orthologous systems in many other Gram-negative bacteria, such as *Salmonella enterica* BarA/SirA, *Erwinia carotovora* ExpA/ExpS, *Vibrio cholerae* VarA/VarS, *Pseudomonas* spp. GacA/GacS, or *E. coli* UvrY/BarA (Babitzke and Romeo 2007; Cui et al. 2001; Kay et al. 2005; Lenz et al. 2005; Suzuki et al. 2002). *letA* and *letS* mutants are nonmotile, noncytotoxic, sodium sensitive, and less proficient in infecting macrophages; however, *letA* mutants still multiply in macrophage host cells (Gal-Mor and Segal 2003; Hammer et al. 2002). Furthermore, *letA* mutants are more sensitive to oxidative and acid stress than the wild type (Lynch et al. 2003), and infectivity of *A. castellanii* is reduced (Hammer et al. 2002; Lynch et al. 2003; Molofsky and Swanson 2004). The LetA/LetS TCS belongs to a family of signal-transducing proteins that employ a four-step phosphorelay to regulate gene expression. Histidine 307 of the LetS protein is the primary site of phosphorylation required to activate LetA (Edwards et al. 2010). Additionally, a threonine substitution at position 311 of LetS generated a *L. pneumophila* mutant with an intermediate phenotype (Edwards et al. 2010), in which gene expression of the flagellar regulon and numerous other loci was delayed when compared to wild-type bacteria (Edwards et al. 2010).

A common feature of this family of TCS is that they regulate the expression of small noncoding RNAs (ncRNAs) that subsequently interact with proteins of the CsrA/RsmA family. Indeed, like in other bacteria where homologues of CsrA and LetA/LetS exist, two small ncRNAs (RsmY and RsmZ) are induced by the activation of LetA, and they link LetA and CsrA (Rasis and Segal 2009; Sahr et al. 2009). RsmY and RsmZ bind CsrA and antagonize its activity by sequestering CsrA. CsrA-mediated repression involves the binding of CsrA to the ribosome binding site of target transcripts, thereby blocking ribosome access to the mRNA. In contrast, activation by CsrA seems to be due to mRNA stabilization, similar to what is described for *Escherichia coli*. Thus, after detection of a yet unknown activating stimuli that trigger the sensor kinase LetS autophosphorylation, LetS activates LetA, leading to an increased transcription of RsmY and RsmZ. Subsequently, CsrA interacts with the specific loop structure of the two ncRNA containing a GGA binding motif and sequestering CsrA, thereby releasing it from its targets. Analysis of the transcriptional programs of the *letA*, *letS*, and *rsmYZ* double mutants in different growth phases revealed that the mutants are both blocked in the replicative phase, while the switch to the transmissive phase is partially blocked as judged by the downregulation of many transmission factors, such as Dot/Icm-secreted substrates. One major difference between the *letA*, *letS*, and *rsmYZ* double mutants was that the latter synthesizes flagella (Sahr et al. 2009). A regulatory link between the two RNA-binding proteins CsrA and Hfq has been proposed (McNealy et al. 2005), and both CsrA and Hfq are under the control of LetA and RpoS. Thus, it can be speculated that in *Legionella* – as it is described for *E. coli* – CsrA and Hfq might bind to the same ncRNA in a competitive or concomitant way. Also involved in the regulatory network governed by the TCS LetA/LetS is LetE, a small, *Legionella*-specific protein (Bachman and Swanson 2004; Hammer et al. 2002). *L. pneumophila* possesses also a homologue of CsrD of *E. coli*, which accordingly might be required for the decay of the regulatory RNAs sequestering CsrA.

The Regulatory Network Controlled by ppGpp and RpoS

The LetA/LetS two-component system of *L. pneumophila* probably responds to ppGpp (Bachman and Swanson 2004; Hammer et al. 2002; Molofsky and Swanson 2003). In accordance, the entry in the transmissive phase is initiated by a mechanism called "stringent response" (Jain et al. 2006). It was proposed that under conditions of nutrient starvation, signaled probably by low amino acid levels (Hammer and Swanson 1999), RelA synthesizes the alarmone molecule ppGpp (Hammer and Swanson 1999; Hammer et al. 2002; Zusman et al. 2002). In addition to RelA, *Legionella* encodes SpoT, a bifunctional enzyme with ppGpp hydrolysis and weak synthesis activity. SpoT senses perturbations in the fatty acid synthesis by binding to the acyl carrier protein ACP (Dalebroux et al. 2009). Thus, RelA, which senses amino acid starvation, and SpoT that monitors fatty acid biosynthesis together control the biphasic life cycle of *L. pneumophila* and promote expression of traits dedicated to the transmissive phase. *relA* mutants are unable to accumulate (p)ppGpp and transcribe several phase-dependent traits like motility or pigmentation only poorly even when reaching transmissive phase, but they survive and replicate efficiently in host cells (Zusman et al. 2002). In contrast, a *relAspoT* double mutant is strongly diminished in infectivity, indicating that a complete loss of (p)ppGpp has a severe effect on virulence and cytotoxicity and that transmissive phase bacteria require SpoT to reenter the replicative phase (Dalebroux et al. 2009). Many of the physiological effects of ppGpp are mediated through interactions with RNA polymerase (RNAP) in cooperation with the RNAP secondary channel interacting protein DksA (Potrykus and Cashel 2008). Transcriptional profiling of a *L. pneumophila dksA* mutant revealed that during transmission, alarmone accumulation increases the mRNA for flagellar and type IV secretion components, secreted host effectors and regulators, and decreases transcripts for translation, membrane modification, and the ATP synthesis machinery. DksA is critical for the life cycle switch, since mutants are defective for stationary phase survival, flagellar gene activation, lysosome avoidance, and macrophage cytotoxicity. Thus, DksA is thought to respond to the level of ppGpp and other stress signals to coordinate *L. pneumophila* differentiation (Dalebroux et al. 2010). Interestingly, LetA and RpoS are described to be regulated by (p)ppGpp (Abu-Zant et al. 2006; Hammer and Swanson 1999), connecting the second messenger and regulation of the mRNA-binding protein CsrA to a complex regulatory network.

RpoS (σ^S) is playing a dominant role during the life cycle of *L. pneumophila* as it has been shown to regulate a number of known virulence factors including many Icm/Dot effectors. RpoS is required for intracellular multiplication in amoeba and primary macrophages but not in macrophage-like cell lines, probably because of their reduced antimicrobial capacity (Abu-Zant et al. 2006; Hales and Shuman 1999b). Transcriptional profiling during exponential and postexponential growth of an *rpoS* mutant compared to the wild-type strain showed that RpoS affects distinct groups of genes that contribute to intracellular multiplication of *L. pneumophila* (Hovel-Miner et al. 2009). In particular, RpoS affects the expression of many genes encoding Icm/Dot substrates as well as genes encoding regulators required for intracellular multiplication. Furthermore, these analyses revealed that the arginine repressor ArgR is required for efficient intracellular multiplication (Hovel-Miner et al. 2009). Subsequent characterization of the *L. pneumophila* ArgR regulon by global gene expression analysis showed that ArgR repression is dependent upon the presence of L-arginine and demonstrated that ArgR-regulated genes are derepressed during intracellular growth. In addition to amino acid metabolism, the categories transport and binding, Icm/Dot-translocated substrates, nucleotide metabolism, and detoxification and stress adaptation were affected. These results suggest that L-arginine availability functions as a regulatory signal during intracellular growth (Hovel-Miner et al. 2010).

Most importantly, RpoS regulates the transcription of several regulators important for intracellular multiplication.

First, expression of the two ncRNAs RsmY and RsmZ in *L. pneumophila* that regulate CsrA activity is RpoS dependent (Hovel-Miner et al. 2009; Sahr et al. 2009). Furthermore, RpoS affects the expression of FleQ and FliA (sigma 28) that are together with the sigma factor RpoN the major regulators of flagellum biosynthesis (Albert-Weissenberger et al. 2010). In a hierarchal and possibly temporal ordered mechanism, these two factors coordinate the synthesis and assembly of the different components indispensible for motility like FliA that is regulating the transcription of the most abundant flagellar protein, FlaA (Albert-Weissenberger et al. 2010). Additional important regulators under the control of RpoS are the TCS Cpx and Pmr. CpxA/CpxR directly control the regulation of *dot/icm* virulence genes (Altman and Segal 2008), and PmrA/PmrS is a global regulation system implicated in the regulation of the Dot/Icm type IV secretion system and intracellular growth of *L. pneumophila* (Al-Khodor et al. 2009; Zusman et al. 2007). Finally, it was shown that RpoS (and to a lesser extent also LetA) control LqsR (Tiaden et al. 2007). LqsR is part of a gene cluster homologous to the *Vibrio cholera* CqsAS quorum-sensing system. DNA microarray experiments revealed that LqsR regulates the expression of genes involved in virulence, motility, and cell division, consistent with a role for LqsR in the transition from the replicative to the transmissive phase. *lqsR* mutants are deficient in pathogen-host interactions and entry into the replicative growth phase (Tiaden and Hilbi 2012; Tiaden et al. 2007, 2008, 2010a). Taken together, RpoS is controlling regulatory elements for motility and virulence to express them both in a concerted manner. Thus, the ppGpp-RpoS-LetA network comprises the regulatory systems CsrA, LetE, PmrA, CpxR, or LqsR, which are all together of major importance for *L. pneumophila* to achieve optimal gene expression at each step of its life cycle.

The *L. pneumophila* Flagellum and the FliA (σ^{28}) Sigma Factor

Transcriptomic analyses of *L. pneumophila* wild type showed that in in vitro and in vivo conditions, the flagellin-encoding gene *flaA* is upregulated up to 100 times in transmissive phase cells as compared to replicative cells. Similarly, several genes encoding proteins implicated in flagellum biosynthesis (e.g., *fliS*, *fliD*, *fliN*, *flgBCDEFGHIJKL*, *fhF*, *fleN*) and the *fliA* gene, encoding the sigma factor FliA (σ^{28}) that regulates *flaA* gene expression, are strongly upregulated in the late phases of growth (Heuner et al. 1997). FliA is thought to be a main regulator of flagellar genes. Indeed, further transcriptional analysis of a *fliA* mutant as compared to the wt identified several FliA targets implicated in flagellum biosynthesis or motility (*flaA*, *fliD*, *fliS*, *motY*) (Brüggemann et al. 2006). Upstream of these genes, a FliA-binding consensus sequence was identified, suggesting direct regulation of these genes by FliA. From the expression profiling results, it appears that FliA controls only few targets. However, FliA also controls the expression of genes that were predicted to affect the first steps of cell invasion such as EnhA homologues or a GGDEF/EAL regulator, which may explain lower invasiveness and cytotoxicity of *fliA* mutants and points to an implication of this sigma factor in the infection process (Brüggemann et al. 2006). Further analyses of FleQ and RpoN mutants identified the enhancer-binding protein FleQ as the master regulator of the flagellar regulon (Albert-Weissenberger et al. 2010). Expression of FleQ is probably transcriptionally controlled by the σ70 factor and posttranscriptionally controlled by an unknown factor. Together with the σ54 factor RpoN, FleQ enhances flagellar class II gene transcription, and FleR and RpoN seem to couple protein biosynthesis and metabolism to the requirements of flagellar biosynthesis. Transcription of flagellar class III genes in *L. pneumophila* is solely enhanced by FleQ. As last step in flagellar biosynthesis, expression of the σ28 factor FliA (encoded by *fliA*) induces expression of flagellar class IV genes coding, for example, for flagellin which leads to the completion of the flagellum (Albert-Weissenberger et al. 2010; Brüggemann et al. 2006; Heuner et al. 1995, 1997, 2002; Heuner and Steinert 2003). FliA in *L. pneumophila* seems also to be responsible for a negative feedback loop on flagellar class II and III genes (Brüggemann et al. 2006). This negative control, as a response to the completion of the flagellum, may be an important mechanism used by the cell to turn off flagellar gene expression once the gene products are no longer needed.

The Transcriptional Map of *L. pneumophila* and Identification of Noncoding RNAs

The important implication of noncoding RNAs (ncRNAs) in regulatory processes in bacteria is becoming more and more evident as it has been shown in the last years that ncRNAs control adaptive responses and pathogenesis by regulation of gene expression via transcription interference and termination, translational interference, effects on the stability of target RNA, and interaction with RNA-binding proteins (for a recent review, see Storz et al. 2011). Noncoding RNAs are also important for *L. pneumophila* virulence as shown with the crucial role of RsmY and RsmZ in virulence and life cycle regulation and the implication of 6S RNA (Faucher et al. 2010; Faucher and Shuman 2011; Rasis and Segal 2009; Sahr et al. 2009). First studies attempting to identify noncoding RNAs in *L. pneumophila* used bioinformatics tools and microarray analyses (Faucher et al. 2010). This led to the prediction of 143 putative small RNAs, of which 22 were shown to be expressed as revealed by microarray analyses and six were confirmed by Northern blot and RACE (Faucher et al. 2010). One of these ncRNAs is the widely distributed 6S RNA, which was shown in *E. coli* to bind to RNA polymerase holoenzyme σ^{70} to inhibit transcription, leading to altered cell survival, perhaps by redirecting resource utilization under nutrient-limiting conditions (Wassarman 2007). The 6S RNA of *L. pneumophila* has also an important role, as it is necessary to optimize intracellular replication in regulating positively genes encoding type IV secretion system effectors (Faucher et al. 2010).

Recently, deep sequencing of cDNAs has emerged as a powerful tool to explore these ncRNAs and also to

establish the complete transcriptional landscape of different organisms in a genome-wide manner (Wang et al. 2009). Weissenmayer and colleagues were the first to apply deep sequencing technologies to *L. pneumophila*, which allowed identifying 48 new ncRNAs (Weissenmayer et al. 2011). Thirty-three of these ncRNAs were at least partially complementary to genes encoding proteins, some being known virulence factors like Lpr0020 that is encoded antisense to a gene encoding a homologue of RtxA involved in intracellular survival and modification of trafficking (Cirillo et al. 2001, 2002). However, in addition to cis-encoded sRNAs, also trans-encoded sRNAs have been identified, and the next step will be now to identify the targets of these sRNAs.

A differential RNA sequencing approach was applied to establish the complete transcriptional map of *L. pneumophila*. The *L. pneumophila* operon map contains 623 transcriptional units comprising 1,791 genes organized in two to 32 genes, with an average operon size of 2.8 genes. 2561 primary transcriptional start sites (TSS) were identified. Interestingly, 187 of the 1,805 TSS of protein-coding genes contained tandem promoters, of which 93 show alternative usage dependent on the growth phase. Furthermore, 713 ncRNAs were identified, of which over 60 % are phase dependently regulated (Sahr et al. 2012). This study also identified a new ncRNA regulated by LetA that is part of the LetA-CsrA regulatory cascade, which was named RsmX (Sahr et al. 2012). In the near future, it will be important to characterize the specific roles of sRNA in *L. pneumophila* biology and to identify the sRNA targets and the phenotypes of the mutants that are defective in the expression of these sRNAs.

Metabolomics

L. pneumophila utilizes amino acids as carbon and preferred energy sources (George et al. 1980; Ristroph et al. 1981; Tesh and Miller 1981; Tesh et al. 1983). Underscoring the importance of amino acid metabolism for *L. pneumophila*, bacterial or host cell amino acid transporters are required for intracellular growth (Sauer et al. 2005; Wieland et al. 2005). However, *L. pneumophila* also utilizes carbohydrates as carbon sources. In agreement with this notion, glucose was found to have no effect on growth of *L. pneumophila* in defined media (Pine et al. 1979; Warren and Miller 1979), yet early radiorespirometry studies already suggested that [^{14}C]glucose is consumed and metabolized to pyruvate primarily by the Entner-Doudoroff (ED) and/or the pentose phosphate (PP) pathway, rather than by the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (Tesh et al. 1983). Recent data indicate that intracellular *L. pneumophila* indeed partakes in glucose utilization (Eylert et al. 2010; Harada et al. 2010). Finally, fatty acids are another important determinant of intracellular growth (Edwards et al. 2009).

The completed genome sequences of *Legionella* spp. provided insights into how these bacteria metabolize carbohydrates (Cazalet et al. 2004, 2010; Chien et al. 2004; D'Auria et al. 2010; Glockner et al. 2008; Gomez-Valero et al. 2011; Kozak et al. 2010; Moliner et al. 2009b; Schroeder et al. 2010). The genomes of *L. pneumophila* as well as *L. longbeachae* encode complete pathways required for metabolism of carbohydrates, including the EMP, the ED, and the PP pathway. Moreover, transcriptome studies revealed that during the replicative phase in infected *Acanthamoeba castellanii*, *L. pneumophila* upregulated genes of the ED pathway in addition to components of the respiratory chain and amino acid (Brüggemann et al. 2006). Thus, intracellular bacteria apparently utilize not only proteins and peptides as nutrients but also host carbohydrates.

The catabolism of glucose by *L. pneumophila* grown in broth was studied by isotopologue profiling (Eylert et al. 2010). Using [U-$^{13}C_6$]glucose, these metabolomic experiments revealed that the carbohydrate is indeed used as a carbon source, and the label accumulated in different amino acids, as well as in the storage compound poly-3-hydroxybutyrate (PHB). Moreover, the distinct labeling patterns obtained with [1,2-$^{13}C_2$]glucose identified the ED pathway as the predominant route for glucose amino acid utilization. Accordingly, *L. pneumophila* lacking key components of the ED pathway failed to metabolize glucose. An *L. pneumophila* strain deleted for glucose-6-phosphate dehydrogenase encoded by *zwf*, the first gene of an operon comprising the genes of the ED pathway (*zwf-pgl-edd-glk-eda-ywtG*), did not incorporate label from glucose and was outcompeted by the wild-type strain in coinfection experiments using *A. castellanii* (Eylert et al. 2010). In line with these observations, *L. pneumophila* lacking other components of the ED pathway, either glucokinase (*glk*), phosphogluconate dehydratase (*edd*), 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase (*eda*), or a putative sugar transporter (*ywtG*), was no longer able to metabolize glucose and was defective for growth in *Acanthamoeba culbertsoni* or mammalian cells (Harada et al. 2010). Therefore, the ED pathway is essential for glucose metabolism and intracellular growth of *L. pneumophila*. The results also implicate that the conditions prevailing in host cells within LCVs do not allow *L. pneumophila* to solely grow on amino acids as carbon and energy sources.

Legionella spp. also degrade more complex carbohydrates. *L. longbeachae* harbors a number of genes likely involved in cellulose degradation (Cazalet et al. 2010), and *L. pneumophila* contains genes putatively involved in the degradation of cellulose, chitin, starch, and glycogen (Brüggemann et al. 2006). Moreover, as outlined above, *L. pneumophila* secretes via the T2SS a chitinase, as well as an endoglucanase, which metabolizes carboxymethyl cellulose (DebRoy et al. 2006b). An endoglucanase (CelA) was indeed found to degrade cellulose (Pearce and Cianciotto 2009, p. 48), and a eukaryotic-like glucoamylase (GamA) degraded carboxymethyl cellulose, glycogen, or starch (Herrmann et al. 2011). Yet, neither CelA nor GamA was required for growth of *L. pneumophila* in amoebae. In summary, insights from metabolomics, genomics, transcriptomics, as well as biochemical experiments indicate that *L. pneumophila* utilizes simple and complex carbohydrates as important sources of carbon and energy during extra- and intracellular growth.

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10 Listeria

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Abstract
The genus *Listeria* is a member of the family *Listeriaceae* within the order *Bacillales* and currently comprises ten distinct species. *Listeria* spp. are gram-positive, nonsporulating, facultatively anaerobic bacteria of low G+C content that are present ubiquitously in nature and have been isolated from diverse environmental sources. The genus *Listeria* harbors two pathogenic species, *Listeria monocytogenes* and *L. ivanovii*, and eight nonpathogenic species that include *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. marthii*, *L. rocourtiae*, *L. fleischmannii*, and *L. weihenstephanensis*. Based on their 16S rRNA sequences, *L. fleischmannii* and *L. grayi* branch deeply and are clearly separated from the remaining species. Even though classified as mesophilic bacteria, *Listeria* spp. are characterized by their ability to grow at low temperatures and have high tolerance ranges to both salt concentrations (10%) as well as a broad range of pH. Listeriosis, the infectious disease caused by *L. monocytogenes*, is an extremely serious, life-threatening foodborne disease with high mortality rate documented by several outbreaks. Contamination with *L. monocytogenes* is critical for the food industry and is among the leading microbiological causes of food recalls. The pathogenic bacterium *L. monocytogenes* is morphologically indistinguishable from other *Listeria* species and often causes nonspecific clinical symptoms; therefore, laboratory testing is required to differentiate *L. monocytogenes* from other *Listeria* species and to diagnose listeriosis. Comparative analysis of full genome sequences reveals an open pan-genome and that *Listeria* genome evolution is dictated by limited gene acquisition and gene loss. Based on these analyses, the diversity of modern listerial species evolved through loss of virulence properties rather than virulence gene acquisition.

Taxonomy, Historical and Current

Short Description of the Genus

The genus *Listeria*, together with the genus *Brochotrix*, is a member of the family *Listeriaceae*, the order *Bacillales*, the class *Bacilli*, and the phylum *Firmicutes*. The genus *Listeria* comprises a group of gram-positive bacteria, closely related to the phylum *Firmicutes* including *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, and *Staphylococcus*. *Listeria* are closely related bacteria that share overall morphological and biochemical characteristics. These are small, gram-positive, nonsporulating rods, facultative anaerobic bacteria with an average size of 1–2 µm and are characteristically motile at temperatures below 25 °C (*Fig. 10.1*). *Listeria* test positive for catalase and the Voges-Proskauer reaction and are indole and oxidase negative. *Listeria* can hydrolyze esculin, but not urea or reduce nitrates. *Listeria* exhibit variability in their ability to ferment L-rhamnose, D-xylose, and α-methyl-D-mannopyranoside as well as in the expression of their hemolytic, lecithinase, and phospholipase C activities. The latter assays are used to distinguish pathogenic from nonpathogenic *Listeria*.

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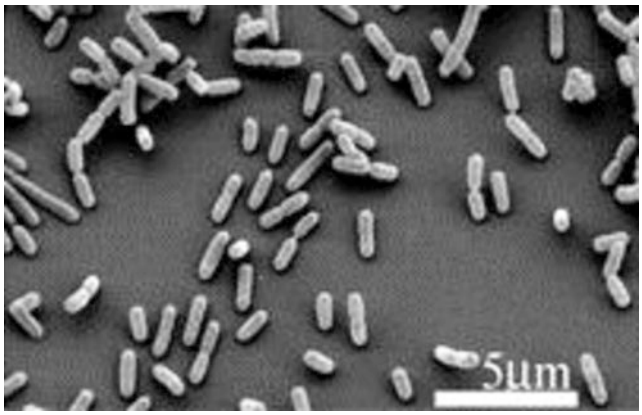

Fig. 10.1
Microscopic morphology of *Listeria monocytogenes* showing smooth rod-shaped cells

In 1911, Hülpers described the identification of gram-positive bacteria from a liver necrosis in a rabbit that were pathogenic for mice. He was unable to classify this bacterium with methods then available and named it *Bacillus hepatica* following its isolation site (Hülpers 1911). In 1926, Murray, Webb, and Swann isolated bacteria from dead laboratory rabbits and guinea pigs which they subsequently named *Bacterium monocytogenes* because of the monocytosis it caused in the animals (Murray et al. 1926). One year later, a bacterium was isolated from wild gerbils with "Tiger River Disease" in South Africa and named as *Listerella hepatolytica* to honor Lord Joseph Lister and as it induced the typical liver infections in experimentally infected animals (Pirie 1927). In Denmark in 1929, Nyfelt reported the first confirmed isolation from humans (Nyfelt 1929). In the 1940s, the present name *Listeria monocytogenes* was established for taxonomical reasons (Pirie 1940).

Prior to the 1980s, listeriosis was a rarity. Since then, the number of reports on the association of *Listeria* isolates with clinical disease increased, and a number of epidemic outbreaks established listeriosis as an important foodborne infection (Bille 1990; Fleming et al. 1985; Linnan et al. 1988; McLauchlin 1987). The first cases of human listeriosis were reported in 1929 in Denmark (Nyfelt 1929). Two basic forms of listeriosis can be distinguished: perinatal listeriosis and listeriosis in the adult patients. The incidence of listeriosis is quite low with 2–15 cases per million population per year, but the high mortality rate of 20–30% in those developing listeriosis (pregnant women, elderly, and immunocompromised persons) makes *L. monocytogenes* a serious human pathogen (Farber and Peterkin 1991; Mead et al. 1999). *L. monocytogenes* causes serious localized and generalized infections. The clinical symptoms often manifest as meningitis, meningoencephalitis, septicemia, abortion, and prenatal infection (Vazquez-Boland et al. 2001). The majority (99%) of the infections caused by *L. monocytogenes* are thought to be foodborne (Mead et al. 1999; Swaminathan and Gerner-Smidt 2007).

Listeria spp. are isolated from a wide range of environmental sources, including soil, water, effluents, food products, decaying vegetation, and feces of animals and human beings. The natural habitat is suspected to be decomposing plant material, where they live as saprophytes in conjunction with other soil bacteria and many invertebrate species. This ubiquity combined with tolerance of *L. monocytogenes* to high concentrations of salt (10% NaCl), broad range of pH (pH 4.5–9), and the ability to multiply at ambient and refrigeration temperatures (0–45 °C) makes *Listeria* a serious threat to food safety. The high mortality rate has resulted in the implementation of stringent regulation to reduce the occurrence of *L. monocytogenes* in the food production chain. In the United States, the presence of $\geq$1 *L. monocytogenes* in 25 g of ready-to-eat food requires a recall (Orsi et al. 2011). The annual costs of recalls due to *L. monocytogenes* food contamination are estimated to be between USD 1.2 and 2.4 billion in the USA (Ivanek et al. 2004). Also, the incidence of listeriosis in Europe is on the increase since 2000 (Allerberger and Wagner 2010). Particularly, the incidence among those >65 years old appears to have increased over the last years. The causes of this increase are not clear but may be related to the relative increase in elderly and immunocompromised populations, use of biologicals in treatments of chronic diseases, and changes in food processing and consumption.

Phylogenetic Structure of the Genus and Its Species

The genus *Listeria* (Group 19, Bergey´s Manual 19th Edition) are facultative anaerobic, nonsporulating gram-positive rods, with low G+C content that have no capsule and are motile at 10–25 °C (Collins et al. 1991; Rocourt 1996; Sallen et al. 1996). Today, the genus *Listeria* comprises of ten species: *L. monocytogenes*, *L. innocua*, *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. marthii*, and *L. rocourtiae* (Seeliger and Jones 1986; Graves et al. 2010; Leclercq et al. 2010). Recently two new additional species, *L. fleischmannii and L. weihenstephanensis*, isolated from cheese from a ripening cellar and from the water plant *Lemna trisulca* from a freshwater pond, respectively, have been described (Bertsch et al. 2012; Lang et al. 2012). These strains are nonhemolytic and have not been associated with cases of human or animal disease.

The relatively small number of species comprising the genus *Listeria* and the clear differences that separate pathogenic from nonpathogenic species make these bacteria attractive as models to examine for the evolution of pathogenicity in this genus. Also, the spectrum of ecological niches occupied by these bacteria, i.e., from the abiotic environment to the intracellular compartments of the infected host cell, raises questions as to how these features have evolved within these species.

Molecular Analysis

Phylogenetic analysis determined by 16S-rRNA sequencing of *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*,

L. seeligeri, *L. ivanovii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grayi*, and *L. fleischmannii* revealed four main groups of the genus. The first group consists of three subgroups indicating the closely related species *L. monocytogenes* (serotypes 1/2a and 4b) and *L. innocua*, the second subgroup is *L. monocytogenes* (serotype 4a) and *L. marthii*, and the third subgroup consists of *L. welshimeri* which reveals the deepest branching of this main group. *L. seeligeri* and *L. ivanovii* occupy the second group, and the third group consists of *L. rocourtiae* and *L. weihenstephanensis*. The fourth major group harboring the recently identified species *L. fleischmannii* and *L. grayi* seems to be very distant from all three groups, and this loose phylogenetic association could warrant the creation of a new genus (Fig. 10.2).

As the pathogenic species *L. monocytogenes* is morphologically closely similar to other nonpathogenic *Listeria* species and often causes nonspecific clinical symptoms, the availability of rapid, specific, and sensitive diagnostic tests capable of distinguishing *L. monocytogenes* from other *Listeria* species is essential for the effective control of listeriosis. Additionally, the fact that *L. monocytogenes* comprises several strains with variations in virulence and pathogenicity (Liu et al. 2003; Roche et al. 2003) makes the development of subtyping procedures critical in order to determine the pathogenic potential of *Listeria* isolates and is essential to limit the spread of listeriosis outbreaks and reduce unnecessary recalls of food products and may render antibiotic therapy unnecessary.

Ribotyping and DNA Hybridization

Using rDNA hybridization analysis on digested chromosomal DNA isolated from listerial strains comprising all serotypes enabled the identification of distinct hybridization patterns, namely, ribotypes, that can be used to automatically analyze and subtype *Listeria* (Wiedmann et al. 1997). Microarray-based profiling of different listerial genomes revealed the presence of four distinct lineages of *L. monocytogenes* that differ in their history of apparent recombination events. *L. monocytogenes* isolates can be grouped into four different evolutionary branches (Brosch et al. 1994; Graves et al. 1994; Ward et al. 2008). Lineage I contains serotypes 1/2b, 3b, 4b, 4d, and 4e that account for more than 98% of all the cases of infection by *L. monocytogenes*, whereby the serotype 4b has been multiply associated with the recent outbreaks of listeriosis. Lineage II contains serovars 1/2a, 1/2c, 3a, and 3c which are clinical isolates but have never been associated with epidemics. Lineage III consists mainly of serotypes 4a and 4c which are significantly more common among isolates from animals with clinical symptoms to human clinical cases (Jeffers et al. 2001). To date, only few isolates from lineage IV exist with a majority of these strains being isolated from ruminants (Ward et al. 2008; Orsi et al. 2011). Studies based on ribotyping and allelic analysis of the virulence genes *hly*, *act*A, and *inl*A as well as on the *prf*A virulence gene cluster of *L. monocytogenes* revealed that strains belonging to lineage I and lineage III are sister groups (Wiedmann et al. 1997; Ward et al. 2004). While it appears that different lineages of *L. monocytogenes* can share habitats, they appear to differ in their histories of horizontal gene transfer events.

Genomic content analysis deriving from a pan-*Listeria* DNA microarray has also revealed that *L. monocytogenes* serovars belonging to lineage I and lineage III are more closely related to each other (Doumith et al. 2004). The similarity of lineage I strains to both lineage III strains (in terms of the genome content and in terms to virulence gene cluster (vgc) sequence) indicates that lineage I is closest to the

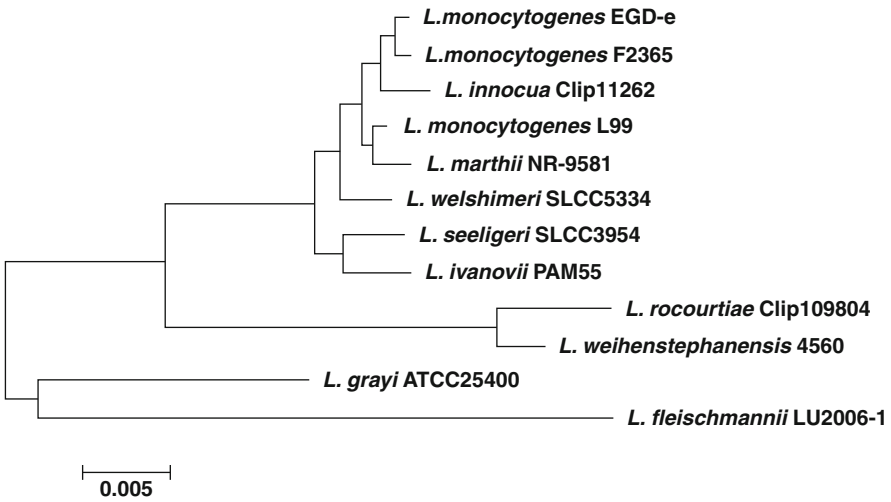

Fig. 10.2

Phylogenetic tree of currently identified species of genus *Listeria* (including 3 lineages of the species *L. monocytogenes*). The tree was based on 16S rRNA gene sequences using the following criteria: partial 16S rRNA genes (~1387 nt each), Clustalw2 alignment, minimum evolution phylogeny (Mega 5), and distance = base substitutions per site (0.005 = 0.5 % = 7 nt)

common progenitor. Lineage II strains vary from the lineage I strains through changes basically associated with the virulence gene cluster.

MLST

Sequence analysis of multiple genes such as genes coding for virulence factors and housekeeping genes is the basis for multilocus sequence typing (MLST) schemes. The phylogenetic structure of *L. monocytogenes* was determined by sequencing internal portions of seven housekeeping genes (3,288 nucleotides) in 360 representative isolates. Fifty-eight of the 126 disclosed sequence types were grouped into seven well-demarcated clonal complexes (clones) that comprised almost 75% of clinical isolates. Each clone had a unique or dominant serotype (4b for clones 1, 2, and 4; 1/2b for clones 3 and 5; 1/2a for clone 7; and 1/2c for clone 9), with no association of clones with clinical forms of human listeriosis. Homologous recombination was extremely limited ($r/m<1$ for nucleotides), implying long-term genetic stability of multilocus genotypes over time. Bayesian analysis based on 438 SNPs recovered the three previously defined lineages, plus one unclassified isolate of mixed ancestry. The phylogenetic distribution of serotypes indicated that serotype 4b evolved once from 1/2b, the likely ancestral serotype of lineage I. Serotype 1/2c derived once from 1/2a, with reference strain EGD-e (1/2a) likely representing an intermediate evolutionary state. In contrast to housekeeping genes, the virulence factor internalin (InlA) evolved by localized recombination resulting in a mosaic pattern, with convergent evolution indicative of natural selection toward a truncation of InlA protein. This work provides a reference evolutionary framework for future studies on *L. monocytogenes* epidemiology, ecology, and virulence.

MALDI-TOF

Differentiation of *L. monocytogenes* from other *Listeria* species and subtyping of *L. monocytogenes* are important tools for epidemiological investigations of food-related illnesses. Species identification is performed traditionally by phenotypic testing, and PFGE is used to subtype isolates into three clonal lineages: lineage I (serotypes 1/2b, 3b, 4b, 4ab, 4d, 4e, and 7), lineage II (serotypes 1/2a, 1/2c, 3a, and 3c), and lineage III (serotypes 4a and 4c). Barbuddhe et al. demonstrated that MALDI-TOF MS accurately identified 146 strains of *Listeria* (representing six species) at the species level and correctly subtyped all strains of *L. monocytogenes*, with the results corresponding to PFGE classifications (Barbuddhe et al. 2008) (Fig. 10.3). These data have been repeatedly validated, and rapid diagnosis of *Listeria* species is now also made by employing mass spectroscopic methods that enable taxonomic characterization down to the level of individual species (Böhme et al. 2011; Farfour et al. 2012).

Comparative Genomics

Over the last decade, *Listeria* genome-sequencing projects have generated an increasing number of *publicly* available closed and

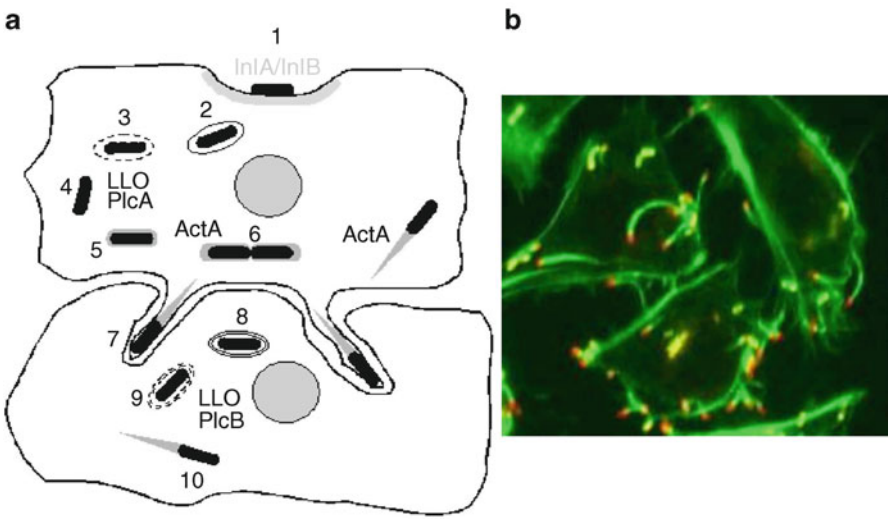

Fig. 10.3

(a) Schematic representation of the cell infectious process by *L. monocytogenes* and the bacterial factors involved (Adapted from Tilney and Portnoy 1989). The entry of *Listeria* into nonphagocytic host cell is triggered by the surface proteins InlA and InlB (other factors are probably involved) (1 + 2). Lysis of the phagocytic vacuole is mediated by LLO and PlcA (3 + 4). The surface protein ActA triggers the actin-based motility of intracellular *Listeria* (5 + 6) and cell-to-cell spread (7 + 8). Lysis of the two-membrane vacuole is achieved by the activities of LLO and PlcB. (b) Immunofluorescence microscopic image of cells (PtK2 epithelial cells) infected with wild-type *L. monocytogenes*

draft genome sequences. Currently, the genome sequences of *L. monocytogenes* (34x) (http://www.ncbi.nlm.nih.gov/genome) and *L. innocua* (4x) (Glaser et al. 2001; den Bakker et al. 2010) are available. Additionally, the genome sequences of individual strains of *L. marthii* (den Bakker et al. 2010), *L. welshimeri* (Hain et al. 2006), and *L. grayi*, as well as three strains of *L. seeligeri* (Steinweg et al. 2010; den Bakker et al. 2010) and three strains of *L. ivanovii* (den Bakker et al. 2010; Buchrieser et al. 2011) have been recently determined.

All currently sequenced listerial genomes are circular chromosomes with sizes that range between 2.7 Mb and 3.2 Mb in which 89% of the genome sequences comprise of coding sequences. All *Listeria* genomes revealed a highly conserved genome synteny, although strain- and serotype-specific genes could be identified. Extrachromosomal DNA such as plasmids is present in both pathogenic and apathogenic *Listeria* species. There are very few inversion events or large shifts of genome segments within the species which could be attributed to the low occurrence of transposons and insertion sequence (IS) elements observed in all sequenced *Listeria* genomes (Hain et al. 2007).

To study in depth the evolution of *Listeria* pathogenicity, genomes of representative strains of three lineages, I, II, and III, of *L. monocytogenes* have been compared (Hain et al. 2012). The strains analyzed in that study included *L. monocytogenes* EGD-e serotype 1/2 a strain, the L99 serotype 4a strain, and two strains of serotype 4b (F2365 and CLIP80459). Strains belonging to the lineages I (serotype 4b) and II (serotype 1/2a) retained all known virulence-associated gene loci, whereas L99 serotype 4a showed extensive virulence gene loss and both reduction and loss of several important surface proteins. The lineage I strains are characterized by the loss of prophage genes, but they share commonly the monocin locus with other *L. monocytogenes* genomes, EGD-e serotype 1/2a and L99 serotype 4a. The essential nature of this prophage for virulence was indicated by the demonstration that chromosomal deletion of mutants in the monocin region of lineage II strain EGD-e serotype 1/2a, which resulted in attenuation of virulence in mice (Hain et al. 2012).

The increasing number of around 46 *Listeria*, both closed and draft genomes, (http://www.ncbi.nlm.nih.gov/genome) has made this bacterium an ideal model microorganism for pan-genomic analysis. Deng and colleagues compared the genomes of 26 *L. monocytogenes* strains (draft and closed genomes) representing the three lineages, I, II, and III, by a comparative genomics approach and by whole genome-based hybridization analysis of a pan-genomic DNA microarray array (Deng et al. 2010). The study showed an open pan-genome pool that contains more than 4,052 genes and a core genome of 2,330–2,456 genes defining the species of this foodborne pathogen (Deng et al. 2010). In a separate study, next-generation sequencing technology was used to assemble seven draft genomes and compare them to 6 publicly available closed genome sequences of *L. monocytogenes* to produce an analysis of the listerial pan-genome (den Bakker et al. 2010). The study indicated that the pan-genome consists of 2,032 core and 2,918 accessory genes and that *Listeria* genome evolution is distinguished by both limited gene acquisition and gene loss. Finally, it was concluded that diversity of modern listerial species evolved through loss of virulence strains rather than virulence gene acquisition (Chakraborty et al. 2000; den Bakker et al. 2010).

Phages

Phages (bacteriophages) are viruses that have the capacities to infect specific bacterial hosts and kill them by sequential internal replication and lysis. Several bacteriophages specific for the genus *Listeria* have been reported, the majority of them deriving from lysogenic *Listeria* strains (Loessner 1991, 1994a, b). Previously, a bacteriophage-based typing system for *L. monocytogenes* was used in epidemiological studies to track the origin and course of listeriosis outbreaks (Audurier et al. 1984). The availability of genome sequences of different *Listeria* strains revealed that bacteriophages cover up to 7% of coding regions (Glaser et al. 2001; Nelson et al. 2004). Comparative genomic sequencing of six *Listeria* bacteriophages from the *Siphoviridae* (A006, A500, B025, P35, and P40) and *Myoviridae* (B054) families has provided detailed information on virion structure, genome organization, bacteriophage integrases, as well as specificity of host insertion sites (Dorscht et al. 2009). This information was used to develop an identification scheme based on the cell wall binding domains (CBDs) of the *L. monocytogenes* bacteriophage endolysins (Eugster et al. 2011). However, more sequencing data is still required to gain detailed insight into the distribution, evolution, and variability of bacteriophages in *Listeria*.

Plasmid Biology

Several species of the genus *Listeria* harbor extrachromosomal DNA such as plasmids (Perez-Diaz et al. 1982). These plasmids can be found in apathogenic species such as *L. grayi* (Perez-Diaz et al. 1982) and *L. innocua* (Glaser et al. 2001) as well as in pathogenic strains of *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, and 4b (Kuenne et al. 2010; Canchaya et al. 2010). Overall, an average of 30% of all *L. monocytogenes* strains harbor plasmids (11–15). Work by Lebrun et al. and McLauchlin et al. showed that plasmid was overrepresented in environmental and food isolates compared to clinical isolates (Lebrun et al. 1992; McLauchlin et al. 1997). Serotype 1 strains were more frequently associated with plasmids than serotype 4 strains. It was also shown that the majority of *L. monocytogenes* plasmid harboring isolates exhibited cadmium resistance, but antibiotic resistances were found to be associated with only two strains (Poyart-Salmeron et al. 1990; Hadorn et al. 1993). A comparative study of 14 *Listeria* plasmids revealed that the common replicon type is related to the theta-replicating plasmid pAMbeta1 (Kuenne et al. 2010). Phylogenetic typing using the replication initiation protein RepA and the gene content of the respective plasmid branches revealed two distinct groups. The listerial plasmids are

characterized by an extended number of different mobile genetic elements and a translesion DNA polymerase gene, both of which might be involved in evolution of plasmids. sRNAs were detectable for some plasmids with homologous genes on the chromosome indicating that plasmids are also responsible for the spread of noncoding RNA molecules via conjugation. In addition, several genes were located on the plasmids conferring heavy metal resistance to cadmium, copper, or arsenite as well as multidrug efflux pumps (MDR, SMR, or MATE) (Kuenne et al. 2010). These genes are responsible for efficient survival in nature and might be acquired to counteract the selective pressure of disinfectants widely used in food-processing plants. Multidrug efflux pumps have also been shown to be involved in transport of cyclic di-AMP which triggers the immune response in the infected eukaryotic host (Woodward et al. 2010). This fact sheds a new light of the role of extrachromosomal DNA due to infection.

Phenotypic Analyses

Biochemical Methods

Listeria species show common biochemical features, i.e., they are catalase positive and indole and oxidase negative and can hydrolyze esculin, but not urea. These characteristics have been used for the delineation of *Listeria* species from other bacteria. Variations in the ability to hemolyze horse or sheep red blood cells and to ferment L-rhamnose, D-xylose, and α-methyl-D-mannopyranoside can be used for species-specific identification of *Listeria* (Robinson et al. 2000) (*Table 10.1*). The pathogenic species *L. monocytogenes* and *L. ivanovii* can be differentiated by the double zone of hemolysis on sheep or horse blood agar and the strong lecithinase reaction with or without charcoal in the medium by *L. ivanovii*. *L. monocytogenes* requires charcoal for its lecithinase reaction (Ermolaeva et al. 2003). Similarly, *L. innocua* and *L. welshimeri* are distinguished from *L. monocytogenes* by their negative β-hemolysis and fermentation characteristics. Generally, the identification of *Listeria* by biochemical methods is time consuming and costly.

Serological Methods

Listeria species possess 15 somatic (O) antigen serotypes (I–XV) and four flagellar (H) serotypes (A–D). Through characterization of group-specific *Listeria* O and H antigens, several serotypes have been recognized in different *Listeria* species (Seeliger and Jones 1986; Kathariou 2002). Serotyping methods are highly discriminative and revealed very early that *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b are responsible for ~99% of human listeriosis cases. However, due to antigen sharing among different *L. monocytogenes* serotypes, a definitive identification of some *L. monocytogenes* serotypes can be challenging. As serotyping methods depend on phenotypic features of *Listeria*, they have been largely replaced by more specific and sensitive molecular methods.

Molecular Methods

This procedure offers more sensitivity than the phenotype-based biochemical and serological methods because it exploits differences at the genetic level. The most common technique for nucleic acid amplification is the polymerase chain reaction (PCR). PCR has the ability to selectively amplify specific targets present in low concentrations (down to a single copy of DNA) and offers both excellent specificity and sensitivity. Additionally, PCR extends the identification to both cultured and noncultivable organisms. Using a two-step PCR with nested primers, one colony-forming unit (CFU) of *L. monocytogenes* could be directly detected in 25 mL raw milk (Herman et al. 1995). There are currently many commercially available kits for both *Listeria* detection and identification. The development of PCR-based serotyping procedures by using group-specific PCR primers has provided additional tools for the identification and serotyping of *L. monocytogenes* (Borucki and Call 2003). Differences within *16S* and *23S* rRNA, *hly, inlA, inlB, iap* genes, and other genes can be used to differentiate *L. monocytogenes* from other *Listeria* species (Aznar and Alarcon 2002).

Virulence-Associated Factors

These assays are helpful to distinguish pathogenic from nonpathogenic *Listeria* species. Laboratory procedures include assessment of enzymatic activities, e.g., lecithinase activity or hemolysis, and molecular methods based on the PCR. The hemolytic activity of listeriolysin (LLO) can be estimated by using sheep blood by comparing zone clearing. The lecithinase activity of PC-PLC is assessed in egg yolk agar plates or on chromogenic media harboring various substrates. The application of virulence-associated proteins for distinguishing *L. monocytogenes* strains is not reliable because these proteins exist in virulent, weakly virulent, and avirulent strains. The use of PCR to target virulence-associated genes such as *inl*A, *inl*B, *plc*A, *plc*B, *act*A, and *hly* shows similar limitations. Also many virulence-associated genes are distributed among pathogenic and apathogenic strains, in vitro studies using Caco-2 epithelial cells showed that variations in the expression of virulence genes and protein production result in different invasion abilities for Caco-2 cells (Chatterjee et al. 2006; Joseph et al. 2006). The assessment of the pathogenic potential of *L. monocytogenes* strains requires simultaneous detection of several virulence-associated genes in combination with in vitro pathogenicity tests. New technologies based on RNA-seq and tiling arrays extend the understanding of the transcriptome and the roles of additional regulators and suggest that, e.g., small noncoding RNAs could be developed as new markers in predicting virulence potential (Toledo-Arana et al. 2009; Mraheil et al. 2011).

Virulence Testing in Infection Models

The pathogenicity of *L. monocytogenes* strains varies from highly pathogenic to almost avirulent. To assess the virulence capacities

Table 10.1

Characteristics for phenotypic distinguishing of *Listeria* species

Characteristic	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. ivanovii*	*L. welschimeri*	*L. grayi*	*L. rocourtiae*	*L. marthii*	*L. fleischmannii*	*L. weihenstephanensis*
G+C content mol%	37–39[a]	36–38[a]	36[b]	38[c]	36[b]	41[d]	41[e]	n.d.[f]	39[g]	41.8[h]
Cell length μm	1.9	2.2	2.2	2.2	2.2	2.2	2.8	n.d.	0.7–1.2	3.2
Optimal growth temperature °C	30–36	32–40	34–36	36–38	34–36	34–40	28–34	32–36	30–37	28–34
Hemolysis	+	–	+	+	–	–	–	–	–	–
Acid production from										
Glucose-1-phosphate	+	–	–	–	–	–	–	–	–	–
Glycerol	+	+	–	+	+	+	+	+	–	+
D-Ribose	–	–	–	+	+	+	+	–	+	+
D-Xylose	–	–	(+)	+	+	–	+	–	+	+
D-Galactose	–	–	–	–	–	+	+	–	–	–
L-Rhamnose	+	+	–	–	–	–	+	–	+	+
Inositol	–	–	–	–	–	–	–	–	–	+
D-Mannitol	–	–	–	–	–	+	+	–	+	+
Methyl-αD-mannopyranoside	+	+	–	–	+	+	+	+	+	–
D-Lactose	+	+	–	–	+	+	+	+	–	+
D-Melibiose	–	–	–	–	–	–	+	–	–	–
Sucrose	+	+	–	+	+	–	(+)	+	(+)	–
D-Melezitose	–	–	–	+	+	–	–	–	+	–
Amidon	+	+	–	–	+	+	+	+	–	+
Xylitol	+	+	–	(+)	+	(+)	+	+	+	+
D-Turanose	+	+	–	+	–	(+)	(+)	+	+	–
D-Lyxose	–	–	–	–	–	+	–	–	–	–
D-Tagatose	–	–	–	–	+	–	–	–	–	–

Adapted from Lang et al. (2012)

[a]Ludwig et al. (2009)

[b]Rocourt and Grimont (1983)

[c]Seeliger et al. (1984, 1983)

[d]Rocourt et al. (1992)

[e]Leclercq et al. (2010)

[f]Graves et al. (2009)

[g]Bertsch et al. (2012)

[h]Lang et al. (2012)

of *Listeria* strains, properties such as hemolytic activity invasion and growth assays in tissue culture infection experiments, as well the detection of virulence-associated genes, are often performed.

Animal models have made significant contribution to our understanding of host-pathogen interactions and the cellular response to bacterial infections. Unfortunately, animals naturally infected by *Listeria* are not classical laboratory animals but are generally bovine, ovine, and caprine species. However, the widely used mouse model has been established as an infection model for *Listeria* already in the early 1960s and was instrumental in developing the concept of cell-mediated immunity as an integral component of host defense systems in eliminating and controlling intracellular parasites in immunology (Mackaness 1962, 1969; Miki and Mackaness 1964; North 1970, 1978). The mouse bioassays are usually performed by inoculating mice with various doses of *L. monocytogenes* by mainly intravenous (IV), intraperitoneal (IP), and more rarely subcutaneous, conjunctival, intranasal, or intracerebral. In addition to mice, guinea pigs, rabbits, and rats have also been used as an infection model for *Listeria* infection (Schlech et al. 1993; Gray and Killinger 1966).

As several essential determinants of the innate immune response to microbial infection are conserved between insects and mammals, infection models employing invertebrates to assess microbial virulence have gained interest over the past years. The use of the greater wax moth, *Galleria mellonella*, has emerged as a reliable model to investigate the *Listeria* pathogenesis (Mukherjee et al. 2010; Joyce and Gahan 2010). Using the *Galleria* model to investigate the differences between infections caused by strains with different virulence potentials in the mouse infection model revealed a strong correlation with virulence previously determined by the mouse model (Mukherjee et al. 2010). In comparison to animal models, the rearing costs of the *Galleria* model are very low, and an important advantage of *Galleria* model in contrast to other insect infection models is the possibility to examine bacterial infection at 37°C since it is at this temperature that most human pathogens induce their virulence factors.

Isolation, Enrichment, and Maintenance Procedures

Isolation

Ubiquity, tolerance to high salt concentration, and broad range of pH values and especially the ability to multiply at a broad range of growth temperatures (0–45 °C) make *Listeria* a serious threat to food safety. Historically, it has been challenging to isolate *Listeria* from food or other samples, and this explains why it remained unnoticed as a major food pathogen until recently. One of the earliest enrichment methods was based on the ability of *Listeria* to grow at low temperatures. This cold enrichment technique (Gray et al. 1948) remained the only available method for many years. The samples were incubated on agar plates at 4 °C for long time periods, up to several weeks, primarily to isolate the pathogen from infected animal or human tissue. The key issues, enrichment/isolation time, and the recovery of stressed *Listeria* cells were addressed when further methods of enrichment and isolation were developed.

Several foodborne outbreaks of listeriosis led to the implementation of a zero-tolerance level (absence in 25 g of a food sample). Therefore, tests must be able to detect one *Listeria monocytogenes* per 25 g of food and ready-to-eat food if it is to be approved by the regulatory agencies. To avoid overgrowth by competing microbiota, enrichment media and selective agar for *Listeria* include antibacterial compounds such as nalidixic acid and acriflavine that specifically suppress the growth of competing microflora (Gasanov et al. 2005).

The recovery of *L. monocytogenes* from several sources requires the use of enrichment cultures followed by selective plating, and where injured organisms are likely to be present, a preenrichment step is required (Curtis and Lee 1995). For the detection of *Listeria* in all foods, two culture reference methods are most frequently used. Both methods, the FDA bacteriological and analytical method (BAM) and the International Organization of Standardization (ISO) 11290, are sensitive but often time consuming, laborious, and may take 5–6 days before the result is available.

The FDA-BAM method starts with enrichment of sample (25 g) for 48 h at 30 °C in *Listeria* enrichment broth (LEB) (Lovett et al. 1987) containing the antibacterial agents acriflavine, nalidixic acid, and the antifungal agent cycloheximide followed by plating onto selective agar (Oxford, PALCAM, MOX, or LPM) (Gasanov et al. 2005). The ISO 11290 method employs a two-stage enrichment process: the first enrichment in half Fraser broth (Fraser and Sperber 1988) for 24 h, then an aliquot is transferred to full-strength Fraser broth for further enrichment followed by plating on Oxford and PALCAM agars. Similar to LEB, Fraser broth also contains the selective agents acriflavine and nalidixic acid as well as esculin, which allows detection of β-D-glucosidase activity by *Listeria*, observed as blackening of the growth medium (Gasanov et al. 2005). The concentration of acriflavine and nalidixic acid occur in primary or preenrichment broths are usually lower to support the resuscitation of possibly injured cells. Both primary and secondary broths contain a phosphate-buffering system.

The USDA and Association of Analytical Chemists (AOAC/IDF) methods use a modification of the University of Vermont medium (UVM) (Donnelly and Baigent 1986) containing acriflavine and nalidixic acid for primary enrichment, followed by secondary enrichment in Fraser broth and plating onto modified Oxford (MOX) agar containing the selective agents moxalactam and colistin sulfate (Gasanov et al. 2005).

While the FDA method is optimized for processing dairy products, the USDA method was designed and has been officially recommended primarily for meat and poultry products (Brackett and Beuchat 1989), the latter being slightly superior for detection of *L. monocytogenes* in foods and environmental samples. The change of the final Oxford and PALCAM plating agar to *L. monocytogenes* blood agar (LMBA) improved the detection rate of *L. monocytogenes* in milk using the FDA method (Kells and Gilmour 2004).

Selective or Differential Plating Media

The first *Listeria*-selective agar (LSA) included potassium tellurite to inhibit growth of gram-negative bacteria (Gray et al. 1950). Another *Listeria*-selective agar known as McBride *Listeria* agar (MLA) was developed by substituting phenyl ethanol agar containing lithium chloride, glycine, and blood (McBride and Girard 1960).

The FDA method uses modified McBride *Listeria* agar (MMLA), while the USDA method includes LiCl-phenylethanol-moxalactam agar (LPMA) as isolation agars (Brackett and Beuchat 1989). A set of other media were developed which include Oxford agar (Curtis et al. 1989), LiCl-ceftazidime agar, modified (LCAM) (Lachica 1990), polymyxin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol-egg yolk (van Netten et al. 1989), and enhanced hemolysis agar (EHA) (Curtis et al. 1989).

Chromogenic Media

The pathogenic *Listeria* species *L. monocytogenes* and *L. ivanovii* produce the enzyme phosphatidylinositol-specific phospholipase C. This enzyme hydrolyzes a specific substrate added to the medium and thereby produces an opaque halo around the colonies. Another enzyme, β-glucosidase, is common in all *Listeria* spp. and can be used for the detection of all *Listeria* colonies. The commercially available chromogenic media include "Agar *Listeria* according to Ottaviani and Agosti," BCM *L. monocytogenes* detection system, CHROM agar, and Rapid'L.mono. Chromogenic media are simple, cost effective, and easy to interpret (Gasanov et al. 2005).

Ecology

Listeria is ubiquitously distributed throughout the environment including soil, water, sewage, vegetation, and wild animal feces as well as on the farm and in food-processing facilities (Sauders et al. 2006). Several types of vegetables have been reported to act as vehicles for transmission of *Listeria*. The sources for contamination of these vegetables with *Listeria* are soil contaminated with infected waste of domestic animal. Animals are infected via consumption of spoiled feeds and can transfer this pathogen to the soil environment via their infected feces (Katherine 2006). Generally, vegetables are contaminated during primary production, processing, distribution, and preparation (Moreno et al. 2012; Oliveira et al. 2011). Many of these vegetables are used as salads which increase the risk of foodborne infection with *Listeria* (Cordano and Jacquet 2009). *L. monocytogenes* has the ability to adhere to and grow on a variety of surfaces found in food-processing plants including stainless steel, rubber, glass, and polypropylene (Lunden et al. 2000) resulting in biofilm formation. The cells in a biofilm following detachment may contaminate the processed food products and can survive cleaning and sanitization. Bacteria in biofilms have been reported to be generally more resistant to antimicrobial substances than planktonic bacteria.

Pathogenesis and Clinical Relevance

The infection model of *Listeria* in mouse has made a significant contribution in the investigation of cellular immune response. Studies based on the murine model of listeriosis were the first to indicate the importance of activated macrophages in the elimination of intracellular parasites and that T-cells are required for cell-mediated immunity (Mackaness 1969; North 1970; Shen et al. 1998). Listerial virulence depends on a chromosomal 9-kb cluster harboring six genes (virulence gene cluster, vgc), *prfA, plcA, hly, mpl, actA*, and *plcB*, all of which are known to be under the direct or partial control of positive regulatory factor A (PrfA) (Chakraborty, et al. 1992; Mengaud, et al. 1991). The vgc is generally absent from the nonpathogenic *Listeria* species such as *L. innocua*, *L. welshimeri*, and *L. grayi*. Exceptions include the detection of naturally occurring *L. innocua* strains that harbor all elements of the vgc cluster (Johnson et al. 2004). The products of these genes enable escape from phagolysosomes and promote intra- and intercellular movement (Portnoy et al. 1992). Introduction of the vgc locus into nonpathogenic *L. innocua* renders these bacteria capable of escape from the phagosome as well to exhibit actin-based intracellular motility (Mohamed et al. 2012). The expression of the virulence regulon is modulated via PrfA by several environmental and growth-phase-dependent signals. The activating signals include high temperature (37 °C) (Leimeister-Wachter et al. 1992), stress conditions (Sokolovic et al. 1990), sequestration of extracellular growth medium components by activated charcoal (Ripio et al. 1996), contact with host cells, and the cytoplasmic environment of the infected host cell (Bubert et al. 1999; Freitag and Jacobs 1999; Moors et al. 1999; Renzoni et al. 1999).

The first step in host infection is the internalization of *L. monocytogenes* either by phagocytosis in the case of macrophages or induced phagocytosis (invasion) in the case of otherwise nonphagocytic cells. *Listeria* is able to infect most cell types, including epithelial, hepatocytes, fibroblasts, and endothelial cells (Vazquez-Boland et al. 2001). Bacterial invasion starts by the interaction with the plasma membrane, which progressively enwraps the bacterium. This process usually refers to the "zipper" mechanism in contrast to the "trigger" mechanism used by *Salmonella* or *Shigella*.

Internalins A and B (InlA-B) are cell wall-associated proteins which mediate the invasion to nonphagocytic cells (Braun and Cossart 2000). The InlA protein promotes invasion of *L. monocytogenes* into epithelial cells via engagement of its receptor, the human E-cadherin molecule (Gaillard et al. 1991), while InlB binds to the HGF (hepatocyte growth factor) tyrosine kinase receptor Met to effect entry into a broad range of cell types (Cossart and Lecuit 1998; Braun and Cossart 2000). In addition to the internalins, several other cell surface proteins including Vip, Ami, and ActA have been implicated in

promoting adhesion and entry into various cell types (Cabanes et al. 2002, 2005).

L. monocytogenes is adapted to life in the host cytosol but is unable to replicate in the phagosomal compartment. Early after internalization, the bacteria disrupt the phagosomal membrane of the host by producing a pore-forming toxin, listeriolysin (LLO), and a phospholipase (PlcA) to access the cytoplasm of the host cell. Intracellular movement of the bacteria inside the host cell is mediated by ActA, which polymerizes the host actin molecules and propels itself inside the cytosol of the host cell (*Fig. 10.4*). ActA and InlK have also recently been shown to enable infecting bacteria to escape autophagy (Yoshikawa et al. 2009; Dortet et al. 2012). Spreading from one cell to another is dependent on hemolysin (Hly) and another phospholipase (PlcB) (Portnoy et al. 1992; Vazquez-Boland et al. 2001). Thus, once *Listeria* access the host cell cytoplasm, it can disseminate from cell to cell, circumventing host defenses such as circulating antibodies and complement. This ability to disseminate in tissues by cell-to-cell spreading provides an explanation for the early observation that antibody is not protective and that immunity to *Listeria* is T-cell mediated (Cossart and Lecuit 1998).

Disease in Humans and Animals

Generally, the only species considered pathogenic to humans is *L. monocytogenes*. However, some reports are available implicating *L. ivanovii* (Cummins et al. 1994; Snapir et al. 2006; Guillet et al. 2010) and *L. grayi* as a cause of disease in humans (Todeschini et al. 1998). A severely immunocompromised patient with advanced Hodgkin's disease suffering from bacteremia caused by *L. grayi* has been reported (Todeschini et al. 1998).

L. monocytogenes is an important pathogen in Europe and the United States for neonates where it has high case-fatality rate of around 20–30% (Braden 2003). Rarely, *L. ivanovii* and *L. seeligeri* have also been associated with disease in humans (Lopez et al. 2008). According to data from FoodNet, listeriosis was responsible for 30% of foodborne deaths from 1996 to 2005 and had a case-fatality rate of 16.9% (Barton et al. 2011). A recent outbreak in the United States between September and December 2011 implicated the consumption of contaminated cantaloupes, which involved at least 146 infected individuals with 30 individuals succumbing to severe infection (CDC 2011).

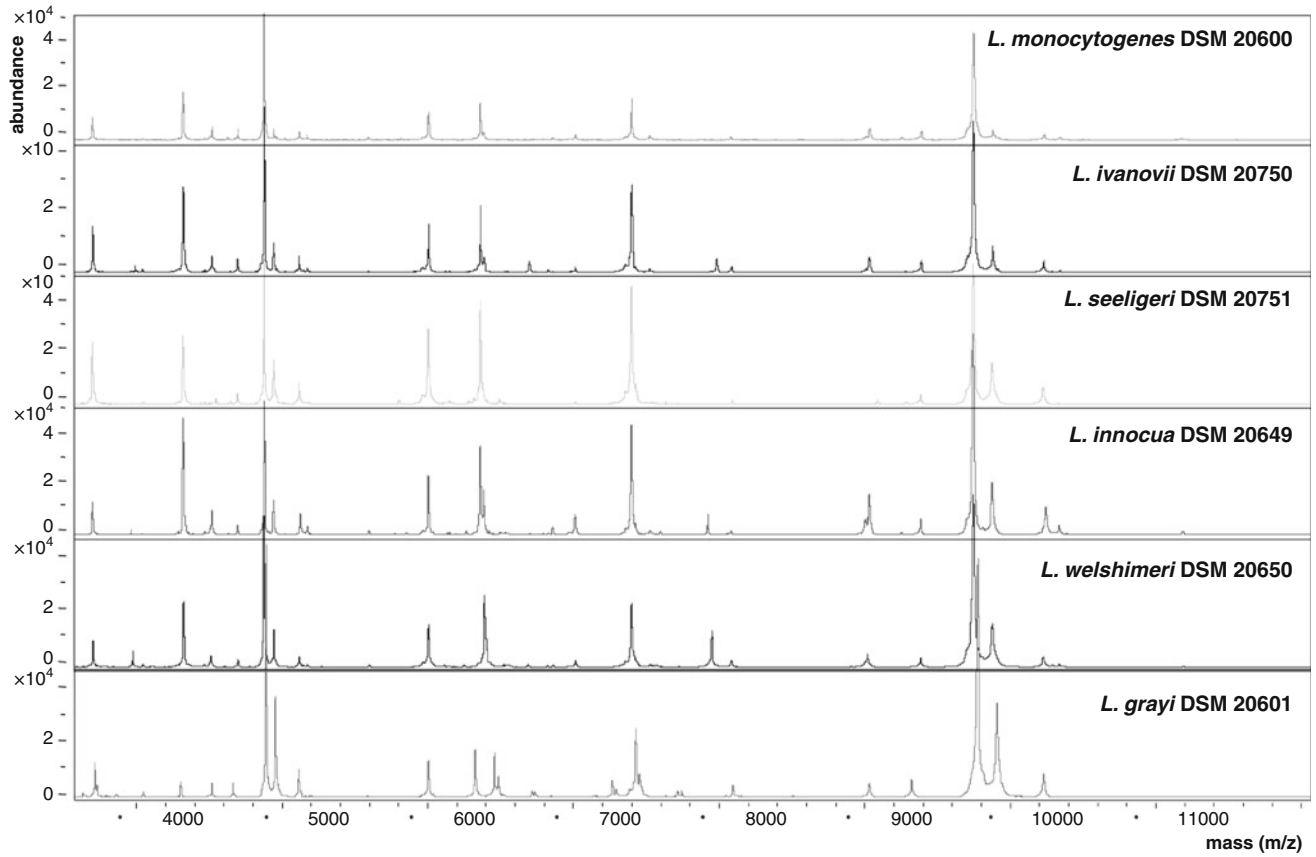

Fig. 10.4

MALDI-TOF MS spectra of whole-cell extracts of *Listeria* reference strains. Spectra representative of the MS profile were obtained from the German Collection of Microorganisms and Cell Cultures. The absolute intensities of the ions are shown on the *y*-axis, and the masses (in Da) of the ions are shown on the *x*-axis. The *m/z* value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular weight of the protein (Adapted from Barbuddhe et al. 2008)

An interesting aspect of this outbreak was the involvement of at least four different outbreak strains (FDA 2011).

Human listeriosis can take several forms including invasive or noninvasive (Nightingale et al. 2005b). For healthy adults, it is mainly manifested as diarrhea and fever; for pregnant women, fever, diarrhea, abortion, or stillbirth; and for newborn septicaemia pneumonia, or meningitis (Todd and Notermans 2011). The noninvasive form results in milder food poisoning symptoms such as fever, headache, and diarrhea, often referred to as febrile gastroenteritis (Berrada et al. 2006). In addition to these, listeriosis may manifest as a local infection including dermatitis, endocarditis, pericarditis, perinephric abscess, pneumonia, peritonitis, arthritis, hepatitis, and endophthalmitis (Gomber et al. 1998; Kida et al. 2007; Revathi et al. 1995; Oevermann et al. 2010). Abscesses in the cerebrum or cerebellum are less common CNS manifestations, and in up to 24% of patients, encephalitis targeting the brain stem (rhombencephalitis) has been reported (Mylonakis et al. 1998; Antal et al. 2005; Armstrong and Fung 1993). Persons with a predisposing condition having immunocompromised status include individuals with cancer malignancies, organ transplant, liver disease, HIV/AIDS, and diabetes and can develop sepsis, meningitis, and serious infections affecting the nervous system (Todd and Notermans 2011). Interestingly in Europe, the incidence of listeriosis has been reported to be increased since 2000 (Allerberger and Wagner 2010), particularly, among those >65 years old. A high prevalence of *L. monocytogenes* peritonitis has been reported in cirrhotic patients of an Egyptian medical center (El Sayed et al. 2011). A large proportion of fecal samples collected from healthy animals with no clinical symptoms of listeriosis may contain *L. monocytogenes* (Wesely 1999).

L. monocytogenes causes invasive and often fatal disease including CNS infection in numerous animal species including farm ruminants, horses, dogs, pigs, deer, South American camelids, and cats (Oevermann et al. 2010). *L. ivanovii* is considered only mildly pathogenic and seems to affect almost exclusively ruminants, causing abortion, stillbirths, and neonatal septicemia, but not CNS infections (Low and Donachie 1997; Vazquez-Boland et al. 2001). Infections with *L. ivanovii* in ovines and bovines have been reported (Alexander et al. 1992; Gill et al. 1997; Chand and Sadana 1999).

Among animals, spontaneous abortions, subclinical mastitis, meningoencephalitis, and endometritis were the commonest forms reported (Barbuddhe et al. 2012). In ruminants, rhombencephalitis is the most common clinical expression of listeriosis and the most common CNS disorder (Oevermann et al. 2008).

L. monocytogenes can be shed in the feces of clinically affected animals but also healthy carriers (Mohammed et al. 2009; Ho et al. 2007; Borucki et al. 2004) contributing to amplification and dispersal of *L. monocytogenes* into the farm environment (Nightingale et al. 2005a). Raw milk and milk products might contain *L. monocytogenes* either as a consequence of bacterial shedding in the milk or due to exogenous contamination from the dairy farm environment (Van Kessel et al. 2004; Pintado et al. 2009; Jayarao et al. 2006).

Isolation in Human Infections

Human listeriosis is principally a foodborne infection. Most of the reported outbreaks of listeriosis in humans are attributed to the consumption of contaminated products of animal origin (Bille et al. 2006; Van Kessel et al. 2004). Risk of infection with *L. monocytogenes* may increase by use of anti-TNF-α drugs used to curb chronic inflammatory states such as in arthritis but has the undesired side effect of promoting of microorganisms. Cases of *L. monocytogenes* meningitis in ulcerative colitis patients under infliximab plus steroids have been reported (Abreu et al. 2012).

Listeria as a Gastrointestinal Pathogen

The gastrointestinal tract is thought to be the primary site of entry of pathogenic *Listeria* into the host by consumption of contaminated food (Farber and Peterkin 1991; Pinner et al. 1992). *L. monocytogenes* uses the internalins InlA and inlB to induce internalization in nonphagocytic cells by binding in a species-specific manner the adhesion molecule E-cadherin and the hepatocyte growth factor receptor Met, respectively (Mengaud et al. 1996; Shen et al. 2000). The InlA-E-cadherin interaction plays a key role for *L. monocytogenes* in the crossing of both the intestinal and the placental barriers. E-cadherin is only expressed by a limited number of cell types, mostly of epithelial origin. It has been reported that InlA (Lecuit et al. 2001), but not InlB (Khelef et al. 2006), plays a critical role in the crossing of the intestinal barrier after infection.

Once the host cells are infected, the bacteria cross the intestinal wall at Peyer's patches and invade the mesenteric lymph nodes and the blood and end up in liver, where the bacteria multiply inside hepatocytes (Vazquez-Boland et al. 2001).

To survive the high acidic environment in the stomach, *Listeria* produce a set of molecules which are under the control of the stress response regulator σ^B. The glutamate decarboxylase system (GAD) plays an essential role in pH resistance in *Listeria*. The passage of *Listeria* from stomach to the small intestine along with food brings the bacteria in an environment of high salinity (0.3 M NaCl). Several studies have confirmed the role of osmolytes in increasing the osmotolerance of *Listeria*. The uptake of osmolytes counteracts the turgor pressure and prevents or suppresses water loss from the cytoplasm (Sleator and Hill 2002). During migration into the intestine, *L. monocytogenes* is exposed to the bactericidal activities of bile salts. In *L. monocytogenes*, Bsh (for bile salt hydrolase) and BtlB (for bile tolerance locus B which possesses bile acid dehydrogenase activity) were identified to be involved in the detoxification of bile salts (Begley et al. 2005; Dussurget et al. 2002). A bile exclusion system, BilE that enables *Listeria* to survive at high bile salt has been identified (Sleator et al. 2005). All three proteins, Bsh, BtlB, and BilE, were found to be regulated by the main virulence regulator PrfA.

Listeria as a Pathogen of the Central Nervous System

CNS infections in humans are often observed as meningitis and meningoencephalitis followed by rhombencephalitis (brain stem infections) as well as brain abscesses. Clinical features of *L. monocytogenes* meningitis may have a subacute course and are observed as changes in consciousness, behavior, and even seizures. CNS infections are common in ruminants and occur through contact with contaminated silage and are usually associated with meningitis and abortions. Clinical signs such as "circling disease" in sheep are lined to lesions of the CNS, and recent data suggest that neurolisteriosis may be very common among ruminants ranging from 200 to 500 cases per million of ruminants. A hematogenous route of infection is thought to be the major pathway leading to meningoencephalitis, while for rhombencephalitis, a neural retrograde pathway has been proposed especially for ruminants.

Applications, Biotechnology, and Vaccines

L. monocytogenes represents one of the most well-studied and characterized microorganisms in bacterial pathogens and is an important model for the study of host-pathogen interactions and bacterial adaptation to mammalian hosts (Cossart 2011). The vast knowledge that has been accrued as to how these bacteria invade cells, move intracellularly, disseminate in tissues and organs, and escape host cell and organ-based immune defenses provides a mine of information which can now be harnessed for biotechnological applications.

As *L. monocytogenes* is an intracytosolic pathogen, it is capable of efficiently inducing both CD4 (+) and CD8 (+) T-cell responses. Thus, it has been viewed as an attractive vector for the delivery of heterologous antigens for both class I and class II antigen-processing pathways (Singh and Paterson 2006). To improve its usefulness as a vaccine vector, several strategies have been developed. These include deletion of virulence genes to generate attenuated vaccine strains (Darji et al. 2003) or the introduction of the virulence gene cluster (vgc) from *L. monocytogenes* into a nonpathogenic *L. innocua* strain to generate a vehicle with potent vaccinogenic properties (Mohamed et al. 2012). Furthermore, dead but metabolically viable *L. monocytogenes* preparations, following psoralen inactivation of an *L. monocytogenes* strain harboring a *uvrAB* mutation, have been demonstrated to be protective (Brockstedt et al. 2005). *L. monocytogenes* has also been used to transfer eukaryotic expression of plasmids to mammalian host cells both in vivo and in vitro. This can be used to induce immune responses toward protein antigens encoded by the plasmid, to complement genetic defects, or even direct the production of protein in targeted organs (Weiss and Chakraborty 2001). Functional transfer of CFP-CFTR (cystic fibrosis transmembrane conductance regulator) to eukaryotic cells using attenuated *L. monocytogenes*-mediated gene transfer (bacteriofection) has been demonstrated (Zelmer et al. 2005; Krusch et al. 2002). These properties show promise for the development of *L. monocytogenes* as novel drug and delivery system capable of use in many different clinical settings because of its ability to induce immunological responses. *Listeria*-based vaccines have been successfully deployed in mouse models to control tumor growth and induce potent antitumor immune responses (Singh et al. 2005). A novel vaccine concept uses modified *Listeria* able to express a number of HIV/SIV antigens as a live bacterial vaccine vector for the delivery of HIV antigens (Paterson and Johnson 2004). Listerial LLO can be used as an efficient vaccine delivery system carrying a viral antigenic protein to generate protective antiviral immunity (Mandal et al. 2004).

Outlook and Perspective

Listeria remains among the deadliest known foodborne pathogens worldwide. Important progress has been made in the detection and classification of *Listeria*, at the species level and even discrimination between strains associated with human and animal infections or even sporadic from epidemic strains. Although *Listeria* is one of the best models in pathogenesis, several aspects of the *Listeria* infectious process remain unclear. The multifaceted virulence factors, e.g., LLO, suggest more complexity in the infection process than previously thought. Furthermore, mechanisms responsible for the persistence in the environment and in food-processing plants and ability to grow at refrigeration temperatures should be elucidated. The advent of functional listerial genomics combined with transcriptional analysis (RNA-seq) is providing new possibilities to comprehensively catalog genes and their products involved in the transition from life in the environment to life within infected cells. The continuously decreasing costs and increasing speed for genome sequencing will provide us with an opportunity to define the evolutionary paths taken to pathogenesis and help us understand the emergence of *Listeria* spp. as major foodborne pathogens. Understanding of the sophisticated strategies used by *Listeria* to overcome host cell defenses will lead to novel biotechnological applications. Indeed, the term "patho-biotechnology" has been coined to exploit our understanding of the valuable traits exhibited by these pathogenic bacteria during their growth in the environment or during infection into applications in biotechnology, medicine, and food processing (Sleator and Hill 2007).

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11 Diphtheria

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Abstract
Diphtheria is a paradigm of an infectious disease caused by the toxigenic bacteria *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*. Classical diphtheria of the upper respiratory tract, which is transmitted by breathing or coughing, is characterized by a thick pseudomembrane, which forms as a result of cell damage and fibrin deposition. In severe cases, obstruction of airways results in suffocation and death. In tropical and subtropical regions respiratory tract diphtheria is outnumbered by diphtheria of the skin. Common sites for this cutaneous form, which is characterized by lesions covered by a smeary coating, are the lower legs, feet, and hands.

Today the infection can be eliminated easily using antibiotics and the detrimental exotoxin can be neutralized by an antitoxin. Nevertheless, immunization using a highly effective toxoid vaccine is the mean of choice for diphtheria control. Despite the advances made in diphtheria therapy and control, outbreaks reaching even epidemic proportions have been observed during the last decades. Today, several thousand diphtheria cases per year are reported to the World Health Organization, showing that diphtheria is not completely eradicated and that reservoirs exist. These are, for example, groups of people with insufficient access to medical care and also animals seem to play a role as reservoirs of the infection. Isolation of *C. diphtheriae* strains were reported from domestic cats and horses and animal reservoirs are even more common, when *Corynebacterium ulcerans* is taken into consideration, which has been detected as a commensal in various domestic and wild animals.

Historical Retrospective

Diphtheria has been frightening people since ancient times due to its high mortality rate and the dreadful death by suffocation caused by this disease. As an additional terror for parents, especially infants up to 5 years of age were preferred victims of this infection, which was also designated as "strangling angel of children." Documents describing diphtheria are known already from Babylonian and Sumerian times and can be found in the Talmud and in writings by Hippocrates. Most likely, diphtheria was disseminated from the Middle East to Europe and later to all other continents. Numerous waves of epidemics occurred in Europe, often in cycles that include gaps of 100 years and more (English 1985). Epidemic outbreaks of diphtheria were reported, for example, from Germany in several regions in 1517, from Paris in 1578, from Toledo (Spain) in 1610–1614, and from Southern Italy in 1610. Other reports are from Sweden in the 1760s and from Berlin (Germany) in 1758 and 1759. In the eighteenth century, diphtheria reached the east coast of North America. Diphtheria outbreaks were reported during the independence war, and in 1799, George Washington also became a victim of this disease. From ancient times to the nineteenth century, there were almost no means to cure diphtheria and its occurrence was unpredictable.

The situation changed when in the late nineteenth century, diphtheria became a paradigm of an infectious disease and provided key evidence for Koch's postulates on the germ theory (Dolman 1973; Levy 1973; English 1985; Murphy 1996). In 1871, Oertel showed the formation of a pseudomembrane in a rabbit's throat after swabbing it with material from a human diphtheria patient, and in 1883, Klebs noted two different types of bacteria within the pseudomembrane, bacilli and cocci, delivering the first description of *Corynebacterium diphtheriae* (Klebs 1883). Based on Klebs' work, Loeffler was the first to grow *C. diphtheriae* in pure culture (*Fig. 11.1*) and found that bacilli and cocci observed by Klebs cause inflammatory symptoms but only the bacilli evoke diphtheria. He was able to pass the disease from guinea pig to guinea pig and found that even after more than 20 passes, diphtheria-like symptoms were still developed by the animals. Loeffler additionally recognized that *C. diphtheriae* colonizes the membranes of the nasopharyngeal cavity but not deeper parts of the body and correctly postulated that the secretion of a toxin by *C. diphtheriae* is causing the often fatal damage

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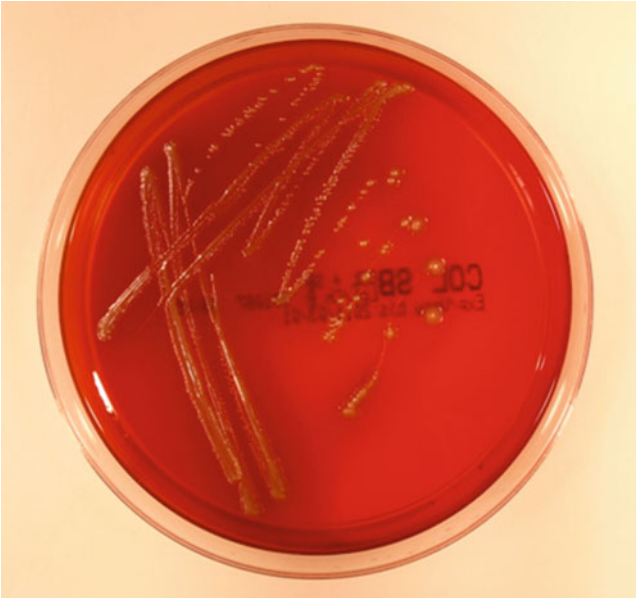

Fig. 11.1
Streak out of *Corynebacterium diphtheriae* on an agar plate containing 5 % sheep blood

of internal organs (Loeffler 1884). This hypothesis was supported by experiments carried out by Roux and Yersin (1888), which showed that sterile filtrates of *C. diphtheriae* cultures can evoke organ damages that are indistinguishable from that of human diphtheria after injection in animals.

It was also Loeffler who delivered the first epidemiological study on diphtheria showing that 5 % of the schoolchildren in Berlin (Germany) were carriers of *C. diphtheriae* without showing diphtheria-like symptoms. Interestingly, diphtheria became more prevalent within the next three decades (English 1985; and references therein) and developed into a leading cause of infant mortality. Up to four fifth of children infected with diphtheria died. This situation improved dramatically after the development of an antitoxin by von Behring and the introduction of vaccines.

In 1890, von Behring published his pioneer work on diphtheria and tetanus and suggested antitoxin application as a means of diphtheria treatment (von Bering 1890; for overview, see Grundbacher 1992). He was also the first who used antitoxin from a horse to successfully treat diphtheria. In 1913, von Bering developed the vaccine against diphtheria toxin, and widespread vaccination started in 1920. The basis of the vaccine is the diphtheria toxoid, a formaldehyde-inactivated diphtheria toxin, produced and secreted in vast amounts by strain Park-Williams no. 8 (PW8), originally isolated from a mild case of diphtheria (Park and Williams 1896; Iwaki et al. 2010). Immunization with the inactivated toxin that remains antigenically intact is extraordinarily effective, and vaccination of infants changed diphtheria from a former main cause of infant mortality to an extremely rare disease, which will never be seen by most pediatricians or other physicians (English 1985). Today, diphtheria is almost eradicated with only a handful of cases occurring per year in industrialized countries of Western Europe or Northern America but still a health threat in mainly Asian countries with between 3,000 and 11,000 annual cases in the years 2000–2008 according to the World Health Organization.

While classical diphtheria of the respiratory tract became rare with the introduction of vaccination programs in the first decades of the twentieth century, during World War II, cutaneous diphtheria was a serious problem among American and European troops stationed in the Mediterranean, South Pacific, and Asian regions (Liebow et al. 1946; Livingood et al. 1946; Höfler 1991). The waning immunological protection against the toxin in adults and the infection with non-toxigenic strains might be an explanation of this phenomenon.

Transmission and Clinical Manifestation of Diphtheria

Diphtheria is an infectious disease which is typically spread from person to person by close physical and respiratory contact. Bacteria are distributed by respiratory droplets produced by coughing or sneezing. Establishment of the disease after infection takes 2–5 days. Untreated patients are infectious for 2–3 weeks; application of antibiotics (see below) usually renders patients noninfectious within 1 day. Besides asymptomatic carriers and humans with skin infections, animals also might be a reservoir of diphtheria (see below).

Diphtheria is most common an infection of the upper respiratory tract that causes sore throat, low fever, and malaise (*Fig. 11.2*). Symptoms of classical pharyngeal diphtheria range from mild pharyngitis to severe hypoxia due to airway obstruction by pseudomembrane formation. In severe cases, the air passages might be completely blocked. In advanced cases, strong swelling of the neck tissue (bull neck diphtheria) is also observed, due to development of soft tissue edema and swelling of lymph nodes (Murphy 1996). Affected lymph nodes might be enlarged, appear blackish red, and be hemorrhagic (Hadfield et al. 2000).

The infection starts with the colonization of epithelial cells of the upper respiratory tract (*Fig. 11.3*); unusual sites of infections are buccal mucosa, upper and lower lips, hard and soft palate, and tongue (Nikolaeva et al. 1995; Hadfield et al. 2000). Typically, *C. diphtheriae* stays at these entry ports and multiplies on the surface of the mucous membrane but does not advance into deeper parts of the body. Tissues infected show inflammatory symptoms; later, edema and necroses develop due to the detrimental action of the diphtheria toxin. The toxin is also responsible for inflammation of deeper lying capillaries, which results in fibrin secretion into the damaged epithelium. Released fibrin protein, components of the destroyed epithelial cells, and bacteria together form the so-called pseudomembrane, a grayish or yellowish-white coating. The damage of the capillaries due to inflammatory processes causes bleeding, and due to decaying erythrocytes, the color of the pseudomembrane can turn into

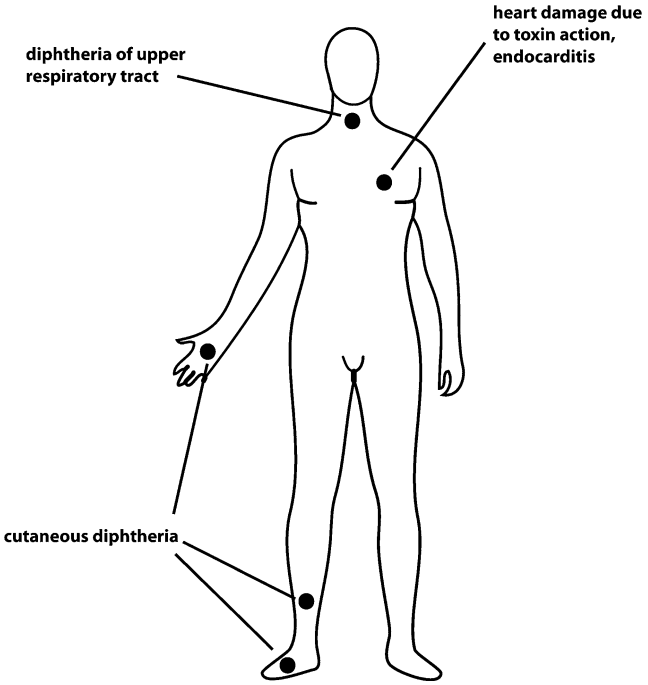

Fig. 11.2
Tissues and organs affected by diphtheria. Transmission of diphtheria occurs from person to person by close physical contact. Respiratory diphtheria is mainly disseminated by respiratory droplets; however, also smear infections are possible. Entry points for cutaneous diphtheria are skin lesions; often, insect bites are colonized. When diphtheria toxin is distributed by the circular system, the myocardium is a main target. Additionally, the heart can be damaged by a systemic *C. diphtheriae* infection leading to endocarditis (Kindly provided by Susanne Morbach, Friedrich-Alexander-Universität Erlangen-Nürnberg)

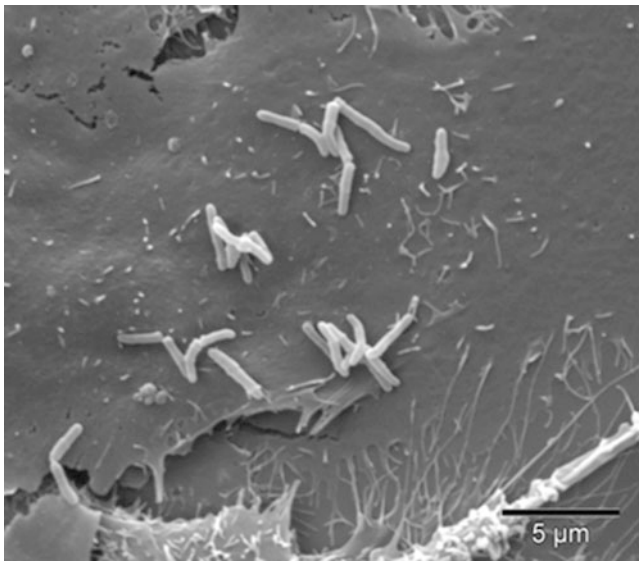

Fig. 11.3
Scanning electron micrograph of *Corynebacterium diphtheriae* adhering to the surface of a pharyngeal cell (Courtesy of Lisa Ott, Friedrich-Alexander-Universität Erlangen-Nürnberg)

a dirty brown, and the breath of the patients becomes sweetish putrid. For experienced physicians, this smell is a characteristic indication of diphtheria. With progressive pseudomembrane formation reaching the larynx, a barking cough develops. As a result, the voice of the patients becomes affected; hoarseness is observed, which can even end in voicelessness. Later, trachea, the main bronchi, and also the smaller bronchi may be covered by firmly or loosely attached pseudomembranes. At this stage, breathing renders difficult for the patients, and their lips turn blue (cyanosis). With increasing dyspnea, the patients become restless, their pulse beats become faster, and the look of their faces becomes timid and frightened. Sometimes, coughing can remove parts of the pseudomembranes at this stage of the disease, easing the situation of the patient temporarily. After several of these fits of coughing, the pseudomembrane might even be completely removed and healing might be achieved in some cases. More often, suffocation results in death agony, and finally, the patients faint and heart failure results in death.

Typically, the colonization of the human host by *C. diphtheriae* remains localized to the upper respiratory tract. Satellite infections may occur in the esophagus, stomach, or lower airways. The severe complications in later stages of infection described above are the result of the diphtheria toxin absorbed and distributed by the circulatory system to remote tissues and organs.

Diphtheria toxin is synthesized depending on the iron (Fe) concentration in the environment. When Fe becomes limiting, as it is the case in the human host, the bacteria start to synthesize the toxin, which is then secreted into the extracellular medium as a single polypeptide chain (Pappenheimer 1977; Holmes 2000). In this form, the toxin is inactive, can be absorbed into the circulatory system, and disseminated to distant parts of the body. When binding to its receptor, the uncleaved precursor of the heparin-binding EGF-like growth factor (Naglich et al. 1992) can enter the cell by endocytosis. Once inside the endosome, the inactive diphtheria toxin, consisting of an A and B chain, is cleaved, and the A chain is released into the cytoplasm, where it is activated by further cleavage into the active toxin and inactivates its cellular target elongation factor 2 (EF-2) by ADP-ribosylation (Pappenheimer 1977; Murphy 1996; Lord et al. 1999; Falnes and Sandvig 2000).

Especially myocardium and peripheral motor neurons are affected by diphtheria toxin (Murray and Noble 1985; Hadfield et al. 2000; Perles et al. 2000). Up to two thirds of patients show some evidence of myocarditis, and 10–25 % of cases develop clinical cardiac dysfunctions. Cardiac symptoms are directly correlated to the extent and severity of local *C. diphtheriae* infection in the patient's upper respiratory tract and often may prove fatal weeks later during convalescence (MacGregor 1995; Hadfield et al. 2000; Perles et al. 2000). Histological changes in the heart differ significantly from patient to patient and may

include edema, congestion, infiltration by mononuclear cells, and presence of neutral fat drops. The myocardium may show areas of granular degradation, hyaline degradation, necrosis, inflammation, and loss of cross striation (Kline and Kaplan 1998; Hadfield et al. 2000; Perles et al. 2000). The heart may be dilated, pale, and flabby (Hadfield et al. 2000). Electrical disturbances are common and include bradyarrhythmia, tachyarrhythmia, and atrioventricular and bundle branch blocks (Perles et al. 2000) as a result of toxin damage to the cardiac conduction, muscle, and nervous system (*Fig. 11.2*).

The nervous system is – besides the heart – a main target of the toxin. About three fourths of patients with severe diphtheria infection develop neurologic complications. First symptoms of neuropathy are paralysis of the soft palate and posterior pharyngeal wall, resulting in regurgitation of swallowed fluids through the nose. Additionally, other cranial neuropathies such as oculomotor paralysis and dysfunction of facial, pharyngeal, or laryngeal nerves, resulting in typical symptoms of diphtheria, are observed. In later stages, the nerves of trunk, neck, arms, and hands might be affected leading to paralysis. Histological changes include paranodal and segmental demyelination, resulting in degeneration of myelin sheaths and axons (Baba et al. 1984; Hadfield et al. 2000).

Diphtheria of the skin is more common in tropical and subtropical regions, where it prevails over respiratory tract infections (Höfler 1991) and is still endemic, for example, in African and Asian countries. Besides the climate, overcrowding, poverty, poor hygiene, and frequent, slowly healing wounds and skin lesions such as insect bites favor the infection. Transmission of skin infections occurs by contact with respiratory droplets of a patient infected with respiratory tract diphtheria or by contact with infected skin lesions, exposure to dust, or clothing contaminated with *C. diphtheriae* (Höfler 1991). Compared to diphtheria of the upper respiratory tract, cutaneous diphtheria is more contagious. It is characterized by skin lesions usually covered by a smeary grayish-brown coating. Later, the pseudomembrane might change color to a dirty or dark reddish brown. However, the appearance of cutaneous diphtheria can be extremely variable due to the various skin lesions that can be colonized by the bacteria (dermatitis, eczema, impetigo, insect bites, pyoderma, surgical wounds, etc.). Furthermore, coinfections of the lesions by different other bacterial pathogens are rather common (Hadfield et al. 2000).

The most typical clinical manifestation of diphtheria of the skin is the ulcerative form or *ecthyma diphthericum* (Höfler 1991). Common sites for cutaneous diphtheria are the lower legs, feet, and hands (e.g., see Hamour et al. 1995; Connell et al. 2005; *Fig. 11.2*). The painful infection starts with a pustule, which is filled with fluid. This pustule breaks down quickly and progresses as a punched-out ulcer. The diameter of the ulcer might range between a few millimeters to centimeters. During the first weeks, lesions are covered by the pseudomembrane described above. Later, the lesions become anesthetic and the pseudomembrane falls off, leaving a hemorrhagic base with a surrounding of edematous grayish-white-, pink-, or purple-colored tissue (Höfler 1991; Hadfield et al. 2000). Spontaneous healing takes several weeks to months; cases lasting 1 year have been observed (Höfler 1991). This long healing time might favor dissemination of the disease and might explain the extremely high infection rates of skin lesions with *C. diphtheriae*. In some African and Asian countries, a frequency rate of up to 60 % was observed (Liebow et al. 1946; Livingood et al. 1946; Bezjak and Farsey 1970a, b; Höfler 1991).

Systemic Infections by *C. diphtheriae*

C. diphtheriae is not only the etiological agent of classical diphtheria of respiratory tract and skin but can cause systemic infections as well. Although generally rare, cases of bacteremia, endocarditis, hepatic and splenic abscesses meningitis, mycotic aneurysm, osteomyelitis, pneumonia, as well as septic arthritis caused by non-toxigenic and toxigenic *C. diphtheriae* were reported (Isaac-Renton et al. 1981; Puliti et al. 2006; Hirata et al. 2008; Homna et al. 2009; Muttaiyah et al. 2011; and references therein). The best documented systemic infections are related to *C. diphtheriae* endocarditis.

Endocarditis as a result of *Corynebacterium* infection has been described as aggressive disease often requiring surgical intervention (Mishra et al. 2005). Typically the left heart of adult males is infected, and underlying valvular disease is frequently found. Up to 28 % of patients require valve replacements, and more than 40 % die (Belmares et al. 2007). Endocarditis can be the result of infection with different *Corynebacterium* species besides *C. diphtheriae* such as *Corynebacterium amycolatum*, *Corynebacterium hemolyticum*, *Corynebacterium jeikeium*, and *Corynebacterium pseudodiphthericum*. The various species show different gender and age prevalence, and *C. diphtheriae* has especially been connected to cases where children are involved (Davidson et al. 1976; Belmares et al. 2007; Hirata et al. 2008; Menon et al. 2010; Schnell et al. 2010); from other studies, it was concluded that *C. diphtheriae* infections may be on the rise and change their epidemiological pattern (Mishra et al. 2005). In fact, a recent review of case series showed a high proportion of adult patients (Muttaiyah et al. 2011).

Diagnosis

The diagnosis of respiratory tract diphtheria is – as in former times – still based on the classical symptoms of this disease, sore throat, formation of a pseudomembrane, and the typical sweetish-putrid smell of the patients' breath. With the identification of *C. diphtheriae* as its etiological agent by Loeffler (1884) and the development of modern biochemistry and molecular biology, the diagnostic toolbox was constantly improved. Besides different screening and identification tests, the classical Elek test (Efstratiou et al. 1998) for toxicity testing is most commonly used (for an overview of tests and quality evaluation, see Neal et al. 2009). Furthermore, also a number of other toxigenic *Corynebacterium* species (see below) can be reliably identified today (Schuhegger et al. 2008; Sing et al. 2011).

Treatment and Control

Before the introduction of antitoxin and antibiotics, physicians were restricted to means preventing suffocation. Tracheostomy was introduced by Bretonneau in 1825, intubation first by Bouchut in 1859 and later by O'Dwyer in 1885 (English 1985). Nevertheless, mortality stayed high, since – besides the severe side effects of the proposed treatments – the detrimental action of the toxin could not be avoided by these methods. The situation improved dramatically with the introduction of antitoxin and with the discovery of antibiotics. In contrast to other corynebacteria such as *Corynebacterium jeikeium*, which causes severe infections in intensive care units, multiresistance against antibiotics is not the problem in *C. diphtheriae* and penicillin and erythromycin are first-line antibiotics used for its eradication (Begg 1994; Kneen et al. 1998; Pereira et al. 2008; Zasada et al. 2010). In cases of cutaneous diphtheria, additionally, local application of drugs such as bacitracin or gentian violet is recommended (Höfler 1991). The therapy with antibiotics might become more difficult in the future with the emergence of multidrug resistant strains in some countries. While a recent study on antimicrobial resistance found no multidrug resistant strains among isolates circulating in Poland (Zasada et al. 2010), a considerable number of isolates resistant against one or more antibiotics were observed among Brazilian *C. diphtheriae* strains and reservations about the use of penicillin were risen (Pereira et al. 2008).

To avoid complications due to action of diphtheria toxin, for example, myocarditis, typically, antitoxin is administered. However, even if properly treated with antibiotics and antitoxin, 5–10 % of cases can end fatally. Therefore, mass immunization of the entire population is the best means to control diphtheria.

Immunization with diphtheria toxoid, formaldehyde-inactivated diphtheria toxin that remains antigenically intact, is extraordinarily effective (*Fig. 11.4*). With increasing levels of antitoxin immunity, the frequency of isolation of toxigenic *C. diphtheriae* strains decreased and the annual incidence of diphtheria dropped to 0.1–0.2 per million (Kwantes 1984; Höfler 1991; (Murray and Noble 1985; Vitek 2006; for a comparison of diphtheria cases in the United States, see Roush et al. 2007). Diphtheria toxoid is widely used as a component of DPT (diphtheria, pertussis, tetanus) vaccine, and immunization typically starts with early childhood. After four doses of the vaccine within the first 2 years, immunization against diphtheria is effective up to 97 %. Since antibody titers wane over time, a large percentage of adults in the United States and Europe have antitoxin levels below the protective level and are at risk when traveling to outbreak areas (Murray and Noble 1985; von Hunolstein et al. 2000). Therefore, booster immunization of adults is recommended.

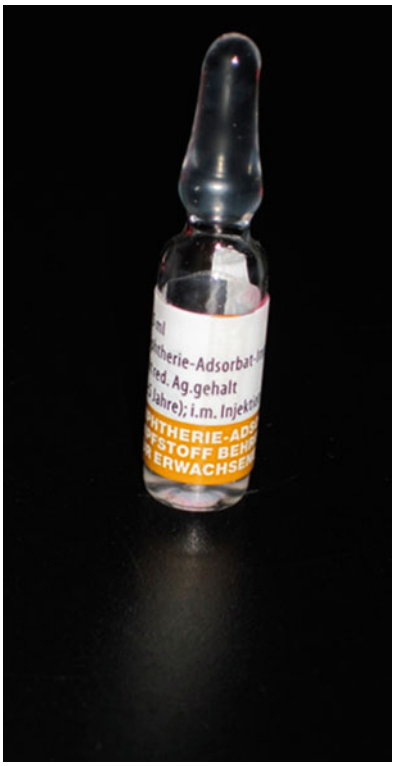

Fig. 11.4
Immunization of infants and adults is extremely effective to prevent diphtheria. The photograph shows a diphtheria toxoid preparation (in combination with tetanus toxoid, pertussis, and polio vaccine) with reduced antigen for booster immunization of adults

Outbreaks of Diphtheria in the Vaccine Era

While diphtheria used to be a major cause of morbidity and mortality in infants a century ago, introduction of immunization programs reduced the number of cases dramatically. The last major epidemic before an outbreak in the states of the former Soviet Union – described in detail below – occurred in the 1940s. Thereafter, only local and relatively small epidemics both in developing and industrialized countries were observed with an all-time low incidence of only 623 cases reported by the World Health Organization in 1980. However, a large-scale outbreak started in Eastern Europe in 1990. The Russian Federation and Ukraine were the center of this epidemic with 15,211 diphtheria cases reported for the Russian Federation and further 2,987 cases for Ukraine in 1993 (Galazka et al. 1995; Eskola et al. 1998; Popovic et al. 2000). The outbreak spread quickly to neighboring countries and cases were also reported for Azerbaijan, Belarus, Estonia, Finland, Kazakhstan, Latvia, Lithuania, Poland, Tajikistan, Turkey, and Uzbekistan (Galazka et al. 1995). The most characteristic feature of this epidemic was the infection of adolescents and adults instead of infants, which was especially pronounced in the countries of the western new independent states Belarus, Russia, and Ukraine and the Baltic states Estonia, Latvia, and Lithuania, where diphtheria cases among people from 15 years and older ranged from two thirds to four fifths of total cases (Hardy et al. 1996; Dittmann et al. 2000). In the first years of the outbreak, routine

immunization of children and adults belonging to risk groups was not very effective; therefore, mass immunization of adults was started in 1993, leading finally to an expiration of the epidemic.

Besides the unusual variance in the age of infected humans, an interesting feature of this *C. diphtheriae* epidemic was the observed periodicity of strains. Following the outbreak by a unique clonal group of *C. diphtheriae* in Russia in 1990 (Popovic et al. 2000), a rising heterogenicity of circulating strains after the epidemic, emergence of new toxigenic variants, and persistence of invasive non-toxigenic strains were observed (Mokrousov 2009).

From the epidemic in the former Soviet Union in the 1990s as well as from previous outbreaks, it can be concluded that socioeconomic instability, large-scale population movements, inadequate information policy, and a breakdown of health infrastructure favor the spreading of this disease. Furthermore, a rapid diagnosis as well as fast and aggressive means for control of the epidemic are crucial with mass immunization programs being extremely effective (Dittmann et al. 2000).

The outbreak of diphtheria in the states of the former Soviet Union as well as case reports from various countries indicate that *C. diphtheriae* might persist in a population for long time (e.g., see Marston et al. 2001). In many cases, the reservoirs are unknown; sometimes, these are groups of people with insufficient access to medical care including vaccination programs (John 2008) or drug abusers (Lowe et al. 2011). For inhabitants of industrialized countries, a major risk factor for infection with *C. diphtheriae* might be travel to a country where diphtheria is endemic (Connell et al. 2005; Bonmarin et al. 2009; Wagner et al. 2010).

Besides humans, animals seem to play a role as a reservoir of this pathogen. Isolations of *C. diphtheriae* strains were reported, for example, from domestic cats (Hall et al. 2010), cows (Corboz et al. 1996), and horses (Henricson et al. 2000; Leggett et al. 2010). The existence of reservoirs in animals is even more common, when other toxigenic *Corynebacterium* species are taken into consideration (Bonmarin et al. 2009).

Toxigenic Corynebacteria as Etiological Agents of Diphtheria

C. diphtheria is the classical etiological agent of diphtheria and the type strain of the genus *Corynebacterium* (Lehmann and Neumann 1896; Barksdale 1970). However, 16S rDNA-based taxonomic analyses revealed that *C. diphtheriae* is closely related to two further distinct *Corynebacterium* species, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* (Riegel et al. 1995). The close phylogenetic relationship between these three species is also highlighted by their common repertoire of potent toxins (Riegel et al. 1995). All three species can produce diphtheria toxin, which is the result of infection of the bacteria with *tox* gene carrying corynebacteriophages (Groman 1984; Buck et al. 1985; Cianciotto and Groman 1996), and in addition, *C. ulcerans* and *C. pseudotuberculosis* produce phospholipase D, the so-called *ovis* toxin (Barksdale et al. 1981; Groman 1984).

C. ulcerans has been detected as a commensal in domestic and wild animals (Schuhegger et al. 2009; Dixon 2010; Sykes et al. 2010) that may serve as reservoir for zoonotic infections. During the last decade, the frequency and severity of human infections associated with *C. ulcerans* appear to be increasing in various countries. Toxigenic *C. ulcerans* strains produce a diphtheria toxin, which is similar to that encoded by toxigenic strains of *C. diphtheriae* (Sing et al. 2003; 2005), since similar lysogenic β-corynephages are involved in the conversion of *C. diphtheriae* and *C. ulcerans* from non-toxigenic to toxigenic (Pappenheimer 1977). Respiratory diphtheria-like illnesses caused by toxigenic *C. ulcerans* strains are increasingly reported from various industrialized countries (Wagner et al. 2001; Hatanaka et al. 2003; De Zoysa et al. 2005; Tiwari et al. 2008) and became even more common than *C. diphtheriae* infections in the United Kingdom (Wagner et al. 2010). Human infections with toxigenic *C. ulcerans* can be fatal in unvaccinated patients and usually occur in adults, who consumed raw milk (Bostock et al. 1984; Hart 1984) or had close contact with domestic animals (Wagner et al. 2010); no person to person transmission was reported up to now. Besides respiratory diphtheria-like illnesses, *C. ulcerans* can also cause extrapharyngeal infections in humans, including severe pulmonary infections (Dessau et al. 1995; Nureki et al. 2007; Mattos-Guaraldi et al. 2008), and seems to have a high arthritogenic potential (Dias et al. 2011).

C. pseudotuberculosis is the etiological agent of caseous lymphadenitis that is prevalent in sheep and goat populations throughout the world (Dorella et al. 2006; Baird and Fontaine 2007). Infections due to *C. pseudotuberculosis* are rare in humans. Despite the putative presence of a diphtheria toxin, diphtheria of respiratory tract or skin is in contrast to *C. ulcerans* not observed, but this pathogen is occasionally recovered from cases of suppurative lymphadenitis in patients with a classical risk exposure of close contact with sheep. *C. pseudotuberculosis* is a facultative intracellular pathogen that is able to survive and grow in macrophages, thus escaping the immune response of the host (McKean et al. 2005; Dorella et al. 2006).

Infections with Non-toxigenic Strains

With rising immunization against the diphtheria toxoid, not only the number of clinical diphtheria incidences decreases but also the number of isolated toxigenic *C. diphtheriae*. In contrast, an increase in the isolation of non-toxigenic strains is observed (Zuber et al. 1992; Gilbert 1997; Hadfield et al. 2000; Wagner et al. 2011). The shift in population of the pathogen might be the result of immunization programs directed against diphtheria toxin and, since these strains often evoke systemic infections, might lead to a development of *C. diphtheriae* as a reemerging pathogen. This development might be further supported by the fact that non-toxigenic strains are persistent in different

populations (Romney et al. 2006; Lowe et al. 2011; Shashikala et al. 2011) and continued surveillance was proposed as a control measure (Edwards et al. 2011).

Open Questions

Although characterized in respect to clinical manifestation and details of toxin distribution and function, for decades, diphtheria and its etiological agents *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* still impose a number of unanswered questions including the resurgence of epidemics, reservoirs of these pathogens in humans and animals, the mechanisms of acquiring natural immunity against diphtheria, and the shift in age of patients. Furthermore, variations of genome and population structure of *C. diphtheriae* isolates such as the plasticity of pathogenicity islands (Soares et al. 2011) and the relative increase in the occurrence of non-pathogenic *C. diphtheriae* strains (Galazka 2000) are not well characterized. Additionally, in comparison to other pathogens, knowledge of molecular details of the different steps of host colonization and host-pathogen interaction is low. Best investigated are the functions of pili (for recent review, see Rogers et al. 2011) on adhesion, the acquisition of heme-bound iron by *C. diphtheriae* (for recent papers see Allen and Schmitt 2009; Bibb and Schmitt 2010), and the role of members of the NlpC/P60 protein family DIP1281 and DIP1621, which seem to be involved in bacterial surface organization and separation (Ott et al. 2010; Kolodkina et al. 2011). The contributions of other genes annotated in the genome sequence as encoding putative virulence factors (Cerdeno-Tarraga et al. 2003) as well as the potential of the organism to produce biofilm, for example, on catheter surfaces (Gomes et al. 2009), await detailed experimental characterization.

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12 Botulism and Tetanus

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Abstract
Botulism and tetanus are two severe neurological diseases in man and animals. While botulism is characterized by a descendant flaccid paralysis, tetanus consists in spastic paralysis. In the severe forms of both diseases, death occurs by respiratory distress. Botulism and tetanus are caused by neurotoxins, botulinum neurotoxin (BoNT), and tetanus toxin (TeNT), respectively, which are produced by anaerobic sporulating bacteria, *Clostridium botulinum* and *Clostridium tetani*, respectively. In contrast to *C. tetani*, which forms a homogeneous bacterial species, BoNT-producing Clostridia are divided into several bacterial species and groups. These Clostridia are widely distributed in the environment, including food notably for *C. botulinum*, where they can survive during long periods in the sporulating forms. BoNTs and TeNT share a common structural organization consisting in a light (L) chain (about 50 kDa) linked by a disulfide bridge to the heavy (H) chain (about 100 kDa). Only a unique TeNT is known, while BoNTs encompass seven toxinotypes (A to G, BoNT/A, B, and E mainly involved in human botulism, and BoNT/C and D mainly responsible for animal botulism), which are subdivided into several subtypes according to amino acid sequence variations. H chain, which contains a C-terminal receptor-binding domain and an N-terminal translocation domain, delivers the L chain into target neurons. BoNTs target the motoneuron endings or neuromuscular junctions, and TeNT is transported to central inhibitory interneurons through a retrograde axonal pathway along motoneurons. Both BoNT and TeNT block the release of neurotransmitter by an L chain-mediated proteolytic cleavage of SNARE proteins (synaptobrevin, SNAP25, or syntaxin) which are involved in the neuroexocytosis process. Blockage of acetylcholine release at the neuromuscular junctions by BoNTs induces a flaccid paralysis, whereas TeNT-dependent inhibition of glycine or

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GABA exocytosis in inhibitory interneurons results in spastic paralysis. Botulism is mainly acquired by ingestion of preformed BoNT in food, but it may also occur subsequently to intestinal or wound colonization by *C. botulinum*. Tetanus essentially results from a wound contamination by *C. tetani*. BoNT/A is the most potent toxin with a long activity duration in neurons, and it is also a therapeutic agent widely used to treat hypercholinergic diseases including localized muscle spasticity, dystonia, autonomic dysfunctions (hyperhidrosis, hypersalivation), and also pain such as migraine headaches.

Introduction

Among the large number of *Clostridium* species, which are Gram-positive, spore-forming bacteria from the environment, some of them produce potent neurotoxins causing neurological disorders in human and animals. *Clostridium botulinum* is defined as a *Clostridium* which produces one or several neurotoxins inducing flaccid paralysis, termed botulinum neurotoxins (BoNT), which are responsible for a rare but often severe disease with a high lethality rate, the botulism. In contrast, *Clostridium tetani* synthesizes a related neurotoxin called tetanus toxin (TeNT), which causes a dramatic spastic paralysis. Both, BoNT and TeNT exert the neurotoxicity by blocking the release of neurotransmitters. While BoNTs inhibit the evoked release of acetylcholine at the neuromuscular junctions causing a flaccid paralysis, TeNT impairs the neuroexocytosis at the central inhibitory interneurons resulting in the prevention of the inhibitory pathways and thus in a spastic paralysis. Albeit BoNT is the most potent toxic compound, it is widely used as therapeutic tool in the treatment of various cholinergic disorders such as dystonia.

Clostridia Producing Botulinum Neurotoxins

Clostridia capable of producing BoNT display heterogeneous bacteriological characters and are divided into several species and groups. The taxonomic position of the *C. botulinum* species was originally based on only one phenotype, the production of a BoNT, and nontoxic variant strains, although genetically related to *C. botulinum*, were assigned to different species such as *Clostridium sporogenes* and *Clostridium subterminale*. It appeared soon that BoNTs are seven different protein neurotoxins which are immunologically distinct and are termed with letters from A to G. More recently, sequence analysis permits to distinguish subtypes within BoNT types (see below and ❯ *Table 12.1*). However, all of them cause the typical flaccid paralysis of botulism in experimental animals, similar to that observed in human patients suffering from botulism. Later on, physiological differences between *C. botulinum* strains were identified, but the production of the different BoNT types does not necessarily correlate with the *C. botulinum* strain phenotypes. The species was divided into four physiological groups (❯ *Table 12.1*) (Smith 1975).

- Group I: *C. botulinum* A and proteolytic strains of *C. botulinum* B and F
- Group II: *C. botulinum* E and glucidolytic strains of *C. botulinum* B and F
- Group III: *C. botulinum* C and D
- Group IV: *C. botulinum* G (or *C. argentinense*).

The latter group which also includes nontoxic strains previously identified as *C. subterminale* and *C. hastiforme* is metabolically distinct from the other groups and has been assigned to a different species called *C. argentinense* (Suen et al. 1988).

The taxonomic position of *C. botulinum* became more ambiguous since it has been found that BoNTs can be produced by *Clostridium* strains clearly distinct from already defined *C. botulinum*, and biochemically and genetically related to different species such as *C. butyricum* and *C. baratii* (Hall et al. 1985; McCroskey et al. 1986, 1991). In each group, all the strains regardless of toxin types are closely related according to their phenotypic properties, DNA/DNA homology and 16s rRNA analysis. All the BoNT-producing *Clostridia* have a G+C content between 26% and 28% (Cato et al. 1986) and belong to the group I of 23S rRNA homology (Johnson and Francis 1975), which contains the type species *C. butyricum*. 16S rRNA comparison corroborated that the group I strains are homogeneous and form a distinct phylogenetic branch. It has been proposed that the genus *Clostridium* should be retained only for this taxon (Hippe et al. 1992; Lawson et al. 1993).

Morphological Aspects

BoNT-producing *Clostridium* is usually straight to slightly curved rods, 0.6–2 wide and 2–22 µm long. They are usually motile and peritrichous, except *C. baratii* which is nonmotile. Spores are oval and subterminal and swell the cell. Type G strains sporulate poorly, and *C.butyricum* spores are central to subterminal and usually do not swell the cell (Cato et al. 1986).

These bacteria grow well in usual anaerobic liquid media with production of gas. Surface colonies can be grown on blood agar plates, incubated under anaerobic conditions; they can be circular (1–6 mm in diameter) or irregular, with a scalloped or lobate margin, translucent to semiopaque, gray-white. They are surrounded by a narrow zone of partial hemolysis, while those of *C. butyricum* are nonhemolytic.

Physiological Properties

Group I (*C. botulinum* A, and Proteolytic Stains of Types B and F)

The strains of this group are characterized by a proteolytic and lipase activity and by the non or weak acidification of carbohydrates (❯ *Table 12.1*). Glucose is acidified weakly. The cell wall contains glucose as sugar. The optimal temperature of growth is 37 °C. The strains of group I usually do not grow and do not form toxin at 10 °C or below. But large inoculum and long incubation period might result in sufficient growth and toxin

Table 12.1

Groups of botulinum neurotoxin-producing Clostridia, produced toxins, and main properties.[a]According to (Peck 2006, 2009; Peck et al. 2011)

Neurotoxin-producing *Clostridium*	Group I	Group II	Group III	Group IV *C. argentinense*	C. butyricum	*C. Baratii*
Toxin type	A proteolytic B, F	E nonproteolytic B, F	C, D	G	E	F
Subtype	A1, A2, A3, A4, A5 B1, B2, B3 bivalent B (Ba, Bf, Ab) F1, F2, F3, F4, F5	E1, E2, E3, E6, E7, E8 nonproteolytic B F6	C, D, C/D, D/C	G	E4, E5	F7
Proteolysis	+	–		+	–	
Lipase production	+			–		
Growth temperature[a]						
Optimum	30–40 °C	25–37 °C	37–40 °C	30–37 °C	30–37 °C	30–45 °C
Minimum	10–12 °C	2.5–3 °C	15 °C		10 °C	
Minimum pH for growth[a]	4.6	5.0	5.1		4.8	
Minimum water activity for growth[a]	0.96	0.97	0.97	0.94		
NaCl concentration preventing growth[a]	10%	5%		6.5%		
Spore thermoresistance[a]	$D_{121°C}$=0.21 min	$D_{82.2°C}$=2.4–231 min	$D_{104°C}$=0.1–0.9 min	$D_{104°C}$=0.8–1.12 min	$D_{100°C}$<0.1 min	
Botulism	Human		Animal		Human, animal	
Related non-botulinum toxin-producing *Clostridium*	*C. sporogenes*		*C. novyi* *C. hemolyticum*	*C. subterminale* *C. proteolyticus* *C. schimacherense*	*C. butyricum*	*C. baratii*

formation at low temperature such as 12 °C within 3–4 weeks (Peck 2006). Growth of group I strains is inhibited by acidic pH (<4.6) and NaCl concentrations ≥10%. The thermoresistance of the spores is usually high (above 120 °C) but varies according to the strains and also of the heating buffer and recovery culture medium (Hinderink et al. 2009; Peck 2009; Smith 1975). Botulism was a major problem at the beginning of the canning industry, and the thermoresistance of *C. botulinum* strains has been extensively investigated in order to recommend safety rules of heat treatment. According to the most heat-resistant strains which require a heating at 121 °C for 0.21 min to reduce the number of viable spores by a factor of 10^{12} (12-D or *D* value), heating at 121 °C for 3 min has been recommended for commercial and low-acid canned foods (Peck 2009).

Group II (*C. botulinum* E and Glucidolytic Strains of Types B and F)

Group II strains acidify various carbohydrate substrates (amygdalin, dextrin, fructose, galactose, glucose, glycogen, maltose, ribose, sorbitol, sucrose, and trehalose but not lactose, mannitol, melibiose, and salicin) and produce mainly butyric and acetic acids from trypticase-yeast extract-glucose broth. They hydrolyze gelatin, but they are nonproteolytic (Cato et al. 1986). The strains of this group contain glucose and galactose as the cell wall sugars. They have a lower optimal temperature of growth (around 25–30 °C), and they can growth and produce toxins at very low temperature (*Table 12.1*). Growth and toxin production have been reported to occur at temperature as low as 2.5–3.0 °C in 5–7 weeks. However, most of the strains grow poorly below 5 °C (Peck 2006, 2009; Peck et al. 2011). Spores are only moderately resistant to heat, not withstanding 10 min at 90 °C (Smith 1992). The highest temperature resistance of spores in phosphate buffer was reported to be 82.2 °C. Spores of group II strains are lysozyme-dependent for their germination. Lysozyme diffuses in the spore coat and induces the peptidoglycan hydrolysis in the cortex permitting the first step of germination. Lysozyme increases the spore recovery after heat treatment and thus increases heat resistance. In the presence of lysozyme, the resistance time to heat treatment at 82.2 °C is 100-fold increased to 231 min instead of 2.31 min without lysozyme (Peck 2009). Moreover, the presence of high protein and fat content in raw material may also confer a higher heat resistance of spores (Lindstrom et al. 2006a). The BoNT produced by group II strains is mainly type E and is not fully activated by endogenous protease. Therefore, trypsinization enhances the toxicity of

these cultures. In addition, strains from group II are most sensitive than proteolytic *C. botulinum* strains to NaCl (inhibitory concentration 5% and above) and pH (no growth below pH 5).

Group III (*C. botulinum* C and D)

The organisms of this group are nonproteolytic or very slightly. They ferment glucose, glycerol, inositol, ribose, and xylose. Their cell wall do not contain sugar or only traces of glucose (Smith 1975). The main end products of metabolism are acetate and butyrate. They grow at higher temperature than the other *C. botulinum* strains, optimum temperature for growth being 37–40 °C, with most strains growing well at 45 °C, and minimal temperature for growth being at approximately 15 °C (Cato et al. 1986).

This group is heterogeneous according to some biochemical properties (fermentation of several sugars, production of indole and hydrogen sulfide). A subdivision into four subgroups have been proposed (Oguma et al. 1986).

C. argentinense (Also Referred as Group IV)

The strains of this species are proteolytic, hydrolyze gelatin and do not ferment any of the usual carbohydrates. Contrarily to the other strains of the *C. botulinum* groups, *C. argentinense* strains do not produce a lipase. They produce acetate, butyrate, isobutyrate, isovalerate, and phenyl acetate as end products of metabolism. The optimum temperature of growth is 30–37 °C. The spores have an intermediate heat resistance like group III *C. botulinum* strains. Phenotypic differences have been observed between toxic and nontoxic *C. argentinense* strains by using cellular fatty analysis and multilocus enzyme electrophoresis (Altwegg and Hatheway 1988; Ghanem et al. 1991). *C. argentinense* strains are phenotypically and genetically closely related to *C. subterminale* strains which are non-neurotoxigenic strains.

C. butyricum

Toxigenic *C. butyricum* strains are phenotypically and genetically related to the typical strains of this species. They are nonproteolytic, they do not hydrolyze gelatin and acidify strongly various carbohydrates including glucose, cellobiose, fructose, galactose, glycogen, lactose, maltose, mannose, melibiose, raffinose, ribose, salicin, starch, sucrose, trehalose, xylose, and pectin. They do not produce lipase neither lecithinase (Smith 1992). They are mesophilic bacteria with optimum growth temperature of 30–37 °C and minimum temperature for growth and toxin production of 10–12 °C. The lowest pH for growth and toxin synthesis was found to be 4.8 (Anniballi et al. 2002). Spore of neurotoxigenic *C. butyricum* are less resistant to heat ($D_{100°C} < 0.1$ min) than the nontoxigenic strains ($D_{100°C} = 4.7$ min) (Peck 2009).

C. butyricum strains producing type E neurotoxin have been isolated from infant botulism and young people in Italy (Aureli et al. 1986; Fenicia et al. 1999; Koepke et al. 2008; McCroskey et al. 1986), as well as from botulism associated with consumption of fermented soybean in China (Fu and Wang 2008; Meng et al. 1997, 1999; Wang et al. 2000) and also in India and Japan (Abe et al. 2008; Chaudhry et al. 1998). Based on toxin gene sequence, toxigenic *C. butyricum* strains from Italy and China are divided in two distinct subtypes, termed E4 and E5, respectively, indicating an independent evolution of *bont/E* gene after transfer in *C. butyricum* (Hill et al. 2007).

C. baratii

Toxigenic and nontoxigenic *C. baratii* strains display similar morphological and biochemical characteristics. *C. baratii* is phenotypically closely related to *C. perfringens*. Both species are nonmotile and appear as thick, straight rods (0.5–1.9 × 1.6–15 μm). Strains sporulate poorly in usual culture medium. They produce a lecithinase but not a lipase. *C. baratii* is readily differentiated from *C. perfringens* by its lack of hydrolysis of gelatin (Cato et al. 1986). Optimum growth temperature is around 30–45 °C and minimum growth temperature at 10–15 °C. *C. baratii* acidifies culture medium containing various carbohydrates.

Neurotoxigenic *C. baratii* strains are genetically related to nontoxigenic counterparts. Botulinum neurotoxin type F gene sequences from *C. baratii* form a different cluster of those from *C. botulinum* type F (Hill et al. 2007).

Neurotoxigenic *C. baratii* type F is involved in a limited number of human cases, mainly by intestinal colonization, in infants or adults (Barash et al. 2005; Gupta et al. 2005; Hall et al. 1985; Harvey et al. 2002; Koepke et al. 2008; McCroskey et al. 1986, 1991).

Sporulation and Germination

C. botulinum, as other Clostridia, forms spores which permit to survive for long periods in the environment under unfavorable conditions such as exposition to oxygen, dryness, high temperature, and deprivation of nutriments. Spores from *C. botulinum* of group I are among the most heat-resistant spores, and this constitutes an important problem in food industry. Germination is triggered by specific molecules, called germinants, and can occur in the presence of oxygen. However, further growth of *C. botulinum* vegetative cells requires a strict anaerobic environment.

In *C. botulinum* strain Hall, 111 genes have been assigned to germination/sporulation (Sebaihia et al. 2007). The gene cascade involved in sporulation has been well defined in *Bacillus subtilis*. In contrast, sporulation steps are not yet completely known in Clostridia. Spo0A is a key player of sporulation in *B. subtilis* as well as in *Clostridia*. Spo0A is a transcriptional regulator: when activated by phosphorylation at an aspartate residue, it binds to specific DNA motifs by its C-terminal part and thus activates and represses genes, thereby controlling the onset of sporulation. Spo0A is conserved in *C. botulinum*. However, the genes encoding the histidine kinases (kinA-E) and the phosphorelay system Spo0F-Spo0B responsible for Spo0A phosphorylation in response to nutrient limitation in *Bacillus* are not found in *C. botulinum* and other known clostridial genomes. In *B. subtilis*, the phosphorelay kinases of Spo0A are orphan kinases. *C. botulinum* A strain Hall encodes

five orphan kinases, which might potentially phosphorylate Spo0A, *C. acetobutylicum* contains six orphan kinase genes, *C. perfringens* 7, and *C. tetani* 3 (Paredes et al. 2005; Sebaihia et al. 2007). The CBO1120 orphan sensor histidine kinase was identified as being capable of phosphorylating Spo0A (Wörner et al. 2006). The environmental factors controlling sporulation in Clostridia are thereby different from those involved in *Bacillus*. The control of intracellular pH seems to be important to initiate sporulation of Clostridia. The organism prevents excessive acidification due to organic acids produced during the fermentation process. Thus, the intracellular pH in anaerobic bacteria is fluctuant and is generally one unit higher than the external pH. The terminal fermentation end products are exported through the cytoplasmic membrane in an undissociated form at low pH. But at higher pH, they dissociate into the cytoplasm decreasing the intracellular pH (Dürre 2005). In *C. acetobutylicum*, it has been suggested that phosphorylation of Spo0A might use butyryl phosphate as phosphate donor through a butyrate kinase (Paredes et al. 2005). The butyrate kinase gene is conserved in all *C. botulinum* genomes. Another difference to *B. subtilis* sporulation is that Clostridia require sufficient levels of carbon source and ATP.

Most of the genes downstream of Spo0A from *B. subtilis* including sporulation specific sigma factors such as the earliest sporulation sigma factor σ^H as well as σ^E and σ^K in the mother cell and σ^F and σ^G in the forespore are conserved in genomes of *C. botulinum* and other Clostridia (Paredes et al. 2005; Sebaihia et al. 2007).

Germination is the first step in the development of dormant spores into exponentially growing bacteria. This process is triggered by interaction of germinant molecules such as amino acids with specific receptors in the inner spore membrane. The most effective germinants consist in L-alanine or L-alanine/L-lactate for proteolytic *C. botulinum* strains and in L-lactate in combination with L-alanine, L-serine, or L-cysteine at neutral pH in 100 mM potassium phosphate for nonproteolytic *C. botulinum* strains. However, strain variations are observed (Peck 2009). The resulting loss of ions (K^+, Na^+, H^+, Ca^{++}) and dipicolinic acid as well as peptidoglycan hydrolysis in spore cortex leads to decreased resistance of spore coats and subsequent hydration and swelling of the spore core, thus permitting metabolism activity. Three tricistronic germinant receptor gene operons (*gerX/A, gerX/B, gerX/C*) and one orphan germinant receptor gene homologue have been identified in *C. botulinum* A strain Hall genome. One of the three operon is flanked by two additional *gerX/B* gene (Sebaihia et al. 2007). GerAA and GerAB are transmembrane proteins of the spore inner membrane, GerAB is a single component membrane receptor, and GerAC is predicted to be a lipoprotein. The three proteins from each operon are required to form a functional germinant receptor. The three operons and orphan gene of germinant receptors of strain Hall are conserved in the other proteolytic *C. botulinum* genomes including *C. botulinum* type A strains, *C. botulinum* type B Okra, and *C. botulinum* type L Langeland. But *C. botulinum* type B Okra and *C. botulinum* type L Langeland strains contain an additional tricistronic germinant receptor operon, possibly permitting to these strains to respond to different germinants. In proteolytic *C. botulinum* B NCTC7273 and *C. sporogenes*, the tricistronic operon (*gerA/A, gerA/B, gerA/C*) has been characterized to respond to L-alanine (Broussolle et al. 2002). In contrast, nonproteolytic *C. botulinum* genomes (C. *botulinum* type B Eklund17B and *C. botulinum* type E Alaska E43) only show one tricistronic germinant receptor operon which is related to that found in proteolytic *C. botulinum* strains Okra (type B) and Langeland (type F) but not to those found in the proteolytic type A strain genomes (Peck 2009). The *gerA* operon from *C. botulinum* B is highly conserved in *C. botulinum* A strain Hall (89–99% amino acid identity of encoded proteins), and the *gerA/A* and *gerA/B* genes from *C. sporogenes* are highly related to another *C. botulinum* A *ger* cluster. *ger* clusters are also present in multicopies in *C. acetobutylicum* (three copies), *C. tetani* (four copies), whereas *C. perfringens* contains only one bicistronic *ger* operon and *C. difficile* lacks genes similar to those from *Bacillus* or other Clostridia, indicating that the initial step of germination in *C. difficile* is different from that of other Clostridia (Sebaihia et al. 2006, 2007).

Clostridium tetani

Tetanus neurotoxin (TeNT) is produced by a uniform group of bacteria belonging to the *Clostridium tetani* species.

Morphological and Cultural Characteristics

The cells of *C. tetani* are usually 0.3–0.6 μm in width and may vary considerably in length between 3 and 12 μm. They are Gram-positive in young cultures, but they lose the Gram coloration upon prolonged incubations. *C. tetani* is usually highly motile by peritrichous flagella, this property being responsible of their swarming growth on agar medium. However, some strains are nonmotile and nonflagellated. These bacteria form spores, which appear as translucent terminal enlargements which give the typical appearance of drumsticks. The sporulation rate is variable according to the strains. At pH 7 or above and at temperature near 37 °C, the sporulation starts within 24 h of culture and continues for 4–12 days or more. Sporulation does not occur above 41 °C, and it is slow at pH < 6. The sporulation process depends on the nature of the culture medium (Bytchenko 1981). Spores generally survive moderate heating (75–80 °C for 10 min) but usually are destroyed within 1 h at 100 °C.

Germination of *C. tetani* spores occurs both under anaerobic and aerobic conditions, but the outgrowth of *C. tetani* which follows spore germination is strictly dependent upon a low oxidation-reduction potential (Smith 1975). In fact, *C. tetani* is an anaerobic bacterium and forms colonies on the surface of agar medium only under anaerobiosis. Motile strains swarm over the entire surface of the agar leading to a transparent film. Discrete colonies (2–5 mm) can be obtained with media containing 3–4% agar. On blood agar, colonies are slightly raised, semitranslucent, gray, with an irregular margin and

surrounded by a narrow zone of hemolysis. *C. tetani* grows fairly well on the usual media containing peptones or tissue extracts.

Most of the usual biochemical tests used for *Clostridium* identification are negative, as no carbohydrates are acidified, and there is no proteolysis nor production of lipase and lecithinase. Gelatin is liquefied slowly (2–7 days), the peptone used in the basal medium is of considerable importance to evaluate the ability of *C. tetani* to liquefy the gelatin. H_2S and indole are usually produced (Smith 1975).

C. tetani strains are sensitive to penicillin and metronidazole. However, intravenous administration of penicillin can be inefficient due to an impaired transport of the antibiotic in the wound, and wound debridement is a required step in the treatment of tetanus (Campbell et al. 2009).

Genetic Characteristics

The G+C content of *C. tetani* is 25–26% (Cato et al. 1986). This species has been classified in the *Clostridium* group II by using 23S rRNA homology (Johnson and Francis 1975). The *Clostridium* genus encompasses more than 100 species which display a wide range of phenotypes and genotypes (Hippe et al. 1992). Recently, phylogenetic analysis using 16S rRNA comparison indicate that the *Clostridium* genus should be restricted to the homology group I, as defined by Johnson and Francis (Johnson and Francis 1975; Lawson et al. 1993). According to these data, *C. tetani* should be classified in a different genus. However, a study based on restriction maps of 16S rRNA showed that *C. tetani* belongs to the same cluster as *C. perfringens, C. sporogenes*, and *C. botulinum* C and G, which are members of the homology group I of Johnson and Francis (Gurtler et al. 1991).

Ten neurotoxigenic and three nontoxigenic *C. tetani* strains studied by Nakamura et al. were homogeneous in DNA/DNA hybridization (85–93% similarity) (Nakamura et al. 1979). *C. tetani* is similar culturally and biochemically to *C. cochlearium* and *C. tetanomorphum*, but it can be distinguished from the two latter species by DNA comparison (Nakamura et al. 1979; Wilde et al. 1989). *C. cochlearium* and *C. tetanomorphum* are nontoxic, and they are difficult to differentiate from the nontoxic *C. tetani* strains according to the bacteriological characteristics, as they do not, or weakly, liquefy gelatin. Contrary to *C. tetani*, *C. tetanomorphum* acidifies glucose and maltose (Cato et al. 1986).

The complete genome sequence of a toxigenic *C. tetani* strain has been determined (Brüggemann et al. 2003). It consists of a 2,799,250 bp chromosome containing 2,372 putative genes and of a 74,082 bp plasmid containing 61 genes. *C. tetani* possesses many genes for peptidases, amino acid, and lipid degradation, whereas genes for sugar utilization are lacking. It contains numerous transport-related genes, in particular 35 genes, for sodium ion-dependent systems indicate that Na^+ gradient is a major driving force in membrane transport. The TeNT encoding gene and seven putative regulatory genes are localized on the plasmid, whereas the tetanolysin (a hemolysin) gene and putative adhesin genes are located on the chromosome. Many genes encoding for putative adhesins have been identified: 2 fibronectin-binding proteins, 11 related surface-layer proteins (SLP), 19 homologues to a *Clostridium difficile* adhesin, and 2 proteins with multiple leucine-rich repeat domains similar to the *Listeria monocytogenes* internalin A (Brüggemann et al. 2003). SLP-A shows important size variation from strain to strain (Qazi et al. 2007). Genome analysis has revealed different sets of surface-associated protein genes in pathogenic Clostridia, which probably mediate their interactions with the environment or host and account why some clostridial species such as *C. tetani* and to a lesser extent some *C. botulinum* strains of group I can develop in wound (Bruggemann and Gottschalk 2008).

Ecology of Neurotoxin-Producing *Clostridia*

C. tetani and BoNT-producing *Clostridia*, as the other *Clostridia*, are largely present in the environment mainly in the form of spores, which are able to survive for very long periods under extreme conditions (including heat, dryness, radiations, chemicals, and oxygen). Spore germination and cell division occur only under anaerobic conditions and in the presence of the appropriate nutritional requirements. This restricts the habitat of the *Clostridium* to anaerobic areas or areas with low oxygen tension and containing sufficient amounts of organic materials. Based on their physiological properties (tolerance or extreme sensitivity to oxygen, requirement of particular pH, temperature, and substrate for growth or spore germination), the distribution of the different *Clostridium* species in nature is not uniform (Hippe et al. 1992). Saccharolytic *Clostridia* such as *C. butyricum* are able to grow on carbohydrates and are mainly found in decomposing vegetables and fruits. Proteolytic and gelatinolytic *Clostridium* such as *C. botulinum* and *C. tetani* are preferentially associated with animal cadavers and soils or sediments rich in organic material. They can also be found in the digestive tract of healthy humans and animals, and, after death, they participate to the cadaver decomposition.

In the Environment

C. tetani

C. tetani is an ubiquitous organism which is commonly found in soil samples in all parts of the world. The frequency of its isolation is variable according to the different investigations. Surveys in Japan, Canada, Brazil, and the United States have yielded 30–42% positive samples (Smith and Williams 1984). Several factors influence the different frequencies of *C. tetani* isolation from soil sample, including pH, temperature, moisture, amount, and type of organic materials. Thus, germination and multiplication of *C. tetani* have been observed preferentially in neutral or alkaline soil, with temperatures >20 °C and humidity reaching 15% (Smith 1975).

Geographical distribution of *C. tetani* shows a higher presence in southern regions, and accordingly the incidence of tetanus is higher in warmer countries (West and Central Africa,

Southeast Asia, India, Pacific Islands, and south of the United States) than in the cooler parts of the world (Canada, Norway, England, Finland, Sweden) (Smith 1975). This bacterium can be found in the intestine of animals, but it does not represent a significant part of the normal digestive flora. Different surfaces and objects contaminated with soil particles, dust or feces may contain *C. tetani*. Toxigenic strains have also been isolated within hospitals from catgut, cotton wool, dust and air samples, human skin, and wounds (Bytchenko 1981).

BoNT-Producing *Clostridium*

C. botulinum is widespread in soils as well in lake and sea sediments in most parts of the world. However, the different toxinotypes of *C. botulinum* are not equally distributed, some of them being restricted to particular ecological areas. The factors responsible for the geographical distribution of the different toxinotypes are still poorly understood. In general, toxinotypes A, B, E, F, and G seem to have their principal habitat in soil and sea and freshwater sediments. The toxinotypes A and B occur more frequently in soil samples, and the regional distribution of these two toxinotypes are different. Toxinotype E is more predominant in sea or lake sediments and fish than in soil. The toxinotypes C and D appear to be obligate parasites of birds and of other animals. Cadavers of animals or birds died of botulism or from healthy carriers are the main source of these organisms. They are seldom encountered in soil samples, except in the areas where the incidence of animal botulism is high (Smith 1975). *C. botulinum* is not usually found in digestive tract of healthy humans, but it can be found in that of animals, particularly *C. botulinum* C and D, particularly in regions where botulism is frequent.

C. botulinum A and B

Numerous investigations have surveyed the prevalence of *C. botulinum*, particularly in the USA (reviewed in (Dodds 1993b; Hauschild 1989)). Types A and B were generally found from neutral to alkaline soil samples poor in organic matter and much more rarely from aquatic sediment. *C. botulinum* type A is predominant in the western part of the United States (west of the Missouri and Mississippi rivers), in soil that was neutral to alkaline (average pH 7.5) with a lower than average organic content. In contrast, type B prevails largely in the eastern part of the United States. This toxinotype was recovered in slightly more acidic soil samples (average pH 6.25) with a higher level of organic matter and mainly from cultivated soils (pasture, fields) (Smith 1978). Other investigations reported the prevalence of type B in cultivated samples, but noted that this type is rare in soils contaminated with manure or animal feces. The fertilization was not considered as a significant factor responsible of the *C. botulinum* type B frequent presence in cultivated soils (Dodds 1993b).

The incidence of *C. botulinum* A and B is very low from aquatic (sea or freshwater) sediments and soil samples in the northern part of America (Alaska, Canada). Fewer investigations have been performed in Central and South America. Surveys from soil samples in Argentina, Brazil, and Paraguay demonstrated the presence of *C. botulinum* A and B, with a prevalence of type A. *C. botulinum* type B is the most common type from soil and sediment samples in Central and South Europe (Great Britain, Ireland, Netherlands, France, Switzerland, and Italy) and from soil samples in Denmark and Ireland. In Great Britain and Ireland, *C. botulinum* is much more frequent in lake and loch sediments than in soil samples, and type B is predominant. *C. botulinum* A and B are also largely diffused in Asia including ex USRR, China, and Taiwan. Contamination level and type detection vary from region to region and depend from several factors: temperature, moisture and organic substance content, and other unidentified factors. Soil from south regions with warm climate or subtropical and tropical areas showed the higher incidence of *C. botulinum* A and B. Even higher populated regions are much more contaminated than desertic areas. Distribution of types A and B varies locally and does not correspond to large geographical areas like in Northern America.

Few reports concern the incidence of *C. botulinum* in Africa. In Kenya, the soil samples examined seemed to be heavily contaminated, with a predominance of type A. In contrast, the contamination in South Africa seemed to be low, *C. botulinum* B has been identified in 3 soil samples out of 102 (Knock 1952).

In Australia and New Zealand, *C. botulinum* A and B have been evidenced, but their presence in the environment is low reflecting a low incidence of human botulism in these areas.

C. botulinum E

The distribution of type E is more regional than of other types. *C. botulinum* E is mainly found in north areas of the Northern Hemisphere: north part of America (Alaska, Canada, Northern United States), North Europe (Island, Greenland, Denmark, Norway, Sweden, coast of Baltic Sea), and North Asia (north part of ex USRR, Caspian Sea, North Japan Islands: Hokkaido, north part of Honshu) from soil that is in contact with water (Smith 1992). The ability of *C. botulinum* E to grow a very low temperature reflects its prevalence in areas with cold temperature. Its frequency decreases considerably in south areas with warm weather. This type is mainly found in aquatic environment (sediment, soil from the shore of lake, sea, and river). Other factors such as organic matter content, salinity, and other unidentified factors influence the distribution of *C. botulinum* E, which varies greatly from one region to other. Thus, this organism is commonly found in the Great Lakes in the north of the United States but more frequently in Lake Michigan than in any of the other lakes, and in Lake Michigan, it was found more frequently in Green Bay than elsewhere. *C. botulinum* E is also very common in the Pacific Northwest. Sediment samples of Lake Washington near Seattle contained 18–25 C. *botulinum* E per g. These particular areas seem to represent the principal habitat of *C. botulinum* E, where it can grow and multiply (Smith 1975). A particular high prevalence of *C. botulinum* E occurs in Baltic Sea. These bacteria are most frequent and spore counting more abundant in sea than in freshwater sediment samples. Low oxygen content, low salinity, presence of biomass, and depth seem to be more important factors than temperature controlling the propagation of *C. botulinum* E in aquatic environment

(Hielm et al. 1998c). The contamination of raw fish in Finland ranges from 10% to 40% depending on fish species (Hielm et al. 1996; Hyytia et al. 1998). *C. botulinum* E was also detected in fish roe (4–14% of the samples), vacuum-packed (5%), air-packed (3%) fishery products, and vacuum-packed hot-smoked whitefish (10%) (Hyytia et al. 1998). Investigations in Finnish trout farms evidenced *C. botulinum* E in farm sediment samples (68%), fish intestinal contents (15%), and fish skin samples (5%°) (Hielm et al. 1998a). Recent investigation in Northern France shows a prevalence of *C. botulinum* of 16.5% from 175 sea fish samples, but the predominant toxinotype was type B (72%) followed by type A (24%) and type E (4%). Only one sediment sample from 25 contained *C. botulinum* E (Fach et al. 2002).

C. botulinum C and D

C. botulinum types C and D are widespread throughout the world. *C. botulinum* C is mainly found in muds, sediments of marshes, ponds, and seashore where botulism in waterfowl is endemic. Outbreaks of botulism in birds and presence of *C. botulinum* C in their environment have been reported in the United States, Great Britain, Denmark, Netherlands, France, and Japan. Intestinal contents and cadavers of susceptible birds seem to be the principal habitat of *C. botulinum* C. This type has also be detected in soil from warm areas such as in Indonesia, Bangladesh, and Thailand (Dodds 1993b; Hauschild 1989; Smith and Sugiyama 1988).

C. botulinum D is more frequently associated with botulism in animals (ruminants, horses). Carcasses of these animals and also from small animals (rodents) constitute the most common source of this organism. *C. botulinum* D can also be identified in soil samples where animal botulism is common (South and Central Africa, Australia, America, Europe) (Smith and Sugiyama 1988).

C. botulinum F and G

The types F and G are much more less frequently encountered than other types. Since the first identification of *C. botulinum* type F from a homemade liver paste responsible of an human botulism outbreak in the Danish island of Langeland (Moller and Scheibel 1960), this type has been demonstrated in marine sediment of the west coast of the United States (Eklund and Poysky 1965) and in marine or freshwater sediments in Brazil, Venezuela, and Indonesia (reviewed in (Hauschild 1989)). *C. botulinum* G was first isolated from a soil sample in Argentina (Gimenez and Ciccarelli 1970), and was identified from necropsy specimens in cases of unexplained death in adults and infants (Sonnabend et al. 1981), and from 5 soil samples out of 41 in close association with cultivated land in Switzerland (Sonnabend et al. 1987b).

C. butyricum

C. butyricum is a very widespread bacteria in the environment including soil, freshwater and marine sediments, cheese, rumen of healthy calves, animal and human feces, and more rarely from clinical specimens (blood, urine, respiratory tract, pleural cavity, abdomen, wound, and abscesses) (Cato et al. 1986). *C. butyricum* has been involved in certain cases of necrotizing enterocolitis in newborns. Artificial feeding and intestinal stasis support abundant proliferation of *C. butyricum* in the intestinal content. The bacterial overgrowth is accompanied by large production of hydrogen and organic acids, mainly butyric acid, which have been recognized as the main virulence factors of *C. butyricum* in the neonatal necrotizing enterocolitis (Popoff 1990; Popoff et al. 1985).

The first two neurotoxigenic *C. butyricum* strains were isolated from infant botulism in 1985–1986 in Rome (Italy) (Aureli et al. 1986; McCroskey et al. 1986). Extensive studies have been carried out in the vicinity of Rome, and no strains producing BoNT/E were isolated; only *C. botulinum* A and B strains were found in 9.6% of 52 soil samples (Creti et al. 1990). Ten years later, two additional cases of toxi-infection with neurotoxigenic *C. butyricum* strains were described in young people in Italy. These isolates were genotypically and phenotypically identical to the former Italian strains (Fenicia et al. 1999). In 1994, several cases of foodborne botulism were reported in China. The implicated food consisted of salted and fermented paste made of soybeans and wax gourds (Meng et al. 1997). *C. butyricum* type E were isolated from soil samples around the patient's houses (Meng et al. 1999). The *bont*/E gene sequences from 11 Chinese *C. butyricum* strains were identical and differ from those of *C. botulinum* E (96.9% identity at the amino acid level) and the Italian *C. butyricum* strain BL6340 (95% identity). Pulsed field electrophoresis, Southern blot hybridization, and random amplified polymorphic DNA permitted to distinguish three clones among the neurotoxigenic *C. butyricum* strains: two corresponding to the Chinese isolates, one of which was responsible for the foodborne botulism outbreak, and one for the Italian strains. This indicates that the neurotoxigenic *C. butyricum* strains are clonally distributed in vast areas (Wang et al. 2000). In contrast, *C. botulinum* type E strains from fish and fish products showed a wide biodiversity (62 different subtypes among 92 isolates) (Hyytia et al. 1999b).

C. baratii

C. baratii is isolated from soil, sediments, normal and rat feces, and occasionally from war wounds, peritoneal fluid, infection of the eyes, ear, and prostate (Cato et al. 1986). The source of the toxigenic *C. baratii* producing a BoNT/F responsible of one case of infant botulism and two cases of adult botulism in the United States has not been elucidated (Hatheway 1993a).

In the Foods

This chapter concerns only *C. botulinum.*

In Fish and Seafood

In general, the contamination of fish and other aquatic animals by *C. botulinum* reflects that of the sediments of the respective areas. The incidence of *C. botulinum* depends on the areas and on the type of fish. In Northern America, the level of

contamination is high in fish from the Pacific Northwest (5% of ocean salmon, 17% of other fish, and 23% of salmon from rivers) (Dodds 1993a) and from the Great Lakes and adjacent bays (1–60% of fish) (Hauschild 1989). In the Pacific Northwest, 8% of coho salmon, 17% of sole and cod, 23% of steelhead trout, 24% of sockeye salmon, 31% of oysters, 23% of clams, and 18% of crabs were contaminated by toxigenic *C. botulinum* (Craig et al. 1968). Gills and intestines of fish are the most heavily contaminated parts. The presence of *C. botulinum* in gills seems to indicate an environmental contamination. *C. botulinum* E is predominant in fish and aquatic invertebrates from north part of America, Europe, and Asia. In general, *C. botulinum* E does not multiply in the intestine of living fish, but, after death, the bacterium can grow and produce BoNT. Dead fish in the sediments contribute to the persistence and multiplication of *C. botulinum* in the aquatic environment (Dodds 1993a) and to large outbreaks of botulism caused by the ingestion of dead fish containing high titer of BoNT causing a self-multiplicating chain reaction (Eklund et al. 1982). The incidence of *C. botulinum* is also high in Baltic Sea, Sweden and Denmark coastal waters, Caspian Sea, and Japan lakes, with a predominance of type E. In Great Britain, *C. botulinum* C was predominant in most fish farms, followed by types B, E, and F (Cann et al. 1975). Type C was also predominant in fish and aquatic animals from Indonesian waters (Dodds 1993a).

The contamination of prepared fish depends on several factors including the preparation method and the processing stages in addition to the possible infection of the harvested fish. The highest contamination was found in salted fish from Caspian Sea (Hauschild 1989) and dressed rockfish from California (Dodds 1993a). *C. botulinum* has been detected in brined, frozen, and vacuum-packed fish. Meat smoking reduces the incidence of *C. botulinum* contamination. In general, *C. botulinum* E is the predominant type in prepared fish, type A is most commonly found in fish from California, and type C followed by types D, A, B, and F in fish from Indonesia and tropical and subtropical waters (Dodds 1993a; Hauschild 1989).

In Meats

Meat contamination with *C. botulinum* is less frequent than that of fish, and it is lower in North America than in Europe. Animals may carry *C. botulinum* in their intestinal tract that may lead to meat contamination during its processing. Toxinotypes A and B are usually predominant, followed by C and more rarely by E. The incidence is variable according to the different investigations and to the samplings (Dodds 1993a). Thus, Robert and Smart reported that 2–6% of vacuum-packed bacon samples out of 140 in two investigations were contaminated by *C. botulinum*, and in one occasion, 20 of 138 samples were positive (65% contained type C and 15% type A, and the rest were untyped) (Roberts and Smart 1976).

In Fruit and Vegetables

Raw fruits and vegetables may be contaminated by *C. botulinum*, as a result of soil contamination. *C. botulinum* has been identified in fruits and vegetables but mainly in those harvested from the soil. Utilization of manure as fertilizer may affect the level of contamination. Cultivated mushrooms in Canada were found to be highly contaminated by *C. botulinum* B, while in other areas, this product was free or contained low level of *C. botulinum* (Dodds 1993a). Type A is predominant in fruits and vegetables from California; type B in vegetables from Italy, Germany, and other European countries; types A and B were detected in the former Soviet Union (Dodds 1993a).

In Other Foods

Honey from various areas (United States, Argentina, Canada, China, Hungary, Japan, Mexico, Spain) may contain *C. botulinum*. The level of contamination is usually low (1–10 spore per kg), but the honey samples associated with infant botulism contained 10^3–10^4 spores per kg. Type A was detected more frequently than type B, C, or D (Dodds 1993a). The presence of *C. botulinum* in other foods such as dairy products or prepared foods (boil-in-bag foods, vacuum-packed foods, pressurized foods, dehydrated and freeze-dried foods) is very rare (Dodds 1993a).

Genetics of Clostridial Neurotoxins

Neurotoxin Gene Organization

The Botulinum Locus

BoNTs are produced by neurotoxigenic strains of *Clostridium* together with several associated nontoxic proteins (ANTPs). BoNTs and ANTPs associate to form large complexes, also known as progenitor toxins. ANTPs encompass a nontoxic-nonhemagglutinin component (NTNH) and several hemagglutinin components (HAs) or OrfX proteins (Oguma et al. 1999; Popoff and Marvaud 1999; Sharma et al. 2003). In botulinum complex, the proteins are not covalently linked, but their association occurs in cultures and naturally contaminated food. The complex is stable at acidic pH but dissociates at alkaline pH ($\geq$ pH 7) (Eisele et al. 2011).

The genes encoding the neurotoxins and ANTPs, which associate with BoNT to form the botulinum complexes, have been cloned and sequenced in representative clostridial strains of each BoNT type. The neurotoxin and ANTP genes are clustered in close vicinity and constitute the botulinum locus. The organization of the botulinum locus is conserved in the 3′ part but differs slightly in the 5′ part in the different types of BoNT-producing Clostridia. The *bont* genes are at the 3′ part of the locus and are preceded by the genes of the NTNH component. *ntnh* and *bont* genes are transcribed in the same orientation (*Fig. 12.1*), whereas HA genes (*ha*33, *ha*17, and *ha*70), which are upstream to the *ntnh-bon*t genes, are transcribed in the opposite orientation. The *ha* genes are missing in the nonhemagglutinating toxinotypes A2, E, and F. The *ha* genes of *C. botulinum* G only comprise *ha*17 and *ha*70. In the toxinotype A2, E, and F, a gene (*p*47) encoding a 47-kDa protein is immediately upstream of the *ntnh* gene, and both genes are transcribed in the same orientation. In addition, two genes (*orfX1* and *orfX2*), which are not related to *ha* genes,

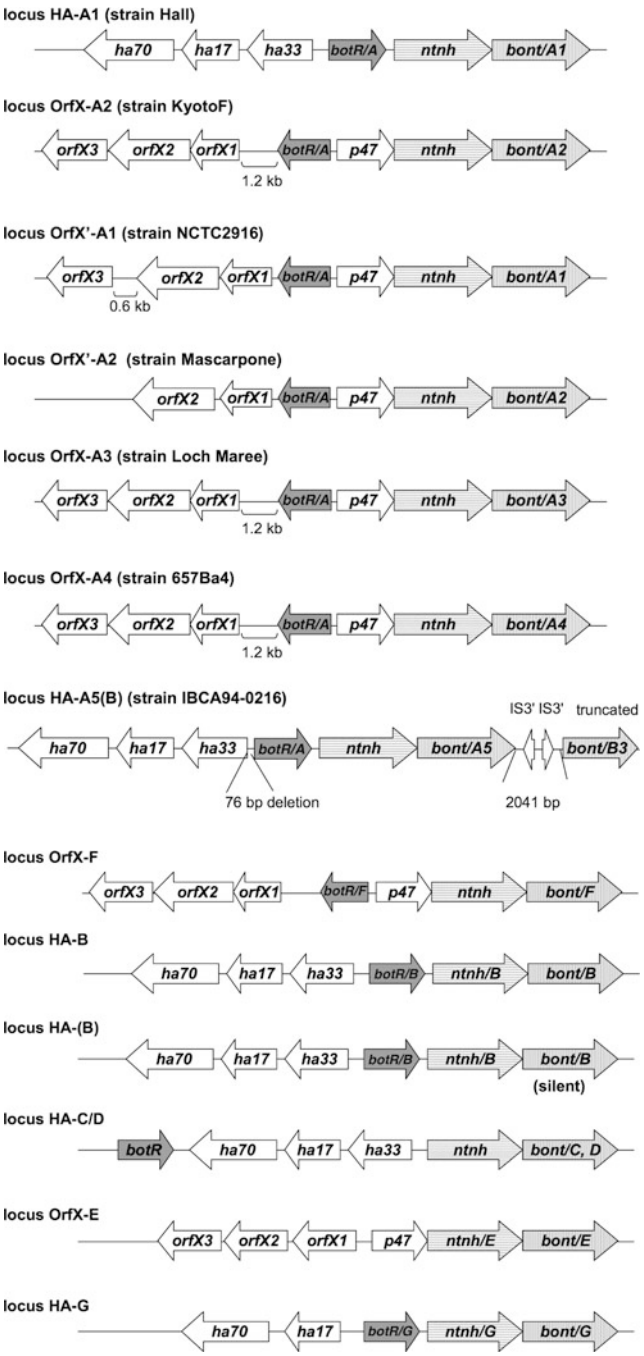

Fig. 12.1
Gene organization of botulinum loci in *C. botulinum* type A to G strains

lie upstream of *p47* in the opposite orientation. An additional gene, *orfX3*, was characterized in *C. botulinum* A2 strain Kyoto-F, downstream the gene *orfX2* and in the same orientation (Dineen et al. 2004). *p47* and the *orfX* operon are also associated with *bont* subtype A1, A3, or A4 (Jacobson et al. 2008a). Indeed, two main types of botulinum locus can be distinguished, the HA locus containing *ntnh* and *ha* genes and the OrfX locus containing *orfX*, *p47*, and *ntnh* genes in addition to *bont* gene (Fig. 12.1). The same *bont* gene can be inserted in a HA or OrfX locus. However, *bont/A1* is the only gene which has been found in either of the two types of botulinum locus.

A gene (*bot*R, previously called *orf*21 or *orf*22) encoding for a 21–22-kDa protein, which encodes for an alternative sigma factor involved in the regulation of botulinum locus gene expression (Raffestin et al. 2005), is present in different positions in different strains of *C. botulinum* (Fig. 12.1). In *C. tetani*, a gene (*tet*R) equivalent to *bot*R (Marvaud et al. 1998; Raffestin et al. 2005) is present upstream of the tetanus toxin (*tent*) gene. No *antp*-like genes have been identified in *C. tetani*

(Brüggemann et al. 2003). It is noteworthy that *bont*/R is not present in botulinum locus containing the *bont/E* gene, in *C. botulinum* or *C. butyricum* type E.

Usually, one clostridial strain produces only one type of neurotoxin and the botulinum locus is present in a single copy on the genome as suggested by Southern blotting of DNA fragments separated by pulsed field gel electrophoresis (Hutson et al. 1996; Lin and Johnson 1995). However, some rare strains synthesize two different BoNTs: BoNT/A-BoNT/B, BoNT/A-BoNT/F, and BoNT/B-BoNT/F producing strains have been isolated (Henderson et al. 1997; Hutson et al. 1996). The A-B strain contains two *bont* genes related to those of *C. botulinum* A2 and proteolytic *C. botulinum* B, respectively (Fujinaga et al. 1995). In such strains, the two neurotoxins are usually produced in different proportions. Thus, in Ba and Bf strains, BoNT/B is produced ten times more than BoNT/A and BoNT/F (Henderson et al. 1997). Some clostridial strains contain silent neurotoxin genes. Several *C. botulinum* A strains isolated from foodborne and infant botulism contain a silent *bont*/B gene. These strains are noted A(b). The characterization of strain NCTC2916 shows that it has two loci, A and B, which are 40 kbp distant within the chromosome. The botulinum B locus consists of *bont*/B, *ntnh*, *orf*21, *ha*33, and *ha*11 genes. The *bont*/A gene is identical to that of *C. botulinum* A1 strains, but the organization of the botulinum A locus is similar to that of *C. botulinum* A2 and F strains (*Fig. 12.1*). The BoNT/B nucleotide sequence is related to that of *C. botulinum* B strains (97% identity), but it has a stop mutation in position 128 and two base deletions (positions 2,839 and 2,944) resulting in reading frameshifts and multiple stop codons (Dineen et al. 2004; Hutson et al. 1996; Jovita et al. 1998). Silent *bont*/B has also been evidenced in nontoxigenic *C. subterminale* strains (Franciosa et al. 1994; Jovita et al. 1998). The strain *C. botulinum* 667 contains also two loci, A and B, 40 kbp distant within the chromosome. The genetic organization is the same than that in strain NCTC2916, and the *bont/B* gene is silenced by mutations and deletions (Hutson et al. 1996).

Genomic Localization of the Botulinum Locus

The genes encoding for the different types of BoNT are present on different genetic elements, including phages, plasmid, or chromosomes depending on the species and strain of Clostridia. In *C. tetani* and *C. argentinense*, the neurotoxin gene is present within a large plasmid (51 and 76 MDa, respectively). Plasmids of various sizes and bacteriophages have been found in *C. botulinum* A, B, E, and F, and previous works have shown that toxigenicity was not associated with the presence of these genetic elements (Eklund et al. 1989; Strom et al. 1984; Weickert et al. 1986). Therefore, the genes encoding for these neurotoxins were assumed to be located on the chromosome. However, it has been recently found that in some strains such as Loch Maree strain (subtype A3), 657Ba (type Ba and subtype A4), Okra (type B1), and Eklund 17B (type Bnp), the botulinum neurotoxin genes are harbored by large plasmids (47–270 kb) (Marshall et al. 2007; Smith et al. 2007). Plasmid location of neurotoxin genes seems common in *C. botulinum* type B strains, mainly in subtype B1, bivalent, and nonproteolytic strains (Franciosa et al. 2009). In the bivalent strain Ba657, the two botulinum locus, locus A and locus B, are located on the same plasmid and are separated by approximately 97 kbp. Similarly, the neurotoxin genes, *bont/B* and *bont/f*, from one Bf strain are located on a same plasmid (pBf),which is very related to pCLJ from 657Ba strain (Hill et al. 2009). Interestingly, none of botulinum plasmids show synteny to *C. tetani* plasmid pE88, which contains the *tent* gene (Hill et al. 2009). In *C. botulinum* type E and neurotoxigenic *C. butyricum* strains, the location of the BoNT/E-encoding gene is located on the chromosome. In *C. botulinum* C and D, it has been clearly evidenced that BoNT is encoded by bacteriophages (reviewed in (Poulain et al. 2006)).

The location of botulinum locus within chromosome or plasmid seems to occur not at random but at specific sites. Indeed, in strains from group I or II, whose genome sequencing is available, three specific sites of botulinum locus integration have been identified. OrfX-A2, orfX′-A1, and orfX-F locus are located in the *ars* operon, which contains 3–5 genes involved in arsenic reduction. OrfX′-A1 and orfX-F locus share a similar integration site at the 5′ end of the *ars* operon, whereas OrfX-A2 locus is inserted between two copies of *arsC* gene. HA-A1 and HA-B locus, which contain a recombinant *ntnh* gene type A and type B strains, are found in the *oppA/brnQ* operon encoding for extracellular solute-binding protein and branched chain amino acid transport proteins, respectively. This operon is lacking in nonproteolytic *C. botulinum* type B, *C. botulinum* type E, and *C. butyricum* type E strains. The third integration site is the *rarA* gene, which contains the OrfX-E locus in *C. botulinum* type E and *C. butyricum* type E strains. *rarA* encodes a resolvase protein involved in recombination or insertion events of transposons. Interestingly, the botulinum E locus is inserted in the same codon (102) of *rarA* gene in both *C. botulinum* type E and *C. butyricum* type E strains, and the inserted botulinum locus contains an additional intact *rarA* gene (Hill et al. 2009).

Two specific sites of botulinum locus location have been identified on plasmids from group I strains, one contains OrfX-A3, OrfX-A4 from Ba strain, or OrfX-F from Bf strain, and the second harbors the HA-B locus from *C. botulinum* B1 strain or bivalent Ba4 or Bf strains. The HA-npB locus is located on a plasmid different from those of group I strains. However, the downstream flanking region of the HA-npB locus contains an IS element, a transposon-associated resolvase, and a site-specific recombinase (Hill et al. 2009).

Genome Characteristics of *Clostridium botulinum*

The complete genome sequences of 10 C. *botulinum* strains from groups I and II have been solved so far. *C. botulinum* genomes consist of a circular DNA chromosome, the size of which (3,659,644–4,155,278 bp) is in the same range than that of other known *Clostridium* genomes (*C. perfringens* 3 031 430 bp; *C. difficile* 4,290,252 bp; *C. acetobutylicum* 3,940,252 bp) except that of *C. tetani* which is considerably smaller (2,799,250 bp). The G+C content (27–28%) is similar

to that of most clostridial species. The main features of the 10 C. *botulinum* genomes are listed in Tables 12.2–12.4. Most strains also possess plasmids which vary in size (16–270 kbp).

The *C. botulinum* A (strain Hall) genome (Sebaihia et al. 2007) shares 1,126 (31%) to 1,565 (43%) genes with the genomes of *C. acetobutylicum*, *C. perfringens*, *C. difficile*, and *C. tetani*, confirming the heterogeneity of the *Clostridium* genus. *C. botulinum* A shares a larger number of orthologous genes with *C. acetobutylicum* (43%), a nontoxigenic *Clostridium*, than with the other toxigenic Clostridia. Among the sequenced toxigenic Clostridia, the most related to *C. botulinum* A is *C. tetani* (40% orthologous genes) which also synthesizes a neurotoxin related to botulinum neurotoxin; the most unrelated is *C. perfringens* (31% orthologous genes).

The shared genes between *C. botulinum* and other Clostridia mainly encode basic functions such as cell division, macromolecule biosynthesis, central/intermediary metabolism, and germination/sporulation. The *C. botulinum* unique genes are spread all around the genome and are not clustered in specific regions. They seem to be involved in accessory functions including transport/binding proteins, energy metabolism, cell surface proteins, and regulation (Sebaihia et al. 2007).

In contrast to the *C. difficile* genome (strain 630) which shows a mosaic organization with multiple horizontal gene acquisitions mainly via mobile elements such as conjugative transposons (11% of the genome) (Sebaihia et al. 2006), *C. botulinum* genomes have a more homogeneous composition without evidence of recent gene acquisition. Only two prophages, two prophage remnants, one functional, and 11 nonfunctional transposase genes were found in the genome of *C. botulinum* A ATCC3502 (Sebaihia et al. 2007), and two prophages were evidenced in *C. botulinum* A ATCC19397. A relatively low proportion (0.8–6.8%) of genes are predicted (Colombo prediction) to be acquired horizontally in *C. botulinum* genomes. However, this portion is significantly higher in group I strains (4.5–6.8% of all genes) than in group II strains (0.8–1.2%).

An overall comparison between complete *C. botulinum* genomes revealed high similarity between genomes from group I strains and their strong distance to group II strains. Four to six large variable genomic regions exist which are scattered around the genomes. These are absent in some strains or show decreased similarity. Whereas most proteins (81–86%) of group I strains share a protein identity of over 90%, only 2–3% of proteins from group II genomes have orthologs encoded in group I genomes with such a high protein identity. Applying a less stringent cutoff (10% protein identity), group I and group II strains share only 48% of their proteome. In addition, there is almost no genome-wide synteny between group I and group II strains, except a region around the origin of replication. The fact that the genomes of six strains of group I (three *C. botulinum* A1, one *C. botulinum* A3, one proteolytic *C. botulinum* B1, and one proteolytic *C. botulinum* F) organisms are highly similar confirms previous genetic analysis, indicating that group I *C. botulinum* strains form a homogeneous bacterial species distinct from the other *C. botulinum* types (reviewed in (Popoff 1995)). This was further confirmed in a set of 5 C. *botulinum* A strains by comparative genomic hybridization microarrays and pulsed field gel electrophoresis (Raphael et al. 2008). However, these strains show limited genomic rearrangements in the botulinum locus (see below). A multiple-locus variable-number tandem-repeat analysis was designed in order to differentiate *C. botulinum* A strains within subtypes. Ten variable-number tandem-repeat (VNTR) regions dispersed throughout the *C. botulinum* A ATCC3502 genome, which are distant from the botulinum locus, permitted to differentiate the strains into subtypes A1-A4 and to distinguish 30 genotypes within 53 strains of subtype A1 (Macdonald et al. 2008). A multiple sequence typing (MLST) based on seven housekeeping genes has also been proposed for *C. botulinum* A subtyping and phylogenetic analysis. Thus, 24 lineages were identified from 73 C. *botulinum* A strains (Jacobson et al. 2008b). Chromosome organization of group I *C. botulinum* A, B, and F strains is also shared by *C. sporogenes* (Hill et al. 2009), confirming that this *Clostridium* species is very close from proteolytic *C. botulinum* strains except the absence of neurotoxin gene.

Two *C. botulinum* E strains (Beluga E1 and Alaska E3) and the nonproteolytic *C. botulinum* B Eklund17B share chromosome synteny, indicating that strains from group II contain a related chromosomal background, which is distinct from that of *C. butyricum* type E (Hill et al. 2009).

Plasmids are common in *C. botulinum* with sizes ranging from 16,344 to 270,346 bp and containing 19–329 genes. Plasmids from strains Loch Maree, Okra, and Ba657, although of different sizes, share large regions that are very similar to each other (Smith et al. 2007). However, they are poorly related to plasmids that do not contain neurotoxin genes. They can replicate independently, since they contain complete DNA polymerase III complex enzymes and DNA helicase II (Smith et al. 2007).

Genomic synteny of one representative type C strain and one representative type D strain indicates a related chromosome organization between these two strains from group III (Brüggemann et al. 2011). Chromosome organization is conserved between isolates, and it is closely related to that of *C. novyi* (Skarin et al. 2011). From group III, their genetic organization is distinct from that of group I and II strains. Botulinum C2 toxin, which is binary toxin involved in actin filament depolymerization (Barth et al. 2004), is encoded by genes located on a large plasmid in *C. botulinum* type C (Barth et al. 2004). Genomes sequencing of *C. botulinum* type D strain 1873 shows that this strain contains two plasmids, one pCLG1 harboring the genes for the enzymatic component and binding components of C2 toxin, and the other, pCLG2, containing genes for clostripain (a cysteine protease) and a thermolabile hemolysin. In addition, *C. botulinum* strains contain phages, which harbor the neurotoxin gene and the C3 exoenzyme gene.

Genetic Diversity of *C. botulinum* Strains and Botulinum Neurotoxin Gene Variation

Genetic analysis by 16s RNA gene sequence comparison or DNA/DNA homology have shown that *C. botulinum* strains form four

Table 12.2

Examples of foodborne botulism outbreaks. Additional botulism outbreaks are listed in (Lindstrom et al. 2006b; Peck 2006, 2009; Peck et al. 2011)

Country	Year	Type	Cases (deaths)	Food	References
Madagascar	1982	E	60 (30)	Commercial pork sausage	Viscens et al. (1985)
USA/Israel	1987	E	8 (1)	Commercial uneviscerated salted, air-dried fish (kapchunka)	Slater et al. (1989)
UK	1989	B p	27 (1)	Commercial hazelnut yogurt	O'Mahony et al. (1990)
Sweden	1991	E	2 (0)	Vacuum-packed hot-smoked rainbow trout	Korkeala et al. (1998)
Egypt	1991	E	>91 (18)	Commercial uneviscerated salted fish (faseikh)	Weber et al. (1993b)
Switzerland	1993–1994	B	12	Ham	Troillet and Praz (1995)
Sweden	1994	E		Vacuum-packed hot-smoked rainbow trout	Korkeala et al. (1998)
Italy	1996	A	7 (1)	Commercial dairy product (mascarpone)	Aureli et al. (2000); Franciosa et al. (1999)
Germany	1997	E	2	Commercial hot-smoked vacuum-packed fish (raucherfish)	Korkeala et al. (1998)
Iran	1997	A	27 (1)	Traditional cheese preserved in oil	Pourshafie et al. (1998)
Argentina	1998	A	9	Meat roll (matambre)	Villar et al. (1999)
Thailand	1998	A	13 (2)	Home-canned bamboo shoots	Control (1999)
Algeria	1998	A	340 (37)	Commercial sausage "halal"	Mesbah (2009)
Morocco	1999	B	78 (20)	Commercial sausage	Kissani et al. (2009); Ouagari et al. (2002)
USA	2001	A	15	Commercial frozen chili sauce	Kalluri et al. (2003)
South Africa	2002	A	2 (2)	Commercial tinned pilchards	Frean et al. (2004)
Georgia	1980–2002	B (85%) E (10%) A (1%)	879 (58)	Home-preserved vegetables (80%), smoked fish (12%), smoked meat (2%)	Gottlieb et al. (2007); Varma et al. (2004)
France	2003	B	10 cases 3 outbreaks	Commercial beef and poultry sausages "halal"	Carlier et al. (2007); Espié et al. (2003)
Germany	2004	E	1	Commercial vacuum-packed smoked salmon	Dressler, (2005)
Italy	2004	ß	28	Green olives (restaurant)	Cawthorne et al. (2005)
Finland	2006	E	1	Commercial vacuum-packed smoked whitefish	Lindstrom et al. (2006b)
Canada/USA	2006	A	6 (1)	Commercial refrigerated carrot juice	Sheth et al. (2008)
Thailand	2006	A	209	Home-canned bamboo shoots	Kongsaengdao et al. (2006); Ungchusak et al. (2007)
USA	2007	E	5	Home-salted uneviscerated fish	Sobel et al. (2007)
USA	2007	A		Commercial hot dog chili sauce	Control (2007)
France	2008	A	2	Commercial chicken enchiladas	King, (2008)
France	2008	A	3	Home pumpkin ham	Mazuet et al. (2011)
France	2009	E	3	Commercial vacuum-packed hot-smoked whitefish (Canadian origin purchased in Finland)	King et al. (2009)
France	2010	A	5 (1)	Home-canned string beans	Mazuet et al. (2011)
Brazil	2000–2008	A, B	27 cases 18 outbreaks	Home-canned meat, commercial canned soybean cheese, home confit of fish, chicken pie	Rowlands et al. (2010)
France	2011	A	8 cases 2 outbreaks	Commercial green olive paste	Pingeon et al. (2011)
Finland	2011	B	2	Commercial olives stuffed with almonds	Jalava et al. (2011)
Scotland	2011	A	3	Commercial "korma" sauce	Browning et al. (2011)

Table 12.3
Examples of indications benefiting from botulinum toxin

Indication	Botulinum toxin is an evidence-based treatment	References
Focal dystonia, spasticity, movement disorders, and related		
Strabismus		Rowe and Noonan, (2009); Scott (1980)
Blepharospasm		Costa et al. (2005a)
Hemifacial spasm	Yes	Costa et al. (2005b)
Cervical dystonia	Yes, type A and B	Albanese et al. (2006); Costa et al. (2005c)
Dysphonia	Yes	Watts et al. (2008)
Writer's cramp	Yes	Dashtipour and Pender, (2008)
Upper limb spasticity	Yes	Fehlings et al. (2010); Hoare et al. (2010); Sheean et al. (2010)
Lower limb spasticity	Yes	Love et al. (2010); Olver et al. (2010)
Masseter hypertrophy		Al-Muharraqi et al. (2009)
Autonomic disorders, Pain		
Sialorrhea (excessive saliva) in people with motoneuron disease/amyotrophic lateral sclerosis	Yes	Young et al. (2011)
Drooling	Yes, level B	Naumann et al. (2008)
Hyperhidrosis (palmar, axillary)	Yes, level B and A, respectively	Naumann et al. (2008)
Gustatory sweating		Naumann et al. (2008)
Neurogenic detrusor overactivity or idiopathic detrusor overactivity	Yes	Duthie et al. (2007); Naumann et al. (2008)
Benign prostatic enlargement		Chuang and Chancellor, (2006); Oeconomou et al. (2008)
Management of pain	Evidence-based conclusions are limited by the availability of data	Naumann et al. (2008); Qerama et al. (2010)
Secondary headaches and cranial neuralgias		Linde et al. (2011)
Others		
Facial aesthetics	Yes	Gadhia and Walmsley, (2009)

distinct clusters which correspond to the physiological groups I to IV (Collins and East 1997; Hutson et al. 1994). Amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis (PFGE) analysis also confirms the classification of proteolytic types A, B, and F strains in group I and the nonproteolytic types B, E, and F strains in group II but can differentiate individual strains into each group (Hielm et al. 1998a, b; Hill et al. 2007; Hyytia et al. 1999a, b; Keto-Timonen et al. 2005; Nevas et al. 2005). These methods have been used in epidemiological studies and are useful tools to investigate relatedness between strains isolated from patients and food. For example, among proteolytic *C. botulinum* strains, PFGE analysis differentiates the toxinotypes A, B, and F at 83–86% similarity level and enables discrimination of most of individual strains. A greater diversity was observed between type A strains than in type B strains (Nevas et al. 2005). These studies also indicate that each *C. botulinum* group is heterogeneous at the genome level.

A high level of similarity was observed between strains from group I by using DNA hybridization with a DNA microarray including 94% of the coding sequences from strain Hall. Two type A strains share 95–96% of the strain Hall coding sequences, and seven other proteolytic strains have 87–91% common coding sequences (Sebaihia et al. 2007). A larger investigation reports that 58 C. *botulinum* strains from group I share 63% of coding sequences with those of strain ATCC3502 (Carter et al. 2009). Interestingly, two *C. sporogenes* strains (physiologically related to *C. botulinum* group I but nontoxigenic) are significantly similar to Hall strain and share 84–87% of the coding sequences (Sebaihia et al. 2007). In another microarray study, three *C. sporogenes* strains show approximately 63% common coding sequences with *C. botulinum* A ATCC3502 (Carter et al. 2009).

BoNT gene has been sequenced from a large number of strains, and sequence comparison has permitted to identify sequence variations in each toxinotype. Thereby, botulinum toxinotypes are divided into subtypes, which are defined as toxin sequences differing by at least 2.6% identity at the amino acid level (Smith et al. 2005). BoNT genes from type A strains show 92–95% nucleotide identities corresponding to 84–90% amino acid identities and are divided into four subtypes termed A1, A2, A3, A4, and A5 (*Table 12.1*). Subtypes A1 to A5 also differ in the botulinum locus composition. Type B genes differ from 2% to 4% at the nucleotide level and 3–6% at the amino acid level. They are classified in five subtypes, B1, B2, B3, bivalent B, and nonproteolytic B. BoNT genes from nonproteolytic type B strains form only one subtype, whereas those from proteolytic strains show a greater variation leading to a four subtype division (*Table 12.1*). Sequences of neurotoxin genes type B show an overall less variation than those of type A, but a more sequence variation is observed within members of each type B subtype compared to bont/A. BoNT/E sequences from *C. botulinum* type E (group II) fit in six subtypes (E1, E2, E3, E6, E7, E8), sharing 99% nucleotide identity and 97–99% amino acid identity, and are more distantly related to BoNT/E sequences from *C. butyricum* strains which are into two subtypes (E4, E5) with 97–98% nucleotide and 95–96% amino acid identities between sequences from both *Clostridium* species. Gene

Table 12.4
Examples of toxin products

Trade name	Manufacturing company	Form	Units/ pack	Toxin load /vial	Active substance	MW (kDa)	Main excipients
Botox®	Allergan Inc. (CA, USA)	Powder	100 Allergan units	~5 ng	Type A complex (neurotoxin + HAs + NTNH)	900	Human serum albumin (0.5 mg), NaCl
Vistabel® Botox Cosmetic®			50 Allergan units	2.5 ng			Human serum albumin (0.25 mg), NaCl
Dysport®	IPSEN Ltd. (GB)		500 Speywood units	4.35 ng		600–900	Human serum albumin (0,125 mg), lactose
Azzalure®	Galderma (manufacturer: IPSEN)		125 Speywood units	1.1 ng			Human serum albumin (0,125 mg), lactose
Neuronox®	Medytox (Rep. South Korea) / Q-Med Inc. (Sweden)		100	?		900	Human serum albumin, NaCl
CBTXA, BTXA Prosigne®	Lanzhou Inst. Biol. Products (Rep. of China)		100 (or 50)	~4–5 ng		?	Porcine gelatin, dextran, sucrose
Xeomin®(NT-201)	Merz Pharmaceuticals Gmbh (Germany)		100 units	0.6 ng	Type A neurotoxin (bi-chainal)	150	Human serum albumin (1 mg), sucrose
Bocouture®			50 units	0.3 ng			Human serum albumin, sucrose
Neurobloc® Myobloc®	Solstice Neurosci. Inc.	Solution	2,500 or more	25 ng or more	Type B	500/700	Human serum albumin, pH 5.6

diversity has also been evidenced in the other parts of the genome as tested by MLST and AFLP analysis, but most of *C. botulinum* E strains are conserved in a same clade. Subtype variation in *C. botulinum* E strains seems to result from recombination events rather than random mutations. High differences (up to 25%) have been found in nucleotide sequences of BoNT/F mainly in the region coding the light chain, and five subtypes have been identified in proteolytic *C. botulinum* F. The low number of strains type C, D, F, and G which were analyzed does not permit to significantly evaluate the neurotoxin gene diversity. In group III, mosaic genes between BoNT genes types C and D can be distinguished from classical types C and D strains. BoNT/F sequences from *C. botulinum* type F form a different cluster of those from *C. baratii* (Carter et al. 2009; Chen et al. 2007; Hill et al. 2007; Macdonald et al. 2011; Raphael et al. 2010).

Signification of sequence diversity in each toxinotype is not yet well known but could be important in diagnostic tests and development of therapeutic agents such as those based on immunotherapy. Thereby, BoNT/A1 and BoNT/A2, which differs by 10% at the amino acid sequence level, show large differences in monoclonal antibody-binding affinity. Among six monoclonal antibodies, which bind to BoNT/A1 with high affinity, three show a marked decrease in binding affinity (500 to more than 1,000-fold) to BoNT/A2. Only combinations of monoclonal antibodies, which tightly bind to toxin subtype, potently neutralize the corresponding toxin in vivo. Association of the three monoclonal antibodies with high affinity binding to subtypes A1 and A2 completely neutralizes A1 or A2 toxin, while replacement of two from three monoclonal antibodies by two having a low binding affinity to BoNT/A2 induces a decrease in BoNT/A2 neutralization (50-fold less) (Smith et al. 2005). The impact of subtype variation in binding and neutralization potency of polyclonal antibodies remains to be determined. Thus, development of therapeutic polyclonal or monoclonal antibodies as well as vaccines based on single toxin subtype needs to be evaluated for their protection ability with the other related subtypes. Although two toxins show a low level of sequence difference, they can have marked difference in activity if amino acid variation occurs in strategic toxin sites. Subtypes A1, A2, A3, and A4 of BoNT/A have been analyzed by sequence comparison, as well as molecular modeling and structure comparison with the crystal structure of subtypes, the impact of which is not known. Ganglioside-binding site is conserved in all subtypes of BoNT/A. The greatest variability was found in the light (L) chain, mainly between subtypes A3 and A4 (76% identity). The enzymatic site of L chain is conserved, but nonconservative mutations are observed in domains involved

in substrate (SNAP-25) recognition. When compared to subtypes A1 and A2, subtypes A3 and A4 show sequence variation in α-exosite and S1′ subsite recognition, respectively, suggesting that these subtypes have a decreased affinity and catalytic efficiency for their substrate (Arndt et al. 2006). Indeed, L chains from subtypes A3 and A4 show different catalytic properties of the substrate SNAP25 compared to L chain from subtypes A1 and A2, which show the same catalytic activity, although all L chain isoforms bind SNAP25 with similar affinity. L chain from subtype A4 and to a lower extent from subtype A3 cleaves less efficiently SNAP25 than L chain subtype A1 (2- and 23-fold less, respectively) (Ahmed et al. 2001; Henkel et al. 2009). Another example of gene variation and toxin activity difference is given by neurotoxins type B. BoNT/B from strain 111 (subtype B2) isolated from infant botulism differs from strain Okra/NT associated with foodborne botulism in Japan by 56 amino acid changes (95.7% identity), from which most occur in the half C-terminal part of the toxin (Ihara et al. 2003). BoNT/B from strain 111 shows an about tenfold lower specific activity than that of strain Okra/NT, and most of monoclonal antibodies which recognize the C-terminus of Okra/NT BoNT/B do not react with BoNT/B of strain 111. Binding affinity of BoNT/B of strain 111 to the receptor synaptotagmin II in the presence of ganglioside GT1b is 4.2 lower than that of Okra/NT BoNT/B. Mutations of 23 residues in the C-terminus of BoNT/B of strain 111 have been attributed to the lower binding affinity of the toxin to its receptor and thus to the lower specific toxicity (Ihara et al. 2003; Kozaki et al. 1998).

Sequence comparison of *bont* genes suggests that they have evolved separately in different genomic backgrounds (Hill et al. 2007). BoNT genetic diversity could also reflect a different geographical distribution of strains or their involvement in different epidemiological situations. *C. botulinum* subtype A2 was first identified in infant botulism in Japan and was found to differ from strains involved in foodborne botulism in adults referred as subtype A1 (Tabita et al. 1991; Willems et al. 1993). However, no correlation was evidenced between strains subtype A1 and A2 isolated from the United States and UK and their clinical origin, foodborne or infant botulism (Cordoba et al. 1995; Johnson et al. 2005). But, strains A1 are more prevalent in the United States, whereas subtype A2 strains are commonly isolated in Europe. Indeed, all the strains from foodborne botulism in the United States which have been analyzed fall into subtype A1, and all 33 C. *botulinum* type A isolated from Italy belong to subtype A2 as well as two strains from infant botulism in United Kingdom (Franciosa et al. 2004; Hill et al. 2007; Johnson et al. 2005). But 18 C. *botulinum* strains type A isolated in France or Europe by Prevot during the period approximately 1950–1960 are of subtype A1 (Hill et al. 2007). Divergent strains of subtype A2 characterized by five amino acid differences in BoNT/A2 and a slightly different botulinum locus organization (locus A2-OrfX′) with a shorter intergenic region between *orfX1* and *botR/A* genes (77 vs. 1,228 nucleotides) when compared to strain A2 Kyoto-F have been identified in Italy such as strain associated with consumption of contaminated cheese (Mascarpone) (◉ *Fig. 12.1*) (Franciosa et al. 2006). Organization of botulinum locus of strain Mascarpone is closely related to that of locus containing *bont/A1* in strain type A(B) NCTC2916 (Dineen et al. 2003; Henderson et al. 1996). Strains Mascarpone and Kyoto-F have probably a common origin and then a distinct evolution including a gene rearrangement in strain Mascarpone with an ancestor of strain NCT2916. Four *C. botulinum* A5(B) strains were isolated from wound botulism in heroin users in UK and one from infant botulism in California (USA) supporting a *bont* gene evolution independent of the geographical location and epidemiological situation (Carter et al. 2009; Dover et al. 2009).

Genetic diversity is also observed in the two FGI regions. Six profiles have been evidenced in 58 proteolytic strains from group I by DNA microarray which correlate with the diversity of flagellin glycan composition as determined by mass spectrometry. The FGI genetic diversity does no match with that of the botulinum locus, indicating an independent evolution of FGI and botulinum locus genes in a relatively stable genomic background of group I *C. botulinum* strains (Carter et al. 2009). In addition to cell wall and surface structure variations, a marked difference in proteolytic strains of group I consists in resistance to toxic compounds. Thereby, group I *C. botulinum* type B strains representative of strains found in North Europe are divided in two clusters BI and BII which differ by 413 coding sequences but contain a same neurotoxin gene of B2 subtype in a HA locus. In contrast to cluster BI strains, cluster BII strains are more resistant to arsenic and more sensitive to cadmium. Moreover, strains from the two clusters show other differences in metabolism, such as cluster BII strains growing at lower temperature than cluster BI strains (Hinderink et al. 2009; Lindstrom et al. 2009). This suggests a differential evolution of these environmental Clostridia in response to adaptation to distinct ecological niches.

Another differential genetic evolution is illustrated by neurotoxigenic *C. butyricum* strains. *C. butyricum* strains producing type E neurotoxin have been isolated from infant botulism and young people in Italy (Fenicia et al. 1999; McCroskey et al. 1986), as well as from botulism associated with consumption of fermented soybean in China (Meng et al. 1997, 1999; Wang et al. 2000). Based on toxin gene sequence, toxigenic *C. butyricum* strains from Italy and China are divided in two distinct subtypes, termed E4 and E5, respectively, indicating an independent evolution of *bont/E* gene after transfer in *C. butyricum* (Hill et al. 2007).

The Cellular and Molecular Mechanisms Involved in Neuroexocytosis: An Overview

An Overview of Neurotransmission

Transfer of information or command between neurons, or neurons and target cells (muscle fibers, endocrine cells, etc.) is most often chemical in nature and occurs at highly specialized contact sites termed *synapses*. Here, the release of neurotransmitter molecules by the presynaptic elements enables activation of receptors localized on the postsynaptic target. Neurotransmitter

molecules are comprised of small organic molecules as acetylcholine (ACh), catecholamines like dopamine or noradrenaline, serotonin (5-HT), glutamate, gamma-aminobutyric acid (GABA), glycine, adenosine triphosphate (ATP), and numerous peptides such as vasointestinal peptide (VIP), substance P (SP), and calcitonin gene-related protein (CGRP). Released transmitter substance(s) can activate ligand-gated ionic channels or metabotropic receptors, thus mediating either transmembrane ionic fluxes or activation of intracellular signaling pathway(s). For example, in the central nervous system, depending on the ion species flowing through the channel, activation of ligand-gated ionic channels can cause depolarization (i.e., excitation) or hyperpolarization (i.e., inhibition) of the postsynaptic plasma membrane, respectively, and the excitation/inhibition net balance determines eventual initiation of action potentials propagated in the neuron until the next synapses. The evoked endplate potential at muscle fibers following stimulation of the motor nerve (and subsequent ACh release) is a depolarization that may reach the threshold for initiating muscle action potential, which itself propagates along muscle fiber ultimately triggering its contraction. In the enteric nervous system, release of neurotransmitter molecules (as VIP) by mucosal nerve endings directly contacting the enterocytes, or indirectly via the activation of enterochrommaffin cells releasing 5-HT, can lead to activation of metabotropic receptors, intracellular activation of the adenylate cyclase and downstream cAMP-dependent pathways, resulting in an active cotransport of ions species (Na^+, K^+, Cl^-) and water efflux for osmotic compensation in the intestine lumen (viz. the molecular mechanisms of diarrhea (Burleigh and Banks 2007)).

In nerve terminals, transmitter molecules are stored into at least two different classes of secretory organelles: the small lucent synaptic vesicles and large dense core vesicles or granules. The small lucent vesicles, approximately 50 nm in diameter, contain small organic molecules and are formed by either budding from the early endosome or recycling of empty vesicles (Murthy and De Camilli 2003; Takamori et al. 2006). The large dense core vesicles are analogous to the secretory granules present in endocrine and exocrine cells and have a biogenesis different from that of the synaptic vesicles. Similar to many cargo vesicles, they are formed by vesicle budding from the trans-Golgi system, followed by homotypic fusion to form larger vesicles. Usually, they mediate release of peptide transmitter or certain catecholamines whose synthesis depends on the presence in the granule lumen of enzymes (as the dopamine beta-hydroxylase). Small synaptic vesicles and certain large dense core vesicles are equipped with a vacuolar-type ATPase that creates a proton gradient which drives specific vesicular transporters, thereby allowing active uptake and storage of neurotransmitter molecules inside these vesicles. Regardless the secretory organelle size, large or small, the release of its content into the extracellular space implicates a fusion of its membrane with the plasmalemma, which then allows passive diffusion of its content into the surrounding medium. This exocytotic process is triggered by a rise in the cytosolic concentration of Ca^{++}, in response either to the arrival of a propagated action potential (i.e., at the neuron nerve endings) or following activation of ionotropic or/and metabotropic receptors located on the plasma membrane of secretory cells (reviewed by (Kasai 1999)).

Mechanisms of Exocytosis and SNAREs

More than 100 proteins are implicated in Ca^{++}-dependent exocytosis. However, only a dozen of them participate in the core machinery required for transmitter release, while the others serve regulatory roles (Jahn and Scheller 2006; Rizo and Rosenmund 2008). Interestingly, several proteins of the release machinery are targeted and disabled by various bacterial toxins. A large body of evidence indicates that the synaptic vesicles do not move freely within the nerve ending cytosol, and regulation of their interaction with actin-based cytoskeleton allows fine-tuning of their movements (Doussau and Augustine 2000; Garner et al. 2000). At the fusion site, they bind to the scaffold proteins associated with a special presynaptic matrix termed "active zone" (Garner et al. 2000). When tethered to the fusion site, the synaptic vesicles are not yet fusogenic and must acquire fusion competence. A key step in this process is the pairing and assembly of a fusion particle comprised of VAMP (vesicle-associated membrane protein, also termed synaptobrevin), SNAP-25, and syntaxin, on the inner face of the plasma membrane (reviewed by (Jahn and Scheller 2006; Rizo and Rosenmund 2008)). These three proteins are also designed as the SNARE proteins (*s*oluble *N*-ethylmaleimide-sensitive factor *a*ttachment protein *re*ceptors). Munc-18 protein is the fourth essential protein partner; it participates in SNARE complex formation and binds to it (reviewed by (Jahn and Scheller 2006; Rizo and Rosenmund 2008)). The SNARE proteins or closely related isoforms are also involved in the fusion of large dense core vesicle or secretory granules. Assembly of the three SNAREs, and possibly munc-18, triggers hemifusion of vesicles and plasma membrane. Termination of fusion is triggered by a rise in intracellular Ca^{++} concentration, which may result from either Ca^{++} influx through voltage-gated channels (e.g., at nerve terminals), Ca^{++} release from intracellular stores, or both (e.g., at many neuroendocrine cells) (Petersen 2003; Rettig and Neher 2002). The principal Ca^{++} sensor for triggering fusion is synaptotagmin, which is an integral synaptic vesicle protein interacting with SNAREs (Bhalla et al. 2006; Chapman 2002; Koh and Bellen 2003; Rizo and Rosenmund 2008). Synaptotagmin is equipped with two "C2 domains," which acquire high affinity for membrane lipids upon binding to Ca^{++} ions but in a pretty high intracellular concentration (10–100 μM). Thus, synaptotagmin is likely sensing the Ca^{++} changes due to activation of voltage-dependent calcium channels. Its Ca^{++}-dependent interaction with plasma membrane leads to changes in membrane curvature and destabilization of the lipids permitting the fusion (Martens et al. 2007). Several SNARE/synaptotagmin complexes (possibly 4–6) must act synergistically to allow fusion of a synaptic vesicle (reviewed by (Jahn and Scheller 2006; Rizo and Rosenmund 2008)). Recently, a role similar to that of synaptotagmin has been assigned to the

Doc2 protein, given the high affinity of Doc2 for Ca^{++}. Thus, Doc2 is likely intervening in spontaneous exocytosis at nerve endings and secretory cells, which is triggered/regulated by minute changes in the resting intracellular Ca^{++} concentration (Groffen et al. 2006).

Actin Cytoskeleton and Small GTPases in Exocytotic Mechanisms

Many other proteins play key roles in synaptic vesicle trafficking and priming of tethered synaptic vesicles. Inside nerve terminals, vesicles traffic along actin filaments, and this implicates molecular motors like myosins II or V, and small GTPases of the Rab family (Rab3 and others) (Augustine 2001; Segev 2001). Reorganization of the actin cytoskeleton is coupled to Ca^{2+}-regulated exocytosis in endocrine cells. However, this is far from being clear in neurons (Bader et al. 2004; Doussau and Augustine 2000; Eitzen 2003). The role of actin in exocytosis seems to consist in governing the vesicle-granule trafficking toward release site (Bader et al. 2004; Eitzen 2003). Organization of the actin-based cytoskeleton is controlled by several proteins, including several small GTPases like Rho proteins (Hall 1998) and ADP ribosylation factor (ARF). Like most small GTPases, ARF and Rho proteins cycle between GDP-bound (inactive) and GTP-bound (active) states, thereby acting as signal transducers that respond to upstream signals. Thus, they activate downstream effector molecules which carry out their biological functions. Rho proteins (Rho, Rac, Cdc42) are widely expressed monomeric GTPases. Their translocation to specific membrane domains enables intervention of distinct biological functions, including: (1) regulation of actin cytoskeletal dynamics, (2) cell cycle progression, (3) gene transcription, (4) membrane transport, and (5) exo/endocytosis (Bader et al. 2004; Hall 1998). In chromaffin and PC12 cells, RhoA associates with secretory granules, whereas Rac1 and Cdc42 are found in the subplasmalemmal region (Bader et al. 2004). Activation of phosphatidylinositol 4-kinase by RhoA promotes the formation of granule-associated actin filaments and/or stabilize the subplasmalemmal actin barrier (Bader et al. 2004). In chromaffin cells, Cdc42 and Rac1 control actin polymerization and secretion (Gasman et al. 2004; Li et al. 2003). In neurons, Rac1 is associated with synaptic vesicles and plasma membrane (Doussau et al. 2000). Rac1 is involved in a post-docking step of neuronal exocytosis during which it controls in an all-or-none manner the functionality of release sites (Doussau et al. 2000; Humeau et al. 2002), possibly via regulation of phospholipase D (PLD) activity (Momboisse et al. 2009). Additional pathways converging on PLD1 implicates ARF6 GTPases (Vitale et al. 2002). Ral-GTPase is abundant in nerve terminals and associates with synaptic vesicles (Bielinski et al. 1993). This molecule apparently plays a key role in neurotransmitter release by regulating the pool size of readily releasable synaptic vesicles (Polzin et al. 2002). Ral has been implicated in regulating PLD activity too (Luo et al. 1998). Downstream from ARF6, Ral, Rho, Rac, and Cdc42, PLD produces PA. PLD is possibly activated by these GTPases upon docking of synaptic vesicles, or secretory granules, at the release sites. PLD activation is an important event for exocytosis in neurons and many secretory cell types (Choi et al. 2002; Humeau et al. 2001b, 2007; Vitale et al. 2001). PLD production of phosphatidic acid (PA) may either signal attachment of some proteins of the fusion machinery to the fusion site or play a role in vesicle fusion. Indeed, PA is a cone-shaped lipid whose local accumulation and possibly destabilization of the lipids at the fusion site (Humeau et al. 2001b, 2007; Vitale et al. 2001) may promote negative curvation of the inner (cytoplasmic) plasma membrane leaflet (Chernomordik and Kozlov 2003).

Structure and Mode of Action of Clostridial Neurotoxins

Structure

BoNTs and TeNT share a common structure. They are synthesized as a precursor protein (about 150 kDa), which is inactive or weakly active. The precursor which does not contain signal peptide is released from the bacteria possibly by a yet misunderstood cell wall exfoliation mechanism (Call et al. 1995). The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases such as digestive proteases in the intestinal content. The active neurotoxin consists of a light chain (L, about 50 kDa) and a heavy chain (H, about 100 kDa), which remain linked by a disulfide bridge. The structure of BoNTs shows three distinct domains: L chain containing α-helices and β-strands and including the catalytic zinc-binding motif, the N-terminal part of the H chain forming two unusually long and twisted α-helices, and the C-terminal part of the H chain consisting of two distinct subdomains (H_{CN} and H_{CC}) involved in the recognition of the receptor (◉ *Fig. 12.2*). While the three domains are arranged in a linear manner in BoNT/A and BoNT/B, both the catalytic domain and the binding domain are on the same side of the translocation domain in BoNT/E. This domain organization in BoNT/E might facilitate a rapid translocation process (Breidenbach and Brunger 2005; Emsley et al. 2000; Fotinou et al. 2001; Fu et al. 2006; Kumaran et al. 2009; Lacy and Stevens 1999; Lacy et al. 1998; Stenmark et al. 2008; Swaminathan 2011; Swaminathan and Eswaramoorthy 2000; Umland et al. 1997).

The overall sequence identity at the amino acid level between BoNTs and TeNT ranges from 34% to 97%. Several domains are highly conserved which account for the common mode of action of these toxins. Thereby, the central domains of L chains are related in all the clostridial neurotoxins and contain the consensus sequence (His-Glu-X-X-His) characteristic of zinc metalloprotease active site. The half N-terminal domain of the H chains is also highly conserved, and it is involved in the translocation of the L chain into the cytosol. Thus, a similar mechanism of internalization of the intracellular active domain into target cells is shared by all the clostridial neurotoxins. In contrast, the half C-terminal parts of H chain, mainly the H_{CC}

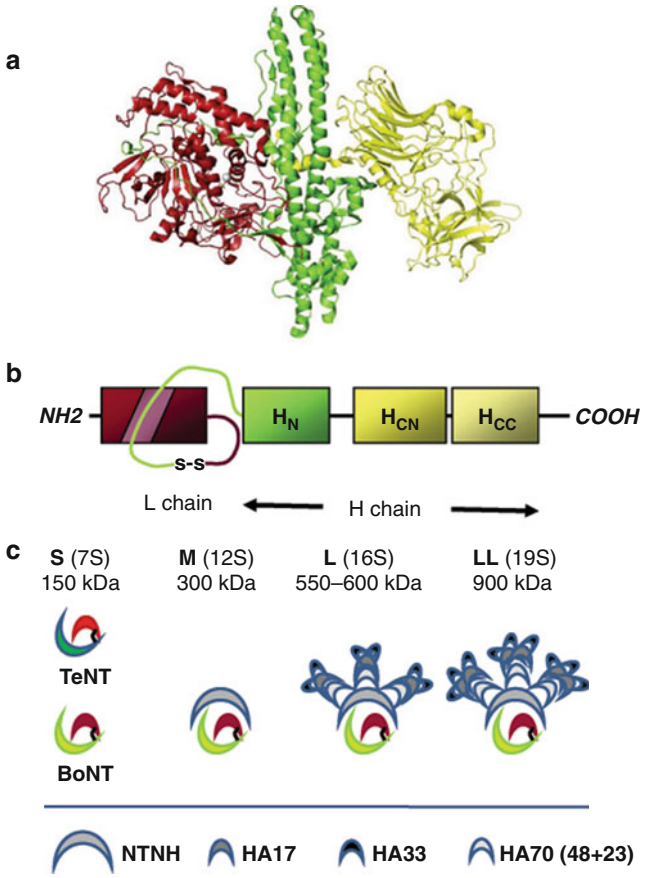

Fig. 12.2

Structure of BoNT or TeNT and conceptual scheme of the botulinum toxin complexes (a) 3D structure of BoNT type A, with (b) the corresponding organization in functional domains. L and H denote light and heavy chains, respectively, H_N and H_c denotes the N- and C-terminus halves of H chain and H_{CN} and H_{CC} N- and C-terminal portions of H_C. (c) The BoNTs associate with ANTP proteins (NTNH and several HA of various MW) to forms complexes of various size (M, L, and LL). Intermediates forms are omitted. TeNT does not make complex with companion proteins and exists only under the S form

subdomains, are the most divergent (Popoff and Marvaud 1999; Poulain et al. 2008). This accounts for the different receptors recognized by the clostridial neurotoxins (see below).

Mode of Action

Although BoNTs and TeNT use different routes to enter their final neuronal targets, they display a similar intracellular mechanism of action. BoNTs enter by oral route or are produced directly in the intestine subsequently to a *C. botulinum* intestinal colonization and then undergo a transcytosis across the digestive mucosa (Ahsan et al. 2005; Couesnon et al. 2008; Jin et al. 2009; Maksymowych and Simpson 2004, 1998; Matsumura et al. 2008). After diffusion into the extracellular fluid and bloodstream circulation, BoNTs target motoneuron endings. In contrast, TeNT is formed in wounds colonized by *C. tetani*. TeNT diffuses in the extracellular fluid and can target all types of nervous endings (sensory, adrenergic neurons and motoneurons), but it is mainly retrogradely transported through the motoneurons (see below) (Manning et al. 1990; Rossetto et al. 2001; Wellhöner 1989) (*Fig. 12.3*).

Each type of BoNT and TeNT recognizes specific receptors on demyelinated terminal nerve endings, mainly through the H_{CC} subdomain. BoNT/A, /C, /E, /F exploit the three isoforms of the vesicle protein SV2 as specific receptors, while BoNT/B and /G bind to synaptotagmin I or II (Dong et al. 2006, 2007, 2008; Mahrhold et al. 2006; Nishiki et al. 1994; Rummel et al. 2004a, 2009). The GPI-anchored membrane protein Thy-1 has been proposed to act as a TeNT receptor (Herreros et al. 2001; Munro et al. 2001), but this has not been confirmed. Albeit SV2 has not been defined as a receptor for TeNT, SV2A and SV2B are involved in the uptake of TeNT into central neurons (Yeh et al. 2011). Ganglioside-binding sites have been characterized in the H_{CC} subdomain. Interestingly, TeNT and BoNT/D exhibit two carbohydrate-binding sites, whereas BoNT/A and BoNT/B show only one (Rummel et al. 2003, 2004b, 2007; Strotmeier et al. 2010; Swaminathan and Eswaramoorthy 2000). Accordingly, TeNT can bind simultaneously to two gangliosides (Chen et al. 2009). BoNT/C and BoNT/D interact with gangliosides (GD_{1b}, GT_{1b}) and phosphatidylethanolamine, respectively, by their H_{CC} subdomain (Tsukamoto et al. 2008). Gangliosides (GD_{1b}, GT_{1b}, and GD_2) and SV2A/B/C also mediate the entry of BoNT/D into neurons but by a different mechanism than that used by BoNT/A and BoNT/E (Kroken et al. 2011; Peng et al. 2011). The role of H_{CN} subdomain, which may interact with phosphatidylinositol phosphates (Muraro et al. 2009), is still unclear. Overall, whatever the considered clostridial neurotoxin, the identified protein receptors are not neurospecific and are expressed on several cell types including intestinal crypt epithelial cells in the intestine (Couesnon et al. 2008). Distribution of the gangliosides recognized by BoNTs differs from that of the protein receptors. Thus, the high affinity of BoNTs and TeNT for presynaptic membranes probably results from multiple and synergistic interactions with the ganglioside and protein parts of receptor, and binding to gangliosides which induces conformational changes in the Hc domain probably facilitates subsequent binding to protein receptor (Chen et al. 2008; Yowler and Schengrund 2004). Copresence of the *ad hoc* ganglioside(s) and protein receptors likely facilitates the identification of cell subset targeted by TeNT or BoNTs at very low concentrations encountered in the physiological medium during the disease. At higher concentrations, binding to the protein receptor is likely sufficient for mediating toxin binding. Indeed the number of cell types affected by these toxins expands with increasing toxin concentrations. Therefore, BoNTs can target numerous neurons but not all, as well as nonneuronal cells at high concentrations, inhibiting the release of various compounds.

Neurotoxin bound to its receptor is internalized by receptor-mediated endocytosis. An essential difference between both types of neurotoxins is that BoNTs are directly endocytosed in recycling synaptic vesicles or clathrin-coated vesicles, which, when acidified, trigger the translocation of the L chain into the

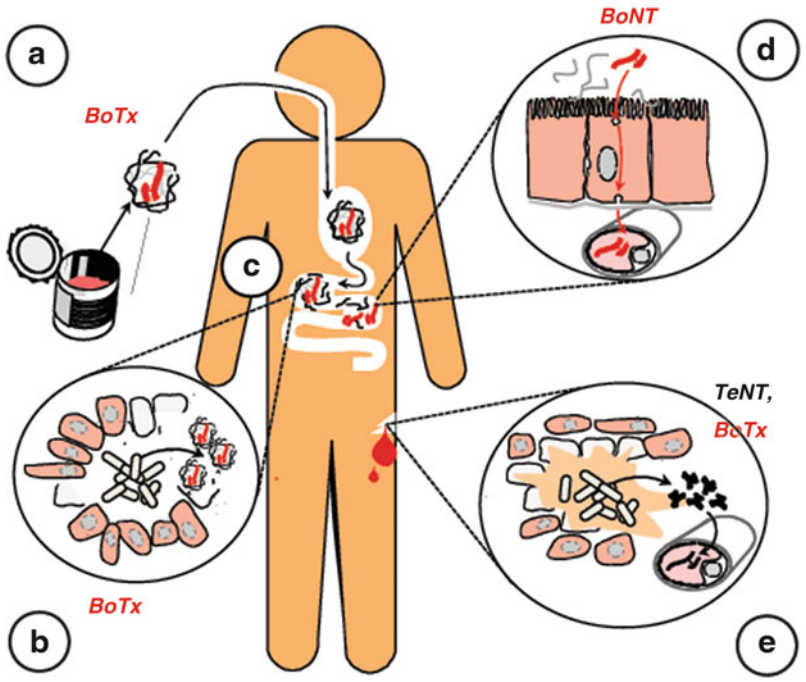

Fig. 12.3

Dissemination of tetanus and botulinum neurotoxin (a) Botulinum toxin (BoTx complex) produced in contaminated food is ingested (foodborne botulism). (b) Under certain conditions, *C. botulinum* spores can grow within the intestine, possibly favored by presence of lesions; this leads to production of BoTx in situ (infant and intestinal forms of botulism). (c) When the BoTx complex arrives in intestinal fluids with near neutral pH (i.e., duodenum, small intestine, and lower part), BoTx complex dissociates and releases the neurotoxin (BoNT). (d) BoNT undergoes transcytosis (passage) in the intestinal epithelial cells and is release in circulatory compartments (lymphatic system and bloodstream), making its dissemination in the whole body. (e) Colonization of anaerobic, necrotic wound by *C. tetani* (tetanus) or, rarely, by *C. botulinum* (wound botulism) leads to production of tetanus neurotoxin (TeNT) or BoTx complex, in situ, followed by dissemination of TeNT or BoNT

cytosol. Therefore, BoNT L chain is delivered in the peripheral nervous system to neuromuscular junctions where it blocks the release of acetylcholine leading to a flaccid paralysis. In contrast, TeNT enters different endocytic vesicles, which are not acidified. The vesicles retrogradely transport the toxin in a microtubule-dependent manner to the cell body of neurons in the spinal cord. Like nerve growth factors, TeNT is transported by tubulovesicular organelles characterized by the presence of neurotrophin receptor such as $p75^{NTR}$ (Bohnert et al. 2006; Bohnert and Schiavo 2005; Deinhardt et al. 2006a; b; Lalli et al. 2003; Lalli and Schiavo 2002). The C-terminal fragment of TeNT drives the retrograde transport of the toxin and can be used to transport heterologous protein in the same way (Li et al. 2001; Maskos et al. 2002). When released in the extracellular space, TeNT carries out a transsynaptic migration and reaches the target neurons, which are inhibitory interneurons involved in the regulation of the motoneurons. TeNT enters target inhibitory interneurons via vesicles that are acidified, thus permitting the delivery of the L chain into the cytosol, where it inhibits the regulated release of glycine and GABA. Overall, the mechanism of translocation is not completely understood for BoNT and TeNT. Acidification of the vesicle lumen triggers a conformational change of the neurotoxin and subsequent translocation of the L chain into the cytosol. H chains form tetramers and insert into lipid membranes, thus forming cation selective channels permeable to small molecules (<700 Da). It remains unclear whether a single H chain or a tetramer of it forming a transmembrane channel mediates the passage of defolded L chain. The N-terminal part of H chain mediates the translocation of L chain into the cytosol at acidic endosomal pH by modifying the electrostatic interactions with the phospholipids without detectable conformational changes. In addition, the disulfide bond between the two chains has a crucial role in the translocation process (Fischer and Montal 2007; Fischer et al. 2008; Galloux et al. 2008; Koriazova and Montal 2003). Then, the L chain refolds in the neutral pH of the cytosol. Cytosolic translocation factors such as β-COPI are possibly involved in this mechanism, as it has been found for diphtheria toxin (Humeau et al. 2000; Meunier et al. 2002b; Ratts et al. 2005; Schiavo et al. 2000).

L chains of all clostridial neurotoxins are zinc metalloproteases that cleave one of the three members of the SNARE proteins. TeNT and BoNT/B, D, F, and G attack synaptobrevin (or VAMP), BoNT/A and E cleaves SNAP25, and BoNT/C1 cut both SNAP25 and syntaxin. The cleavage sites are different for each neurotoxin except BoNT/B and TeNT, which

proteolyze synaptobrevin at the same site. Cleavage of SNARE proteins occurs only when disassembled. Since VAMP, SNAP25, and syntaxin play a major role in the regulated fusion of synaptic vesicles with the plasma membrane at the release sites, their cleavage induces a blockade of the neurotransmitter exocytosis.

SNAP25 cleavage by BoNT/A or BoNT/E deeply decreases both SNAP25 and Ca^{++} binding to synaptotagmin and subsequently the fusion process of exocytosis (◉ *Fig. 12.2*) (Gerona et al. 2000; Lynch et al. 2008; Sakaba et al. 2005; Tucker et al. 2004). Removal of the nine C-terminal amino acids of SNAP-25 by BoNT/A deeply disrupts the coupling between Ca^{2+} sensing and the final step in exocytosis (Sakaba et al. 2005). Truncated SNAP-25 can behave as a dominant negative mutant upon the exocytotic process suggesting that after BoNT/A treatment, the block of release is due to both functional elimination of SNAP-25 and accumulation of the cleavage product which competitively inhibits exocytosis (Apland et al. 2003; Gutierrez et al. 1997; Keller and Neale 2001). In contrast, blockade of exocytosis by BoNT/E is only due to cleavage of SNAP-25, not to the production of competitive antagonists of SNARE complex formation. Indeed, inhibition of exocytosis by BoNT/E can be rescued by supplementing the C-terminal portion of SNAP-25 removed by the toxin (Chen et al. 1999, 2001; Schuette et al. 2004). Truncation of SNAP-25 by BoNT/E destabilizes the four-helix bundle of the SNARE complex (Chen et al. 1999, 2001), and SNAP-25 truncated by BoNT/E is not retained by syntaxin (Bajohrs et al. 2004).

VAMP cleavage abolishes the interaction of VAMP with the adaptor protein AP3 and affect synaptic vesicle recycling via early endosomes (Salem et al. 1998). The SNARE cleavage products have also the potential to interfere with fusion processes (Cornille et al. 1995; Tucker et al. 2004). Consistent with synaptophysin-1 controlling specifically the targeting of VAMP2 but not VAMP1 to synaptic vesicles is the observation that the cytosolic cleavage product of VAMP2 but not VAMP1, released upon TeNT or BoNT/B activity, blocks the neurotransmitter release (Cornille et al. 1995). This result suggests an alteration of the exocytosis due to a disturbance of synaptophysin-1/VAMP2 interaction and of coupling between detecting Ca^{++} and synaptic vesicle triggering (Sakaba et al. 2005). Since the synaptic vesicles docked with unproductive complexes cannot fuse or undock, they stay at the fusion sites (with slightly increased numbers) irreversibly plugging the fusion sites that would normally accommodate intact vesicles. This progressively reduces the number of release sites to which exocytosis can occur as recently demonstrated for TeNT at identified *Aplysia* cholinergic synapses (Humeau et al. 2007). When VAMP is cleaved by TeNT and BoNT/B or /G, the VAMP portion (~20 amino acids) remaining in the synaptic vesicle membrane does not contain interaction sites for the other SNAREs. Therefore, the synaptic vesicle membrane is no longer linked to a SNARE complex, and fusion with the plasma membrane cannot occur. When VAMP is cleaved by BoNT/D or /F, the C-terminal fragment remaining in the vesicle membrane is long enough to anchor the synaptic vesicle to the SNARE complex, but fusion cannot occur because the SNARE complex cannot transit into the thermally stable four-helix bundle.

BoNT/C cleaves both syntaxin-1 and SNAP-25, but in vitro cleavage of SNAP-25 by BoNT/C occurs with low efficiency (~1,000-fold difference) versus cleavage by BoNT/A or /E (Foran et al. 1996) (Vaidyanathan et al. 1999). This raises the question which of the two targets is involved in BoNT/C neuroexocytosis blockade? In squid giant synapses, BoNT/C cleaves syntaxin-1, but not SNAP25 (O'Connor et al. 1997), whereas in cultured hippocampal slices or spinal neurons from mammal, BoNT/C efficiently removes nearly all SNAP25 (Capogna et al. 1997; Williamson et al. 1996). Thus, depending on the cell type, the secretory blockade is likely due to syntaxin and/or SNAP-25 cleavage. In addition, BoNT/C mutant, which only retains cleavage activity against syntaxin, blocks the neurotransmitter release further supporting the role of syntaxin in Ca^{++} triggered neuroexocytosis (Wang et al. 2011a). Upon syntaxin cleavage, SNARE complexes are formed but loosely docked to plasma membrane, thus synaptic vesicles remain tethered to plasma membrane and cannot fuse (discussed by (Poulain et al. 2008)) (◉ *Figs. 12.3*, *12.4*, *12.5*).

Although the physiological properties induced by the cleavage of either VAMP, SNAP25, or syntaxin are not equivalent at the neuromuscular junctions, all the clostridial neurotoxins cause a blockade of the regulated neurotransmission, which varies in intensity and duration according to each neurotoxin type. TeNT and BoNT/B share the same molecular mechanism. They are translocated in different subset of neurons (excitatory neuron: BoNTs >> TeNT; inhibitory neurons: TeNT >> BoNTs) which produce strongly different symptoms. This induces different clinical signs (TeNT: spastic paralysis; BoNTs: flaccid paralysis, ◉ *Fig. 12.5*). Indeed the peripheral dysautonomia and flaccid paralysis caused by BoNTs result from preferential inhibition of acetylcholine release. In the spinal cord or facial motor nuclei, TeNT-mediated blockade of glycine or GABA release disrupts the negative controls exerted by the inhibitory interneurons onto the motoneurons turning on excessive firing of the motoneurons and ensuing muscle contraction (review in (Meunier et al. 2002a; Poulain et al. 2006, 2008; Schiavo et al. 2000)).

Duration of Intoxication

The main factor governing the duration of intoxication is the BoNT toxinotype. The half-lives of exocytosis blockade in rat cerebellum neurons are more than 31 days for BoNT/A, more than 25 days for BoNT/C1, about 10 days for BoNT/B, about 2 days for BoNT/F, and less than 1 day for BoNT/E, and these durations correlate with the paralysis duration (Foran et al. 2003; Keller et al. 1999; O'Sullivan et al. 1999). The factors governing the toxin longevity within the nerve terminals are not fully unraveled, yet presence of a N-terminal sequence and a C-terminal dileucine motif in BoNT/A L chain, and not in the other toxinotypes, may explain the retention of BoNT/A L chain to the plasma membrane (Fernandez-Salas et al. 2004). Other key aminoacids have been identified (Wang et al. 2011) allowing engineering of novel forms of botulinum neurotoxins with modified duration effects (Dolly et al. 2011). Other factors

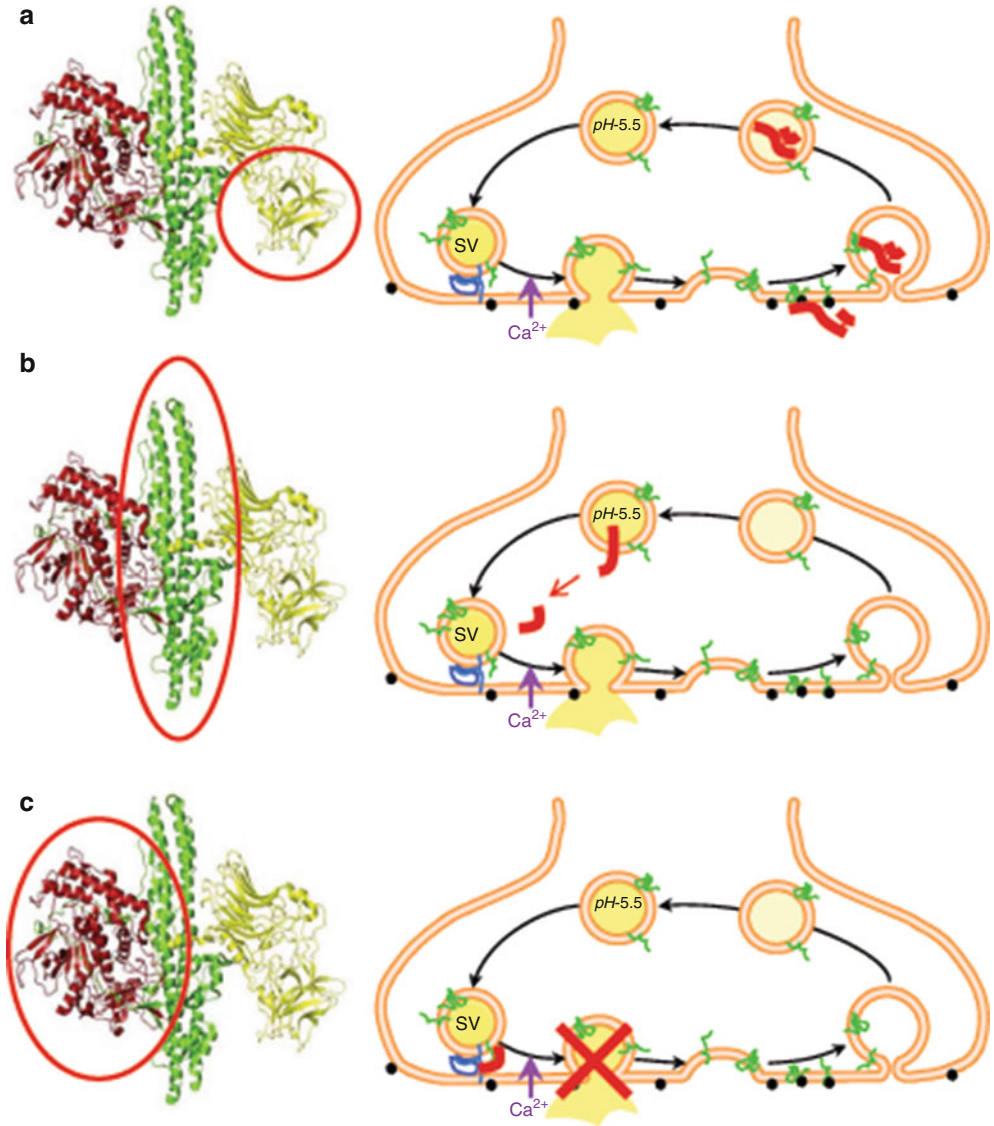

Fig. 12.4

Synaptic vesicles act as Trojan horse in promoting BoNT internalization Left: 3D structure of BoNT. *Right*: schematic representation of a nerve terminal. (a) Upon fusion of synaptic vesicle (SV) with plasma membrane, the luminal domain of their integral proteins (*green*) is exposed at the surface of the neurons nerve ending. Thus, the H_{CC} domain of BoNT molecules (circled in *red*) binds both to gangliosides (*black dot*) and their protein receptor (a SV protein as SV2 or synaptotagmin). Then, BoNT molecules (bound to their receptor) are trapped into nascent recycling vesicles: they are internalized. (b) Refilling of recycled SV with neurotransmitter is mediated by specific transporters which driven force is given by accumulation of protons in the SV lumen. This acidification triggers unfolding of endocytosed BoNT, insertion of H_N chain (circled in *red*) in SV membrane. This allows translocation of L chain into the cytosol. (c). Here the L chain (circled in *red*), which is a Zn-metalloendopeptidase, cleaves its SNARE target, preventing fusion of SV with plasma membrane

should be considered: in contrast to BoNT/E truncation, SNAP25 lacking 9 C-terminal residues, released by BoNT/A proteolysis, remains localized at the membrane in association with syntaxin. Therefore, unproductive SNARE complex at the active zone induces long-term impeding of synaptic vesicle fusion, whereas SNAP25 cleaved by BoNT/E cannot bind to syntaxin and is released into the cytosol (Bajohrs et al. 2004; Fernandez-Salas et al. 2004).

To summarize, despite the scenario that the blocking actions of the various BoNTs differ at the molecular level, they all share several commonalities. Indeed, in all cases following toxin action, the formation or stability of the SNARE complex is compromised (Hayashi et al. 1994; Pellegrini et al. 1995), and it is no longer fusogenic. Thus, synaptic vesicles remain docked at the fusion sites with slightly increased numbers, suggesting

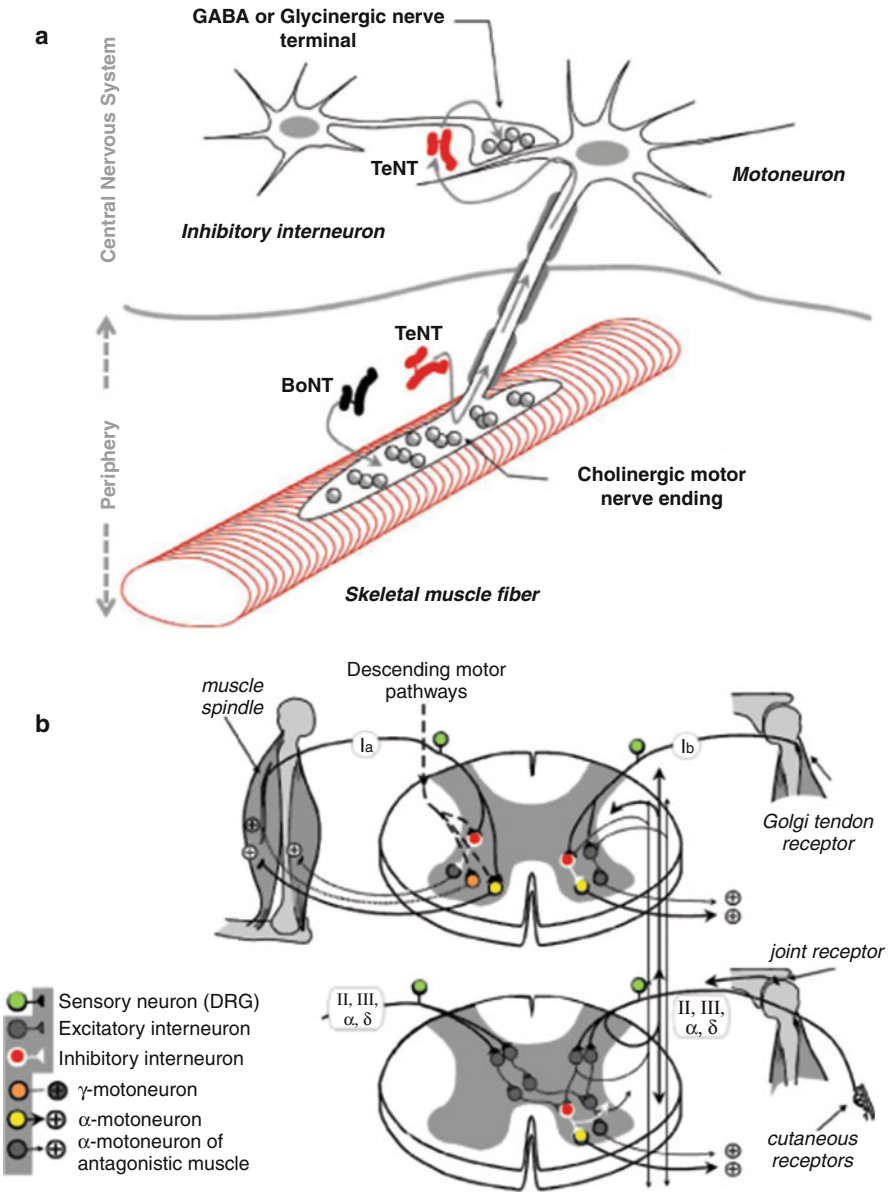

Fig. 12.5

Clostridial neurotoxins and their neuronal targets in periphery and central nervous system (a) *Different sorting of endocytosed BoNT or TeNT into motoneurons*. Neurotoxin molecules taken up in the periphery by the motor nerve endings are sorted in different vesicles. BoNT (*red*), which is into acidic vesicles, is quickly translocated in the cytosolic compartment of motor terminals where it blocks ACh release. TeNT (*black*) is sorted in neutral vesicular cargos undergoing retroaxonal ascent. Then, TENT is exocytosed in the extracellular space surrounding the motor cell body and recaptured by inhibitory (GABAergic, glycinergic) neurons. Here, TeNT is sorted in acidic vesicles allowing is translocation into cytosol and ensuing attack of the release machinery. (b) *The neuronal circuit in spinal cord and clostridial neurotoxins*. Descending pathways, originating from cortical motor areas, project on both motoneurons and interneurons. The neuronal circuit for the myotatic reflex is comprised of the sensory Ia neurons, which cell body (*green*) lies in dorsal root ganglion (DRG) and T-shape axons convey information from the muscle spindle toward (excitatory connections) α- (*yellow*) or γ- (*orange*) motoneurons innervating the homonymous (or synergistic) muscles, and inhibitory interneurons (*white*) neurons downregulating activity of motoneurons (*dark gray*) innervating the antagonistic muscles. Other sensory neurons, which cell somata lies in the DRG (*green*), convey sensory information from tendon, joint and cutaneous receptors and are implicated in more complicated circuits, activating inhibitory or excitatory interneurons. Collateral branches of the interneurons allow activation of interneurons in the neighboring, upper or lower, spinal segments. BoNT action is prominent on nerve endings of the α-motoneurons (*yellow*), while terminals of γ-motoneurons (*orange*) are not exposed due to their insulation by spindle capsule. TeNT can undergo retroaxonal ascent in both α-motoneurons (*yellow*) and sensory neurons (DRG, *green*). In the latter, it stops at the DRG level. Following it transcytosis in motoneurons, it can attack spinal inhibitory neurons (*white* with *red dot*), thus disinhibiting the motoneurons. This results in periphery in cocontraction of muscles and their antagonists

that when tethered to the plasma membrane or docked in an unproductive complex, not only exocytosis but also undocking cannot proceed. Therefore, it is likely that synaptic vesicles docked with unproductive complexes can irreversibly plug the fusion sites that would normally accommodate intact vesicles.

Nonproteolytic Molecular Actions of BoNTs and TeNT

Yet a direct cause-effect relationship exists between the cleavage of the SNAREs and the blockade of neuroexocytosis by BoNTs or TeNT (Humeau et al. 2000; Poulain et al. 2008; Rossetto et al. 2006), a controversial possibility is that BoNTs and TeNT may interfere with exocytosis and other cell functions *via* molecular actions unrelated to their proteolytic activity. Indeed, when mutated in the catalytic site at positions crucial for either Zn^{++} binding (His233 and His237) or cleavage of the Gln-Phe bond in VAMP-2 (Glu234), TeNT L chain cannot cleave VAMP-2 in vitro (Li et al. 1994; Yamasaki et al. 1994). However, several of point-mutated TeNT L chain constructs are able to produce inhibition of neurotransmitter release (His233->Ala233, Leu233 or Val233; Glu234->Ala234; His237->Ala237; Asp237, Gly237 or Val237) albeit with reduced potency as compared to wild-type TeNT L chain (Ashton et al. 1995; Niemann et al. 1994). Such a nonproteolytic mechanism may explain why endopeptidase blockers, which abolish VAMP-2 cleavage in vitro, counteract only partially the inhibitory action of TeNT on neurotransmitter release (Ashton et al. 1995; De Paiva et al. 1993). Moreover, the observation that antagonism of the intracellular action of BoNT/A can be relieved fast by the means of injecting monoclonal antibodies directed against the BoNT/A L chain (Cenci Di Bello et al. 1994) is difficult to conciliate with the proteolytic activity of the neurotoxin protein cleavage being in essence irreversible. The observation that TeNT binds with high affinity to, and strongly activates the GTP-binding protein transglutaminase type II (TGase II) in vitro, suggests that TGase II may participate in the intracellular action of TeNT (Facchiano et al. 1993; Facchiano and Luini 1992). However, the precise contribution of TGase II to the blockade of neurotransmission by TeNT has never been clarified and conflicting data exist in the literature (Ashton et al. 1995; Coffield et al. 1994; Gobbi et al. 1996). TGase II belongs to a large family of bifunctional and Ca^{++}-dependent cross-linking enzymes (Fesus and Piacentini 2002; Lorand and Graham 2003) abundant in neurons and nerve endings (Facchiano and Luini 1992; Maggio et al. 2001) which has been implicated in secretory mechanisms (Driscoll et al. 1997; Pastuszko et al. 1986; Walther et al. 2003). The identification of the vesicular protein synapsin I as one of the two main substrates cross-linked by TGase II needs to be considered for explaining part of the nonproteolytic TeNT-induced decrease in neurotransmitter release. Indeed, synapsin I regulates synaptic vesicle trafficking via interactions with the actin cytoskeleton and participates in post-docking steps of exocytosis (Baldelli et al. 2007; Humeau et al. 2001a). Possibly, TeNT stimulation of TGase II leads to reduced synaptic vesicle availability for release. This view is supported by several observations: (1) the depolarization-stimulated phosphorylation and redistribution of synapsin I are altered after the action of TeNT (Presek et al. 1992); (2) the blocking action of TeNT is diminished after disassembly of microfilaments (Ashton and Dolly 1997); and (3) the amplitude of post-tetanic potentiation, a plasticity paradigm which involves synapsin I in *Aplysia* synapses, is highly reduced after TeNT treatment (Humeau et al. 2001a, 2007). As TeNT can access VAMP-2 only during a defined "physiological window" (Humeau et al. 2000; Poulain et al. 2008), TGase II activation may modulate this access via the modification of proteins involved in regulation of the synaptic vesicle cycle. TGase II substrates include RhoA GTPase, several cytoskeletal proteins (Fesus and Piacentini 2002), SNAP-25 and Synapsin I (Facchiano et al. 1993; 2010). When endogenous TG2 is stimulated by TeNT, Synapsin I and SNAP-25 are covalently modified. TeNT-TG2-induced SNAP-25 modification may explain why TeNT mutants devoid of proteolytic activity against VAMP-synaptobrevin are still able to inhibit neurotransmitter release at Aplysia synapses (Facchiano et al. 2010). TeNT-TGase II-induced synapsin modification may explain why the amplitude of Post-Tetanic Potentiation at Aplysia synapses declines faster than basal neurotransmitter release upon TeNT treatment (Humeau et al. 2007). However, the importance of the proteolytic and nonproteolytic mechanisms of TeNT may be variable from one model systems to other and may depend on differential expression of endogenous TGase II. Contrasting with the observations made using brain synaptosomes or *Aplysia* preparations (Ashton et al. 1995; Niemann et al. 1994), nonproteolytic TeNT mutants have been found ineffective at the mouse hemidiaphragm (Li et al. 1994) or neurohypophysial nerve endings (Dayanithi et al. 1994), and participation of TGase II activation in the blockade of secretion by TeNT has been ruled out at the mouse neuromuscular junction and in NG108 cells (Coffield et al. 1994). In vitro, BoNT/E light chain has been reported cleaving actin, and all the 11 cleavages sites identified involved Arg or Lys residues in *P*'1 position exactly as in SNAP-25 (DasGupta and Tepp 1993). Thus, another unexpected intracellular effect of TeNT is the modification of actin cytoskeleton. This is supported by several observations: TeNT inhibits the rearrangements of subcortical microfilaments that accompany secretion in chromaffin cells (Marxen and Bigalke 1991). Actin cytoskeleton network is altered when TeNT L chain is expressed in Sertoli cells in mice (Eisel et al. 1993). Consistent with the well-documented implication of small GTPases Rho in the dynamics and organization of actin-based cytoskeleton (Hall 1998), BoNT/A has been reported to target RhoB to the proteasome, causing both blockade of exocytosis and actin cytoskeleton disorganization (Ishida et al. 2004). This may relate to a crosstalk between actin cytoskeleton remodeling and SNARE- and Rho-GTPase-dependent mechanisms of exocytosis, as illustrated for Cdc42 and VAMP-2 during insulin secretion (Nevins and Thurmond 2005).

TeNT shows unconventional cellular action time before its classical proteolytic effects became evident. Aguliera and Yavin first reported the in vivo activation and translocation

of protein kinase C in rat brain (Aguilera and Yavin 1990). Concomitantly, an increase of phosphoinositide hydrolysis was observed (Gil et al. 1998). The intracellular pathway activates phospholipase C-1 and other kinases (Gil et al. 2000). Among the different targets these enzymes can attain, the best characterized is the 5-HT transporter which is phosphorylated and has its activity modulated at low toxin concentration (10^{-12} M) in less than 30 min (Inserte et al. 1999; Najib et al. 2000; Pelliccioni et al. 2001). These effects are carried out by the half C-terminal part of the H chain, the portion that carries the binding domain of the toxin to the receptor (Chaib-Oukadour et al. 2004; Gil et al. 2003). It is of interest to note that this portion of the H chain is able to protect from death neuronal cells, in vitro as well as in vivo (Chaib-Oukadour et al. 2004, 2009; Mendieta et al. 2009). Indeed, TeNT Hc activates phosphatidylinositol 3-kinase (PI-3 K)/Akt (a serine/threonine kinase) as well as extracellular signal-regulated kinases 1 and 2 (ERK-1/2) pathways through phosphorylation of tyrosine kinase receptor leading to protection of apoptosis by preventing the proteolytic activation of procaspase-3, cytochrome c release from mitochondria, and chromatin condensation (Chaib-Oukadour et al. 2004, 2009; Gil et al. 2003). Interestingly, TeNT Hc prevents apoptosis induced by 1-methyl-4-phenylpyridinium (MPP+), which is a mitochondrial poison used to reproduce a Parkinson-like disease (Chaib-Oukadour et al. 2009). Since TeNT Hc is retrogradely transported to the central nervous system, it could be used in the prevention/treatment of the Parkinson's disease. In vivo in a rat model of Parkinson's disease, TeNT HC has been found to improve the dopaminergic system and to enhance the survival rate (Mendieta et al. 2009).

Botulism

Main Clinical Forms of Botulism

Three main forms of human botulism are recognized according to the mode of acquisition (◉ *Fig. 12.3*).

Intoxication or Foodborne Botulism. Foodborne botulism is due to ingestion of food in which *C. botulinum* has developed and produced sufficient amounts of BoNT. This is the most common form of disease in adults. Usually, several persons are intoxicated at the same time by sharing contaminated food. In the large majority of outbreaks of botulism, BoNT/A, /B, and /E are involved. Only few cases of human botulism due to type F, type C, or type D have been reported (Hatheway 1993b). It is questionable why types C and D botulism occur so rarely in humans. *C. botulinum* C and D seem to be unable to grow and produce toxin in foods for humans (Hatheway 1993b). Type G has been isolated from autopsy specimens, but its implication in human botulism has not been confirmed (Sonnabend et al. 1981, 1987b).

The incidence and the types of botulism depend on the occurrence of *C. botulinum* in the environment and subsequently in foods and on the cooking practices. The geographical distribution of the different types of botulism corresponds to the distribution of the different toxinotypes of *C. botulinum* in the environment. Type E botulism is mainly found in the colder regions of the Northern Hemisphere, and food based on fish and aquatic animals is usually involved (Hauschild 1989, 1993). Types A and B botulism occur generally in the temperate countries. Type A is predominant in the Western United States, Argentina, and China, and the most frequently implicated foods are fruits and vegetables.

In central and southern Europe, type B is prevalent, and meat has been recognized as the main source of these outbreaks except in Italy and Spain where vegetables are more frequently incriminated. In Italy, 412 cases of botulism have been recorded between 1988 and 1998, 65% were originated from home-canned vegetables (mushrooms, aubergine), 7% from ham and sausage, and 14% from industrial foods (tuna in oil, milk products) (There 1999). An outbreak of type A botulism was due to contamination of a milk product (mascarpone), which is a very unusual type of food substrate (Aureli et al. 1999). In Germany, 177 cases of botulism were reported from 1988 to 1998. Types A and B occurred from ingestion of home-canned vegetables or meat, and several type E cases were related to the consumption of smoked fish (There 1999). The incidence of botulism in France ranges between 10 and 45 annual cases with type B being most frequently involved, but with recent severe type A outbreaks (King 2008; Mazuet et al. 2011). More frequent was type B botulism in Poland with 317 annual cases between 1960 and 1998 (There 1999).

In the majority of outbreaks, botulism is due to home-canned, home-fermented products or home-processed slightly acidic vegetables. Preservation of fish and marine mammal meat by fermentation is the most common source of *C. botulinum* E. Home-canned or home-preserved fruit and vegetables are responsible of the majority of outbreaks in the United States, Spain, Italy, and China. Most of the implicated foods are vegetables home preserved in oil in Italy and fermented vegetables in China. Home-cured smoked ham is the major cause of botulism in several European countries in particular in France, Belgium, Germany, and Portugal (Hauschild 1989, 1993). However, in the recent period, commercial products are more and more involved in botulism outbreaks. Minimally heated, chilled foods such as ready-to-eat foods, ready meals, cook-chill foods, sous-vide foods, and refrigerated processed foods, which correspond to an increased demand of the consumers for high-quality convenience, are at risk for the development of *C. botulinum* notably nonproteolytic strains (Peck 2006). Examples of foodborne botulism throughout the world are shown in ◉ *Table 12.2*.

Botulism by Intestinal Colonization

Infant Botulism. Infant botulism results from ingestion of *C. botulinum* spores that germinate, multiply, and produce BoNT in the infant's gastrointestinal tract. The minimum infective dose for human infant has been estimated to 10–100 C. *botulinum* spores (Arnon 1989). Moderate to high toxin level (10–10^5 mouse lethal dose per gram) and viable *C. botulinum* are recovered from stools of affected infants for

a long period after the onset of the symptoms, as long as 158 days. The seriousness of these diseases varies greatly from a sudden death type of syndrome to a minor flaccid paralysis. The affected infants present constipation, difficulty of feeding, and hypotonia. In the more severe cases, the patient becomes lethargic and loses head control; ptosis and ophthalmoplegia and weak sucking may also be present. The disease progresses to a flaccid paralysis which may extend to respiratory muscles with arrest. Again, type A is generally more dangerous than type B or E, and the recovery time is accordingly longer (Arnon et al. 2001).

Infant botulism has been identified in many developed countries, but most cases were recorded in USA, particularly in California. It is presumed that contamination occurs with *C. botulinum* spores from the environment, and honey feeding has been implicated so far (Arnon 1989; Domingo et al. 2008; Koepke et al. 2008). However, the occurrence of infant botulism is low in Europe (Fenicia and Anniballi 2009; King et al. 2010; Wolters 2000).

Most of the *C. botulinum* strains involved in infant botulism belong to the group I (*C. botulinum* A and proteolytic *C. botulinum* B strains). One of the *C. botulinum* B strain produces also minor amount of BoNT/F. The other implicated *C. botulinum* strains include one *C. botulinum* type C, two BoNT/E producing *C. butyricum*, and one BoNT/F producing *C. baratii* strains (Hatheway 1993b). This could indicate that *C. botulinum* strains from group I are more able to colonize the infant's digestive tract. However, although belonging to the same group, the *C. botulinum* strains from infant botulism and from foodborne botulism could be different. The strains isolated from infant botulism in Japan fall into the group A2; the strains from foodborne and infant botulism from the USA and United Kingdom fall into the groups A1 and A2 (Cordoba et al. 1995). Host susceptibility factors are also involved in the colonization of the digestive tract by *C. botulinum*. In adult mice, the normal intestinal microflora prevents the growth of *C. botulinum* in the digestive tract, while infant mice are susceptible to colonization with *C. botulinum* within the first days of life (7–13 days) (Moberg and Sugiyama 1979). In human infants, most botulism cases have been recorded in newborn between 1 and 6 months of age at onset (Arnon 1989). Moreover, nutritional factors are also involved such as breast-feeding or formula-feeding and subsequent intestinal content pH and composition of the digestive microflora (formula fed infants have less acidic feces (pH 5.9–8) and lower numbers of *Bifidobacterium* than infants fed human milk (pH 5.1–5.4)). Infant botulism with more chronic symptoms seems to be correlated with breast-feeding, and infant botulism form associated to sudden infant death with formula-feeding (Arnon 1989).

Intestinal Toxin Infection in Adults. Colonization by *C. botulinum* and BoNT production in the intestine has been recognized as the botulism origin in certain adults. Predisposing factors such as intestinal surgery, antimicrobial agents, chronic inflammation, and necrotic lesions of the intestinal mucosa could support the growth of *C. botulinum*. *C. botulinum* A and B from group I are generally involved.

C. botulinum types A and B from group I are generally involved. One case with *C. botulinum* type F and two cases with BoNT/F producing *C. baratii* have been documented (Hatheway 1993b; Sonnabend et al. 1987a). Surgical modification of the bowel, achlorhydria, and antimicrobial use has been proposed as predisposing factors. Moreover, chronic inflammation and necrotic lesions of the intestinal mucosa due to underlying disease could support the growth of *C. botulinum* (Tacket and Rogawski 1989).

Wound Botulism. Wound botulism, like tetanus, results from colonization of a wound with *C. botulinum* and subsequent local production of BoNT. However, wound botulism is much rarer than tetanus, despite the fact that the general population is not immunized against BoNT, indicating that *C. botulinum* strains have a low ability to grow in wounds compared to *C. tetani*. A total of 47 cases of wound botulism have been recorded by the Centers for Disease Control (Weber et al. 1993a), caused by toxinotypes A or B from group I. Among the *C. botulinum* strains, those from group I seem to be the most capable of multiplication within the human host (Hatheway 1993b).

Other Forms of Botulism

Rare cases of inhalational botulism have been described, mainly in laboratory workers (Sobel et al. 2004). A few cases have been reported in patients who inhaled cocaine (Roblot et al. 2006). The 50% lethal dose by inhalation has been estimated to 550 mouse lethal intraperitoneal doses (MLIPD) per kg (0.017 μg/kg) for BoNT/A1 and 21,600 MLIPD/kg for BoNT/B in primates (Sanford et al. 2010), and to 0.010–0.013 μg/kg of BoNT/A in human (Arnon et al. 2001). Thereby, BoNT is considered as a potential biological weapon, which could be disseminated by aerosol (Smith 2006: Arnon 2001 #1264). In addition, iatrogenic botulism is a new form of this disease and can result from a hematological spread of therapeutic doses of toxin or from toxin overdoses (Chertow et al. 2006; Coban et al. 2010; Sobel 2005).

Animal Botulism. Botulism is rather common among domestic and wild animals, and types C and D are involved in addition to A, B, and E. Botulism of domestic animals causes economic losses, but it is also a source of a possible risk factor of transmission to human. Animal botulism can be contracted by eating carcass or chewing on bones contaminated with BoNT, by drinking water from contaminated ponds, or by ingestion of forage poisoned by diffusion of BoNT from carcass decomposition of small animals or birds. Among fish and birds, botulism outbreak can involve up to millions of individuals, and the diseases may take the appearance of an epidemic because insect larvae grow in the decomposing cadaver becoming full of BoNT, which is innocuous to insects. Healthy birds and fish are eager of larvae; they become intoxicated and die providing a rich anaerobic medium for the growth of Clostridia and for the deposition of eggs by insects. A self-perpetuating cycle is thus obtained, and rapidly many individuals can die particularly when the animal population is dense as in farms. Where botulism is common, animals often carry in their digestive tract *C. botulinum* which rapidly the carcass becomes highly toxic. Poultry litter often containing chicken carcass are capable of transmitting botulism to cattle (Kennedy and Ball 2011; Payne et al. 2011; Smart et al. 1987; Smith and Sugiyama 1988). In the Netherlands, an outbreak of

type B botulism has been observed in cattles contaminated by eating brewer's grains containing *C. botulinum* B and BoNT/B (Breulink et al. 1978; Notermans et al. 1981). *C. botulinum* B has been recovered in the rumen and feces of the affected animals and also in pasture the cattle were feeding on (Notermans et al. 1981). Silage which is wrapped in plastic and non-acidified can be also a source of botulism for cattle. Although rare, raw milk contamination with *C. botulinum* spores may be responsible for transmission of botulism to human through the dairy chain (Lindstrom et al. 2010).

In France, type D botulism was responsible of important losses in cattle. The animals were contaminated by grazing pasture containing poultry litter which was fed with meat derived from dead animals, including some suspected of botulism. Hen are relatively resistant to BoNT/D (Smith and Sugiyama 1977), and no outbreaks of botulism were observed in the hen farms. *C. botulinum* type D was identified in meat meal, feedstuff for hen, poultry litter, carcass of hen died for unknown reason, and cattle grazing pasture containing poultry litter who died with paralytic symptoms (Popoff 1989; Popoff et al. 1986).

Botulism is also frequent in domestic and wild birds throughout the world. Almost all species of birds are susceptible to botulism, usually type C is involved (Eklund and Dowell 1987). Type C botulism outbreaks have been observed in battery-reared turkeys and chickens in the United States, the United Kingdom (Smart 1983; Smart and Roberts 1977), and in France (unpublished), and in water birds in England (Dorland et al. 1977; Smith and Oliphant 1983), Netherlands, America, Australia, Japan (Eklund and Dowell 1987), and in wild ducks in France (Gourreau et al. 1986). Equine botulism occurs sporadically throughout the world and is commonly due to types C and D, but some type B outbreaks have been reported (Haagsma et al. 1990; Smith and Sugiyama 1988). But animal botulism has also been identified in many other animal species including minks, sheep, monkeys, dogs, sea lions, and lions (Dodds 1993; Smith and Sugiyama 1988).

Clinical Symptoms and Physical Findings During Botulism Pinpoint a Peripheral Action of the Botulinum Toxins

The clinical picture of botulism often initiates as a dysautonomia, followed by motor paralysis. There is no marked distinction in symptoms and physical findings displayed by the various forms of botulism. However, consistent with higher lethality of type A versus type B botulism, the motor findings appear more severe during type A botulism than during type B (Hughes et al. 1981). Initial symptoms of dysautonomia include visual symptoms (blurring, diplopia, and presbytia), followed by dry mouth and constipation. Dysautonomia induced by botulinum toxin can sometimes be the only manifestation of botulism and likely corresponds to a benign form of the disease associated with very low amounts of toxin. The nausea, vomiting, and diarrhea often reported during food botulism may be due to ill-defined enterotoxins unrelated to botulinum toxin. Indeed, foodborne botulism can be considered as the result of the ingestion of a cocktail of several toxins produced by several Clostridia or other bacteria growing together with *C. botulinum* into the contaminated food.

The many symptoms of autonomic dysfunction during botulism are consistent with botulinum toxin acting upon both cholinergic and catecholaminergic neurotransmission but restricted to the peripheral nerve system. Several of the cardiovascular reflexes can be abnormal during botulism. Loss of vagal cardiac control, hypothermia, and urinary retention pinpoint an action of botulinum toxin upon the parasympathetic system. Hypotension without reflex tachycardia, and depressed vasomotor response to postural changes, indicate an action upon the sympathetic nervous system. Effects of BoNTs are exerted at pre- and postganglionic levels, since ganglions are not insulated by the blood–brain barrier. Gastrointestinal troubles observed during foodborne and infant, but not wound, botulism suggest that BoNTs can directly (without hematogenous dissemination) affect neurons of the enteric nervous system.

The second series of manifestations during botulism consists of symmetric weakness or descending paralysis. Observation of this symptom prompts the clinician to diagnose botulism, but, confusion is possible in making diagnosis (Arnon et al. 2001). By contrast with the Guillain-Barre and certain myasthenic syndrome, botulism is characterized by an absence of sensory symptoms. Flaccid paralysis clearly indicates that BoNT effects on the motor system which initially affects striated muscles innervated by cranial nerves producing ptosis, followed by a depressed gag reflex, dysphagia, dysarthria, facial paralysis, tongue weakness, and several neuro-ophthalmological signs. After these early symptoms, general paralysis extends from proximal, to distal, muscles of the neck and limbs. The deep tendon reflex is strongly depressed. When the diaphragm is attacked, respiration is depressed, and the need for mechanical ventilation becomes imperative (reviewed by Hughes 1981; Low 2002; Tacket and Rogawski 1989). Overall, the clinical picture of botulism indicates that flaccid neuromuscular paralysis is due to selective inhibition of neurotransmission, ensuing from the blockade of ACh release, between α-motoneurons and striated muscle fibers. BoNT also blocks transmission between the alpha motoneurons and the muscle spindle, albeit with different kinetics as compared to the motor endplate (Filippi et al. 1993; Rosales et al. 1996). This latter action of the neurotoxin alters the spinal reflexes initiating from the muscle spindle via the sensory Ia fibers.

The large spectrum of application of botulinum toxins in therapy fits very well – at the exception of the potential use of the toxin in pain – with the various manifestations observed during botulisms. Thus, injecting of botulinum toxin produces a local form of botulism, limited in extent to the tissues close to the injection site(s). Both components – dysautonomia and paralysis – of botulism are exploited. Dysautonomic symptoms, as per side effects observed following therapeutic injection of toxin, correspond to hematogenous dissemination of minute amounts of BoNT from the injection site (reviewed by

(Dressler and Benecke 2003)). These latter undesirable manifestations are more frequent with the therapeutic use of type B toxin rather than with type A. Perhaps, this is related to the fact that during type B botulism, the motors symptoms are less marked as compared to type A, whereas dysautonomia manifestations look similar (Hughes et al. 1981), and higher amounts of type B than type A toxin need to be used to produce similar paralytic effect.

Tetanus

Tetanus is a major infectious disease caused by a wound contaminated with *C. tetani*, and it is characterized by persistent tonic muscle spasms with episodes of exacerbation. Several clinical forms of tetanus have been described: local, generalized, cephalic, and neonatal (Bleck 1989). Each form has a clinical relevance because of its prognostic value; however, these clinical entities share common cellular and molecular mechanisms. In all these forms, TeNT is the only etiological cause of neurological symptoms. Other secreted proteins like tetanolysin do not act on nerve cells, but their cytotoxicity may favor abscess formation and growth of *C. tetani* in an anaerobic environment. Although the gut may sometimes offer physiological conditions that promote spore germination of various Clostridia, including *C. tetani*, until now there has been no evidence for an intestinal form of tetanus. It has been hypothesized that the lack of associated protective proteins facilitates toxin degradation in the digestive tract. It is also possible that TeNT cannot cross the gut barrier due to a paucity of receptors that might otherwise enable transepithelial movement (Maksymowych and Simpson 1998).

Dissemination of TeNT and Physical Findings During Tetanus and Poisoning Experiments

Local tetanus consists of persistent muscle spasms in close proximity to the infected site. A rare variant is cephalic tetanus, which is caused by *C. tetani* growth in a wound on the head or neck. The spastic paralysis (i.e., a peripheral physical finding) observed during local tetanus has a central origin with disinhibited motoneurons. TeNT cannot cross the blood–brain barrier composed of tightly sealed endothelial cells that form the spinal cord and brain capillaries. Compelling evidence (Wellhöner 1992) has shown that the entry port of TeNT into the central nervous system is the motoneuron, which essentially acts like a Trojan horse. Indeed, the TeNT molecules first bind to motor cholinergic nerve endings responsible for innervating striated muscles, followed by endocytosis and transport toward the spinal cord (*Fig. 12.5*). Transport studies with 125I-labeled TeNT reveal a retrograde axonal ascent of TeNT along the motor axons. Indeed, 125I-TeNT was found in the ventral roots (i.e., containing motor axons) and ventral horn of the spinal cord (i.e., the area containing motoneuron cell bodies). Apparently, α-motoneurons that innervate striated muscle fibers and γ-motoneurons that innervate the muscle spindle can both take up, as well as transport, TeNT. Toxin labeling of the dorsal root, which contains sensory fibers, has also been reported. However, this toxin transport does not seem relevant for the pathophysiology of tetanus because ligature of the dorsal (sensory) root does not prevent labeling of the spinal cord. Moreover, recent studies with a recombinant, fluor-labeled TeNT-fragment reveal binding, uptake, and transport of TeNT in motoneurons, but only binding without transport in dorsal root neurons (Bigalke and Shoer 2000; Halpern 1995; Wellhöner 1992). Identification of receptors at motor nerve endings that enable internalization of TeNT, as well as the identity of any facilitating molecule(s) implicated in retroaxonal transport, still remain unresolved. Although not presented here, these issues are fully discussed in TeNT molecules, when retroaxonally transported, arrive at the motoneuron soma and dendrites where they are released into the extracellular space. This transcytosis through motoneurons allows the toxin to bypass the blood–brain barrier and reach the vicinity of nerve terminals that are afferent onto the motoneurons. High TeNT concentrations can be found in particular compartments of the central nervous system during tetanus: for instance, release of one TeNT molecule into a small volume like a synaptic cleft ($\sim$ 1 µm $\times$ 1 µm $\times$ 50 nm) leads to a "concentration" of $\sim$30 nM! According to in vitro experiments, TeNT molecules are recaptured by different types of nerve endings, independent of the type of neurotransmitter molecules they release (Bigalke and Shoer 2000; Wellhöner 1992). Consistent with the preferential blocking action of TeNT on GABA and glycine release observed in vitro, TeNT suppresses the inhibitory inputs afferent to motoneurons, while the excitatory inputs, which constantly fire motoneurons, are not modified. Therefore, motoneurons are disinhibited, and their firing rate increases, inducing sustained muscle contractions that lead to spasms and rigidity. Both the inhibitory descending controls and spinal reflexes which converge onto motoneurons are deeply altered. Spindle sensory inputs conveyed by Ia fibers during physiological conditions positively activate the synergistic motoneurons afferent to the same (homonymous) muscle and simultaneously inhibit motoneurons to antagonize muscles through inhibitory interneurons. Therefore, upon TeNT-induced blockade, or when TeNT blocks the inhibition pathway mediated by Ia interneurons, the myotatic reflex is deeply altered muscles working the same joint. TeNT also depresses recurrent inhibition, implicating Renshaw cells. Inverse myotatic reflex involves Ib fibers coming from the Golgi tendon organ and participates in a negative feedback system preventing further development of muscle tension upon reaching the desired point. Alteration of this reflex leads to an uncontrolled increase in muscle tension, causing muscle and tendon damage. In a similar way, the many reflexes based on polysynaptic circuits converging on inhibitory interneurons are disorganized. Therefore, all local stimuli causing an increase in excitatory input (e.g., sensorial or noxious stimuli) leads to local exacerbation of muscle contractions (Bleck 1989; Wellhöner 1992). The situation is not too different in generalized tetanus. TeNT molecules primarily taken up by motor endings can

undergo other transcytotic cycles throughout neurons of second, third, etc. order (Manning et al. 1990). Thereby, TeNT entering a single peripheral site and released into the spinal cord disseminates vertically to proximal spinal segments and horizontally to the contralateral side of the spinal cord, thus resulting in progressive muscle rigidity in all muscles. Retroaxonal transport of TeNT along a chain of functionally connected neurons has been exploited to trace the projection pathways between distant brain structures (Cabot et al. 1991). Furthermore, to study trafficking pathways and connectivity in sensorimotor circuits, several fusion proteins have been engineered using the atoxic TeNT C-subunit (TTC) fused to a reporter gene such as *LacZ*, GFP (green fluorescent protein), or EGFP (enhanced green fluorescent protein) after direct injection of the hybrid proteins (Coen et al. 1997; Miana-Mena et al. 2002), or expression as a transgene in mice (Maskos et al. 2002). When there is a hematogenous portal of entry for TeNT, it enters all motor nerve terminals and reaches the spinal cord at a number of sites. The most dramatic form of the disease, called *generalized tetanus*, then occurs and is characterized by an overall increase in muscle tone, rigidity, and trismus (Bleck 1989) Almost all spinal reflexes based on GABAergic and glycinergic transmission are abolished, leading to horizontal and vertical spreading of the response from any stimulus. Therefore, a local stimulus leads to a general response. For example, application of a cutaneous stimulus on a limb can induce profuse contraction of the face, neck, trunk, arms, and legs. When the spasm affects the diaphragm, respiratory failure and death can ensue. Although TeNT injected directly into the brain can induce epilepsy (Benke and Swann 2004), no central disorder like seizure is observed during tetanus (Bleck 1989). Therefore, the intrathecal dissemination of TeNT remains largely confined to the spinal cord. Several other symptoms are detected during generalized tetanus and indicate that TeNT acts also on the autonomous nervous system (e.g., hypertension due to vasomotor spasm, tachycardia). This hypersympathetic condition is due to disinhibition of the sympathetic reflexes, analogous to that for motoneurons. This is fully consistent with a report that TeNT is also taken up and retrogradely transported in adrenergic neurons. This is followed by toxin delivery to the inhibitory interneurons, whose terminals are afferent to the cell body of adrenergic neurons. Alteration of functions linked to the parasympathetic system has been reported too (Bleck 1989; Wellhöner 1992). All of the above mentioned physical findings observed during local tetanus have a unique origin: TeNT blockade of inhibitory neurotransmitter (GABA and glycine) release.

When the local concentration of TeNT is high, it can also block the release of other neurotransmitters. A high dose of TeNT injected into the lateral rectus muscle of the cat blocks both inhibitory and excitatory synapses simultaneously in abducens neurons (Gonzalez-Forero et al. 2003). At the neuromuscular junction (NMJ), TeNT inhibits ACh release, thereby inducing flaccid muscle paralysis in vitro (Wellhöner 1992) and during disease (e.g., cephalic tetanus in humans) (Bleck 1989). In certain animal species, flaccid motor paralysis may be the only action of the toxin. For instance, application of TeNT into the periphery of goldfish does not produce central actions but it inhibits ACh release from motor nerve endings. This is consistent with motor terminals being the primary binding targets for TeNT. When captured by motoneuron endings, TeNT is preferentially routed to a transport compartment whose neutral pH prevents translocation into the cytosol of motor nerve terminals. This preferential routing is not absolute, and TeNT appears far less potent than BoNTs in inhibiting ACh release by a factor of 100–1,000. This trafficking seems to depend strongly upon the TeNT receptor. Indeed, when the active moiety of TeNT is recombined with the binding domain of BoNT, it acquires a very high efficacy in cholinergic nerve terminals (Wellhöner 1992).

To summarize, the clinical and experimental findings of tetanus reveal a prominent inhibitory action of TeNT on the release of GABA and glycine. Complexity of the neuronal network and an inherent difficulty in dissociating direct, from indirect, effects has not allowed identification of other neuronal targets for TeNT. However, the in vitro experiments clearly indicate that TeNT blocks release of many, if not all, neurotransmitters.

Botulinum Neurotoxin Therapy

Many Potential Indications

Consistent with the well-established action of botulinum neurotoxin on ACh exocytosis, injection of small doses of botulinum toxin into muscles or glands innervated by cholinergic nerve endings is currently used a pharmacological tool to disrupt motor or secretion command in a localized and long-lasting manner. Since the pioneer use of botulinum toxin type A in strabismus (Scott 1980), thousands of papers have addressed an amazing large number of indications potentially benefiting of botulinum toxin. This includes the syndromes related to focal overactivity in skeletal, sphincter, and other muscles (e.g., blepharospasm, hemifacial spasm, cervical dystonia, other dystonia, dysphonia, writer's cramp, limb spasticity secondary to cerebral palsy or stroke, anal fissure, overactive bladder), hypersecretory activity of glands (hyperhidrosis, hypersialorrhea, gustatory sweating as Frey syndrome), drooling associated with Parkinson's disease, and muscle or gland hypertrophy (masseter hypertrophy, benign prostatic enlargement). Consistent with the inhibitory action of botulinum toxin on glutamate and peptides (e.g., CGRP) release, botulinum toxin type A has been assessed in animal to reduce neurogenic inflammation by blocking the release of glutamate from nociceptive fibers (Aδ and C) and ensuing pain (Cui et al. 2004; Favre-Guilmard et al. 2009; Meng et al. 2009). Toxin is tentatively used in the case of numerous pain syndromes in man. Use of botulinum toxin in facial aesthetics is in essence closely related to the above mentioned therapeutic indications: erasing wrinkles is obtained by toxin-induced relaxation of the muscle(s), whose contraction is responsible for skin folding.

Despite the extensive literature produced on many indications (reviewed by (Jankovic 2009; Truong et al. 2009), the consensus assessment studies published in the last decade have

established that botulinum toxin-based therapy is proved to benefit to the patients in a restricted number of indications (Naumann et al. 2008; Simpson et al. 2008); several examples are mentioned in *Table 12.3*). This does not mean that botulinum toxin therapy is unsafe or useless in the case of the other indications: this just indicates that the quality of scientific evidence provided by the clinical studies is not sufficient (too small number of case; presence of bias, marginal statistical validity) to recommend botulinum toxin as an evidence-based treatment.

Toxin Products for Clinical Use

Overall, toxin products available on the market are made from purified toxin or neurotoxin, to which is added several excipients. Although the formulation of the few products approved by the United State and European authorities is known, there exists on the world market non-approved products, the formulation of which remains unclear.

Most of the approved formulations are based on toxin type A and each of them approved for a restricted number of indications in neurology, rehabilitation medicine, ophthalmology, or aesthetic. The formulations with a marketing authorization in the European Union or United States of America contain toxin or neurotoxin type A produced by strains closely related to the strain Hall of *C. botulinum* (Botox®: (Zhang et al. 2003); Xeomin®: (Bigalke 2009), Dysport®: (Panjwani et al. 2008)). Thus, these formulations should be comprised of neurotoxin subtype A1. All the formulations derived from these products (Vistabel® and Botox Cosmetic® derived from Botox®, Azzalure® derived from Dysport®, and Bocouture® from Xeomin®) contain also of the subtype A1. Note that in the early literature, bivalent strain NCTC 2916 A2/B1 has been often incorrectly mentioned for Dysport®. Overall, the other A subtypes and associated complex proteins remain insufficiently characterized, and studies remain at preclinical stage (for recent studies on A2 subtype see (Akaike et al. 2010)).

Botulinum toxin type B has similar clinical gross action as type A but needs to be injected at much higher doses than type A to produce similar myorelaxing effects in man (Sloop et al. 1997). Only a formulation of toxin type B (Myobloc®/NeuroBloc®) is approved, it is based on a toxin produced by the strain Bean of *C. botulinum* and is most probably of subtype B1.

Toxin type C has been tested in the humans (Eleopra et al. 2002, 2006) and found producing similar effects as type A (similar efficacy and duration of paralysis). Although toxin type C has been reported to cause neurodegeneration when applied to cultured spinal cells (Williamson and Neale 1998), no such effect has been reported following its intramuscular injection (Eleopra et al. 2002; Morbiato et al. 2007). To date, too little is known about toxin complex type C to determine whether the cytotoxic associated proteins present in its complex (Jin et al. 2009) make the action of the toxin (complex) type C significantly distinct from that of purified neurotoxin type C.

Lack of epidemiological form of human botulism type D together with the report that botulinum toxin type D fails to induced paralysis of surgically excised human muscles (Coffield et al. 1997) indicates that this serotype is useless in human therapy.

Toxins of types E and F have been tested in humans (Eleopra et al. 1998; Greene and Fahn 1993). As compared to type A toxin, their effect is short lasting (Eleopra et al. 1998; Mezaki et al. 1999).

To this list should be added new products under development that are based on reengineered neurotoxins. They are based on the idea that BoNT has a modular structure, and each module can be either modified or exchanged to produce neurotoxins with new properties. A derivative from type A neurotoxin has been produced in which the C-terminal half of heavy chain has been replaced by lectin from *Erythrina cristagalli* has been produced (Duggan et al. 2002). Since this lectin specifically binds to galactose, this allows retargeting of the neurotoxin biological activity against nerve terminals expressing this sugar on its plasma membrane. This is the case of neurons from the dorsal root ganglion (DRG), whose peripheral axon is specialized in the transfer of noxious information (nonmyelinated C fibers) and central branch makes synapse in the upper lamina of spinal cord. This construct inhibits GCRP release with high efficacy. Its intrathecal administration in rodent induces potent resistance to pain tests (Chaddock et al. 2004). Identification of the key amino acids that determine the intraneuronal life duration of light chain (Fernandez-Salas et al. 2004; Wang et al. 2011b) is exploited to engineer novel forms of botulinum neurotoxins (e.g., derivative of BoNT/E with longer duration effects) to improve and/or extend therapeutic applications (Dolly et al. 2011).

Depending on the manufacturers, formulations include toxin (i.e., complex comprising a neurotoxin, a NTNH, and several HAS) or purified bichain neurotoxin. The complex toxins used may differ in size or by accessory proteins. The different sizes of the complex are indicated in *Table 12.4* (column 7). According to Allergan, Botox®, and derived products Botox Cosmetic® and Vistabel®, should contain only the ~900 kDa (i.e., LL) complex (Zhang et al. 2003). It is suggested that Dysport® and its derived product Azzalure® contain a mixture of the 600 and 900 kDa (i.e., L and LL) complexes (Panjwani et al. 2008). Given the published stoichiometry in B-type complex, Myobloc®/NeuroBloc® is likely containing only complexes of intermediate size L; however the proportion of bichain neurotoxin versus unnicked single chain is unknown. Xeomin® and derived product Bocouture® are based on neurotoxin produced by purification to homogeneity, after dissociation of the toxin (complex) (Jost et al. 2007).

All the formulations available on the market include several excipients, some of which vary from one commercial product to another. The presence of human serum albumin is designed to minimize nonspecific adsorption of the toxin or neurotoxin on the walls of syringes and vials. It is well tolerated by patients. Its amount per vial varies with the formulations, even from the same manufacturer. For example, vials containing 500 units Dysport® and 125 units Azzalure® differ from a ratio of 1–4 for the amount of toxin A but contain the same amount of

Table 12.5
New toxin names according to the USAN COUNCIL

Manufacturer	New name	Product(s)
Allergan Inc. (CA, USA)	OnabotulinumtoxinA	Botox®, Botox Cosmetic®
IPSEN Ltd. (GB)	AbobotulinumtoxinA	Dysport®
Merz Pharmaceuticals Gmbh. (Allemagne)	IncobotulinumtoxinA	Xeomin®, Bocouture®
Solstice Neurosci. Inc.	RimabotulinumtoxinB	Myobloc®

human serum albumin (*Table 12.4*, column 8). For the same purpose, animal gelatin is present in some products instead of human serum albumin. Given the potential allergic effect of animal gelatin, this raises safety questions.

For several years, there has been an increasing diversity of trade names under which the toxin products are distributed, although sometimes based on the same manufactured "toxin." Indeed, a same formulation can receive different trade names in different regions of the world (e.g., Vistabel® and Botox Cosmetic®). Moreover, the biological activity of the various formulations is expressed with systems of units that are not equivalent (see below), thus making the products not interchangeable. To minimize the risk of errors, the FDA USAN Council has introduced in the United States of America a new name for the toxin produced by a given manufacturer, independent of the technical trade names (*Table 12.5*) (FDA-alert-289 2009). This new name is not used abroad.

Toxin Units in Human Therapy

The amount of toxin that is recommended to inject to a patient for a given indication is defined as a number of units of biological activity to be injected in a single or several sites. Depending on the indications, the number of units may range from few tens to several hundreds. The assays used by the manufacturers to measure the biological activity of commercial preparations of toxin/neurotoxin are based on the mouse LD50 assay, with 1 unit of biological activity defined as 1 toxin mouse LD50 (~10 pg purified neurotoxin type A). None of the commercial products contains human serum albumin enough to fully prevent nonspecific absorption of toxin/neurotoxin on the vial and syringe walls (Bigalke et al. 2001), therefore adding or not gelatin to the toxin sample to be assessed by the mouse LD50 assay impacts on the measurement of its biological activity and leads to the situation that a same quantity (amount by weight) of active toxin is associated with a different number of units. For example, Hambleton and Pickett (Hambleton and Pickett 1994) have reported that 100 units of Botox® evaluated with the Speywood's test (i.e., with gelatin (Hambleton et al. 1981)); this test is used to evaluate toxin products from the manufacturer (IPSEN) ended in an assessment of 270–360 units, while 500 Dysport® units evaluated with the assay used by the manufacturer Allergan (i.e., without gelatin) resulted in 267 units. This two to threefold difference corresponds to the conversion factor determined empirically in clinic. Allergan's assay (without gelatin) gives an assessment of the toxin biological activity that can be actually mobilized from the vial in the conditions of clinical practice, whereas the IPSEN-Speywood's assay (with gelatin) measures the amount of biological activity actually present in the vial. The other manufacturers also determine the biological activity of the toxin products by modified mouse LD50 assays. Overall, the units are different, and this makes the products not interchangeable. Misunderstanding these differences may lead to overdosage of botulinum toxin, with the risk of causing iatrogenic botulism.

Spreading and Half-Life of Botulinum Toxin Injected in Therapy

A major difference with generalized forms of botulism and botulinum toxin-based therapy is that the latter remains mostly localized. However, spreading of neurotoxin molecules beyond the injection site can cause adverse manifestations (see below). Muscles and all tissues susceptible to be injected with toxin for therapeutic purpose are comprised of defined compartments (e.g., the different fascia in large muscles) that are insulated each other by tunica or sera. Therefore, the most important pathway for the spreading of toxin from its injection site is the blood capillary network and vascular tree that permit its hematogenous dissemination.

Since the toxin complexes rapidly dissociate (half-time of less than 1 min) at plasma pH (i.e., pH 7.4) (Eisele et al. 2011), the neurotoxin is the only entity responsible for the spreading of the toxin effects beyond the site of injection, whatever is the initial formulation of toxin (purified neurotoxin or complexes of different sizes, see *Table 12.4*). This is supported by observation of no significant difference in the spread of 125-iodine-labeled toxin A and neurotoxin A (Tang-Liu et al. 2003), or Dysport®, Botox®, or Xeomin® in the *tibialis* muscle (as assessed by measuring changes in NCAM expression (Carli et al. 2009)). Also, when neurotoxin (Xeomin®) or toxin complex (Botox®) is injected into the *extensor digitorum longus* muscle, there is similar "diffusion" of the paralytic effects to adjacent muscles (Wohlfarth et al. 2007).

Apart from the small fraction of neurotoxin that is captured by the endings of motoneurons, most of the neurotoxin disappears from the injection site by its dilution in the circulatory system. In the rat, only ~70% of the injected neurotoxin is detectable 30 min after injection (evaluated in the rat gastrocnemius muscle). After 6 h, only 30% remains and only 5% after 24 h (Tang-Liu et al. 2003). In the blood compartment, the neurotoxin half-life is of ~10 h (Al-Saleem et al. 2008; Ravichandran et al. 2006). These values contrast with presence of circulating neurotoxin for several days, or longer than a week, after the onset of botulism. The short life span of extracellular neurotoxin needs to be compared to that reported once captured by the nerve endings. By extrapolating to man the murine data, the long duration of paralysis observed in human therapy (order of several months with types A and C,

several weeks for types B and F, and that of type E even shorter). Montecucco and Molgó (Montecucco and Molgo 2005) suggest an intracellular half-life of several months, and even more: indeed, duration of toxin effect when injected into glands may reach 1 year or more in man.

Botulism Manifestations as Side Events of Botulinum Toxin-Based Therapy

In mammals, the lethal dose of botulinum toxin (900 kDa complex) type A is the order of 1 ng/kg: 1.2 ng/kg ip in mice, 0.5–0.7 ng/kg in guinea pigs, rabbits, or monkeys (Gill 1982). By extrapolation, the lethal dose for humans should be ~90–150 ng of toxin A (complex) injected intravenously or intramuscularly (Arnon et al. 2001; Smith 2006). From experiments carried out in monkeys, and on the basis that 1 unit of biological activity = 1 mouse LD50, Scott and Suzuki (Scott and Suzuki 1988) have suggested that the lowest dose of toxin type A producing a systemic botulism (with respiratory distress) in humans is in the range of 33 unit/kg and the LD50 for humans ranges 38–42 unit/kg (~ 2,800 unit for an adult of ~70 kg). Overall, these evaluations remain above the suggested maximum doses of type A toxin/neurotoxin recommended by the pharmaceutical companies for the approved indications. However, in clinical practice, the dosage of botulinum toxin is determined by the practitioner to adapt on individual patient response, and there is the possibility to inject toxin for not approved indications. The actual injected doses can be very high, for example, in the case of limb spasticity, 800 units (type A toxin) or more are used by certain practitioners.

The adverse effects due to spreading of toxin from the injection site are numerous (Dressler and Benecke 2007), albeit severe case is scarce. The more frequent effects are an extension of muscle paralysis to noninjected adjacent muscles and/or action on the neighboring glands (e.g., dry mouth after injection of toxin in the neck muscles), symptoms of systemic dysautonomia. When large amount of toxin is used (i.e., 800 units), more frequent are the cases of fatigue and long-lasting muscle weakness in numerous noninjected muscles. This is evocative of attenuated forms of iatrogenic botulism. Importantly, however, observation of distant effects of the toxin does not necessarily mean that toxin had spread and exerted its action elsewhere! This will discussed below.

The use of type B toxin in human therapy has shown that this toxin is much less powerful than that of type A to induce myorelaxation in humans (Sloop et al. 1997). Therefore, the dosage recommended for type B toxin to relax overactive muscles is much higher than that of products of type A. This produces a larger spreading of type B toxin beyond the injected muscle. Consistent with observation that botulism type B displays more pronounced autonomic manifestations than motor dysfunction (Hughes et al. 1981; Tacket and Rogawski 1989; Woodruff et al. 1992), there is a higher occurrence of unwanted dysautonomic events associated with the use of toxin B from toxin A (Dressler and Benecke 2007).

Indirect Effects of Botulinum Neurotoxin Observed in Humans

The local administration of botulinum toxin for therapeutic purpose in humans, or in the course of in vivo animal experiments, has been the occasion of revealing several indirect effects of the toxins.

Atrophic Response

Following their paralysis, muscle fibers develop very quickly atrophy (Morbiato et al. 2007; Rosales et al. 1996). This is an indirect effect caused by any situation inducing synaptic silencing. Muscle atrophy is reversible and correlates in intensity to that of the paralytic effect of the neurotoxin (Borodic and Ferrante 1992; Chhetri et al. 2003; Horn et al. 1993; Morbiato et al. 2007; Rosales et al. 1996, 2006). This indirect effect of neurotoxin has been exploited as such to reduce muscle hypertrophy (Al-Muharraqi et al. 2009). Not all muscles are susceptible to atrophy after toxin injection. For example, it is not observed after paralyzing by botulinum toxin the extraocular eye muscles in monkeys (Porter et al. 1991) or rabbits (Ugalde et al. 2005). One reason postulated for this resistance to atrophy is the activation of satellite cells and their rapid fusion with the paralyzed muscle fiber.

Muscle fiber atrophy is associated with significant increases in the sarcolemmal electrical resistance that makes it better responding to motor synaptic inputs. Therefore, although botulinum toxin inhibits ACh release, the better responsiveness of atrophic fibers may compensate for a while the former, delaying the onset of paralysis.

Decrease of synaptic transmission results also in atrophy of the glandular tissue (Oeconomou et al. 2008; Swartling et al. 2004; Teymoortash et al. 2007). This effect participates to reducing the volume of secretion induced by the toxin. Consistent with normal cell proliferation in prostate is under control of cholinergic innervation; several recent studies have explored the possibility of using the toxin to reduce benign prostatic enlargement (Chuang and Chancellor 2006; Oeconomou et al. 2008).

Sprouting

In mammals, muscle paralysis following a single injection of botulinum toxin A elicits within 3–5 days a nerve outgrowth or sprouting that occurs along intramuscular axons at the nodes of Ranvier (nodal sprouting), and at motor nerve terminals of the NMJs (terminal sprouting). The induction of sprouting is not specific of the BoNTs and is also produced by other agents that render the muscle inactive via prolonged blockade of either nerve conduction or muscle nAChRs. Terminal sprouts predominate on nodal sprouts in mature muscles treated with BoNT/A.

Sprouting is greater in younger animals. Generally, it increases with time after BoNT injection and depends upon the contractile properties of the muscle as defined by its innervation pattern. Thus, sprouting is more prominent in slow (e.g., *soleus*), than in fast (e.g., *extensor digitorum longus*), contracting muscles. In addition, sprouting is more abundant in muscles innervated by short axons (reviewed in (Meunier et al. 2002a)). Nerve terminal sprouts evoked by BoNT/A in mouse *levator*

auris longus muscle appear as thin, unmyelinated filaments usually oriented parallel to the longitudinal axis of the muscle extending beyond the original nerve terminus (Angaut-Petit et al. 1990; de Paiva et al. 1999; Molgo et al. 1990). After a BoNT/A injection, the sprouts usually increase in length as well as complexity for about 40–50 days and continue to grow for 30–40 days, despite the recovery of nerve-evoked muscle twitches (Juzans et al. 1996), as confirmed by time-lapse imaging of the same mouse NMJ (de Paiva et al. 1999). The persistence of nerve terminal sprouts has also been reported in the human orbicularis muscle after repeated injections of BoNT/A (Holds et al. 1990). Nerve terminal sprouting was also detected in mouse *levator auris longus* muscle injected with BoNT/D (Comella et al. 1993) and BoNT/C (Morbiato et al. 2007), as well as in mouse sternomastoid muscle injected with BoNT/F (Meunier et al. 2003). Interestingly, no sprouts were visible in the mouse sternomastoid muscle following BoNT/E injection (Meunier et al. 2003), which is probably due to short-lived muscle inactivity since recovery from paralysis starts within 2–3 days after neurotoxin administration.

Distant Effects Unrelated to Hematogenous Dissemination of Toxin

In the course of therapeutic use of the toxin, change in reflexes and central effects of botulinum neurotoxins have been reported (Caleo et al. 2009; Gracies 2004; Gracies et al. 2009; Lim and Seet 2008; Priori et al. 1995). They are consistent with similar observations in animals (Moreno-Lopez et al. 1994; Pastor et al. 1997; Wellhöner 1992). Since the botulinum neurotoxins molecules do not cross the blood–brain barrier, the reported central actions may refer to (1) axonal retrograde ascent of botulinum neurotoxin molecules followed by transsynaptic passage or (2) change in proprioceptive peripheral information and central plasticity.

When applied at very high concentrations, a fraction of the botulinum neurotoxin molecules endocytosed by the motor and sensory nerve endings is routed in a vesicular retroaxonal pathway, leading to its transport up to the cell body of the motoneurons (i.e., located in the ventral horn of the spinal cord), or of the sensory neurons (i.e., in the dorsal root ganglions; DRG) (Antonucci et al. 2008; Habermann 1974, 1989; Wiegand et al. 1976). Experiments carried out in animal have shown that retrogradely transported neurotoxin can also undergo transsynaptic transport (Antonucci et al. 2008; Caleo et al. 2009; Caleo and Schiavo 2009). So, potentially, the neurotoxin injected in the periphery could act directly in the central nervous system. However, after more than 25 years of clinical use of botulinum toxins, no clinical manifestation has been reported demonstrating a direct central action of botulinum toxin when injected for therapeutic purpose. In fact, most of central effects mentioned above may relate to changes in muscular proprioception. The musculoskeletal proprioceptive information conveyed to central nervous system originates from distinct peripheral receptors (muscle spindle, tendon, and joint receptors). Muscle spindles are comprised of sensory fibers type IA and modified muscle fibers that are innervated by cholinergic fibers (i.e., intrafusal fibers) from the γ-motoneurons. The latter are co-activated with the α-motoneurons to adjust the spindle length with that of skeletal muscle. Spindle stretching (e.g., when contraction of skeletal muscles fibers is late as compared to that of spindle organ) leads to activation of IA sensory fibers that in turn reinforces direct activation of homonymous α-motoneurons and inhibitory neurons projecting onto the motoneurons of antagonistic muscles and other inhibitory interneurons (as the Renshaw cells). Although intrafusal cholinergic transmission can be blocked by botulinum neurotoxin (Dressler et al. 2005; Filippi et al. 1993; Rosales et al. 1996; Trompetto et al. 2006) and intrafusal muscle fibers can undergo atrophy (Rosales et al. 1996), such effects have never been demonstrated occurring in the context of therapeutic use of botulinum toxin (Gracies 2004). Indeed, the spindle capsule insulates intrafusal motor nerve fiber from toxin molecules injected in muscle tissue. Thus, in the clinical practice, intramuscular injection of botulinum toxin/neurotoxin effects on the skeletal neuromuscular junctions (i.e., those innervated by α-motoneurons) but the intrafusal ones. The resulting stretching of the spindle leads to increased activity of sensory IA neurons and activation of the inhibitory interneurons projecting onto motoneurons of the antagonistic muscles or other spinal interneurons; therefore, reports on changes in the inhibition by Renshaw cells (Caleo and Schiavo 2009; Gracies 2004) and paralysis of noninjected muscles (Gracies et al. 2009; Wohlfarth et al. 2001) can be explained as the result of a change in the spinal reflex neuronal network induced by myorelaxation of toxin-injected muscles.

The change of proprioceptive information can quickly lead to functional alterations of the upper levels of the motor system. In rats, the position in space of the whiskers is controlled by motoneurons located in the facial nucleus, and their position is detected by sensory fibers. Following injection of botulinum toxin into the whisker pads, whiskers are immobile and proprioceptive inputs dramatically disturbed, indirectly causing quick functional reorganization of primary motor cortex (M1) by synaptic plasticity (Franchi and Veronesi 2004). Functional alterations of the cortical neural network have been also observed in humans in the context of the clinical use of botulinum toxin (Curra et al. 2004). These alterations of central neural network are indirect consequences of the action of the neurotoxin and, in turn, can be manifested in the periphery by change in motor behavior of noninjected muscles.

To summarize, distant manifestations of botulinum toxin deserve careful analysis to distinguish between those issuing from spreading of botulinum neurotoxin molecules from injected sites from the indirect ones mediated by the spinal reflex neuronal network.

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13 Whooping Cough

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Abstract
First described in the sixteenth century, whooping cough or pertussis is a relatively recent disease in human history, although some of the cough syndromes described in antiquity may in fact be pertussis-like diseases. Whooping cough caused by the gram-negative bacterium *Bordetella pertussis* is a severe respiratory disease, especially life-threatening in early childhood. In addition to respiratory symptoms, characterized by paroxysmal cough and whoop, pertussis also manifests itself by a marked leukocytosis, and complications due to superinfections are common. In adolescents, adults, and vaccinated older children, the disease is often atypical. It was a major cause of childhood mortality in the pre-vaccination era. Mass vaccination has tremendously reduced the incidence of the disease, but despite a large global vaccination coverage, we witness a dramatic increase of its incidence in recent years. The pathogenesis of the disease relies on a series of rather well-defined virulence factors, including several adhesins and toxins, whose production is controlled at the transcriptional level by a two-component master regulatory system. Various animal models have helped to decipher the virulence mechanisms of *B. pertussis* and have been instrumental in preclinical testing of vaccines. However, most of them do not reflect all the features of human pertussis, perhaps with the exception of a very recent baboon model. Since the discovery of its etiological agent, different diagnostic methods have been designed, including bacterial culture, serology, and, more recently, polymerase chain reaction. *B. pertussis* is sensitive to several antibiotics. Erythromycin is the drug of choice, and erythromycin-resistant *B. pertussis* isolates are rare. However, unless administered at the early catarrhal stage of the disease, antibiotic treatment is of little help to decrease the severity or to shorten the duration of the disease. Vaccination is today the most powerful tool to combat the disease. Several types of vaccines are currently available, including the whole-cell first-generation vaccines and the newer acellular second-generation vaccines. However, in view of the recent reemergence of pertussis, current vaccine regimens have shown their limits, and new vaccines are urgently needed. In that regard, live attenuated vaccines given very early in life may perhaps be able to protect the youngest and most vulnerable infants during the first months of life.

Introduction and Historical Perspectives

In the history of mankind, whooping cough or pertussis may be a relatively recent disease (Cherry and Heininger 2004). The first clinical symptoms were described in the second half of the sixteenth century as paroxysmal coughing. The disease appears to not have been known during antiquity and the Middle Ages, although Hippocrates described *Perinthus cough* around 400 B.C., and Reginald mentioned *kinkehost* in his Vita Godrici around 1190, and both diseases may be related to pertussis (Versteegh 2005). The first known description by the French physician Guillaume de Baillou was subsequent to an epidemic in 1578 but was published only in 1640 by his nephew. De Baillou called the disease *quinta*. The name *pertussis* was first given by Willis and Sydenham in the seventeenth century to describe the syndrome as a severe cough. The disease has been given several names in different countries describing various aspects of the disease, such as the cockcrow (*coqueluche*) in France, the dog's bark (*tosse canina*) in Italy, the howling of wolves (*Wolfshusten*) in Germany, and the cough of 100 days in China (Long 1997). Some of these names have been used since several centuries. However, since it is impossible to accurately diagnose the disease retrospectively, we will probably never know whether they always refer to what is known today as whooping cough. The word *coqueluche* has been used in France to also

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designate influenza, and pertussis-like symptoms can be caused by a variety of different infectious agents, including viruses.

Since its official first description roughly 400 years ago, pertussis has spread rapidly throughout Europe over the following centuries and has become a major cause of mortality in early childhood. Although the infectious nature of the disease has been rapidly recognized, the principal causative agent, *Bordetella pertussis*, was only discovered in 1900 by Bordet and Gengou (1906). They examined the exudation of a 5-month-old child diagnosed with whooping cough under the microscope and found, in addition to large amounts of leukocytes, a very high number of small coccoïdal, ovoid, or rod-shaped gram-negative bacteria that were colored light blue after staining with Kühne's dye. They appeared mostly singly, sometimes in pairs, and rarely in small clusters. The bacteria could only be observed at the first cough episodes and were difficult to see at later episodes. In addition, they have been very difficult to cultivate, and consistent cultures could only be obtained 6 years later on blood-agar tubes based on glycerinated potato extracts supplemented with fresh rabbit or human blood. The initial inoculation of a very dense fresh exudation from a 2-month-old child at his first cough episode did not result in visible growth after 2 days incubation. However, a secondary inoculation from the first blood-agar tube resulted in a more rapid growth. Morphologically, the bacteria from the secondary culture were indistinguishable from those observed in the fresh exudation. The organism was first named *Haemophilus pertussis*.

However, its identity as the whooping cough agent had initially generated controversy, as it was often confounded by the presence of *Haemophilus influenzae*, easier to culture and often present at the same time as the whooping cough agent. The definitive proof that the Bordet-Gengou bacillus is the whooping cough agent came through elegant experiments demonstrating that antisera from convalescent whooping cough patients were able to agglutinate *H. pertussis*, whereas they did not affect other microorganisms isolated from the respiratory tract. Subsequently, the whooping cough agent was renamed *Bordetella pertussis*, in honor of one of its discoverers.

In the following decades, it became apparent that pertussis-like symptoms can also be caused by other members of the genus *Bordetella*, such as *Bordetella parapertussis*, isolated for the first time by Eldering and Kendrick in 1938 (Eldring and Kendrick 1938) and *Bordetella bronchiseptica*, first isolated from a dog with kennel cough. Other *Bordetella* species have more recently been isolated from patients with respiratory illnesses. They include *Bordetella avium* (Harrington et al. 2009), *Bordetella holmesii* (Yih et al. 1999), and *Bordetella petrii* (Le Coustumier et al. 2011). Although *B. pertussis* and *B. parapertussis* are strictly upper respiratory tract pathogens in humans, the other pathogenic or opportunistic *Bordetella* species may also cause septicemia, be more frequently associated with respiratory infections in other animals, or actually be environmental bacteria (Gross et al. 2010). Finally, some *Bordetella* species, such as *Bordetella hinzii* (Cookson et al. 1994), *Bordetella trematum* (Vandamme et al. 1996), and the most recent species *Bordetella ansorpii* (Fry et al. 2007) may cause wound infections or opportunistic diseases in immunocompromised patients.

Clinical Manifestations

Clinical symptoms of whooping cough depend widely on the age and on the vaccination status of the individual (Hewlett and Edwards 2005; Gregory 2006). Typical illness presents three stages: catarrhal, paroxysmal, and convalescent. Following an incubation period of 6–20 days, the infected individual develops a catarrhal phase with nonspecific cold-like symptoms consisting of nasal congestion, rhinorrhea, sneezing, occasionally conjunctival irritation, and nonproductive cough. At this stage, the diagnosis of pertussis is rarely anticipated. More severe symptoms characterize the next stage, called paroxysmal in reference to the intense bouts of coughing followed by an inspiratory "whooping" sound. Twelve to fifteen episodes of coughing bouts, each lasting 1–2 min, can be experienced every day during at least 2 weeks. The cough is supposed to clear the large amount of thick mucus usually produced in the respiratory tract during the paroxysmal stage, and this attempt can be exhausting for the infected individual. Vomiting and cyanosis after coughing can occur especially in young children.

Additional clinical complications may develop during the paroxysmal phase of whooping cough. The most severe ones are observed in neonates and in young children. These include pneumonia, apnea, hypotension, dehydration, weight loss due to excessive vomiting, brain damage due to oxygen deprivation, and death (Heininger et al. 1997; Smith and Vyas 2000). Autopsy from infants aged less than 4 months suggests that pertussis-related death is associated with marked leukocytosis and bronchopneumonia that result in reduced pulmonary blood flow which aggravates the hypoxic vasoconstriction, eventually leading to refractory pulmonary hypertension, cardiac failure, and shock (Paddock et al. 2008).

In vaccinated older children, adolescents, and adults, the clinical course of whooping cough is often atypical and consequently unrecognized (Cherry 1999). However, adults can also experience a large range of mechanical complications resulting from the intense bouts of coughing, such as nosebleeding, hearing loss, cough syncope, bruised ribs, herniated lumbar disk, and urinary incontinence (Jenkins and Clarke 1981; De Serres et al. 2000; von König et al. 2002). Neurological events have been reported in adults with pertussis, including encephalopathy and seizures (Halperin and Marrie 1991; De Serres et al. 2000), and a clinical case report has suggested a link between pertussis illness and an acute neurological event, a carotid artery dissection leading to stroke and to serious long-term neurological deficits in otherwise healthy adults (Skowronsky et al. 2003). Ear infections such as otitis media and sinusitis frequently develop as a complication of pertussis. During the convalescent stage, which can last up to 3 months, the severity of the symptoms weakens with fewer coughing bouts, but it can take additional months for full recovery.

Severe and long-lasting sequelae from pertussis can be measured using a single metric, the disability-adjusted life years (DALYs). Each DALY is basically equal to 1 year of healthy life lost. In 2000, an estimated 12.7 million DALYs were linked to pertussis worldwide (Crowcroft et al. 2003). This impressive

number exceeded the 11.4 million DALYs due to lung cancer or the 5.8 million DALYs to meningitis.

Epidemiology

For centuries, whooping cough was a major cause of infant mortality rivaling with measles, scarlet fever, and diphtheria (Crum 1915). Data from the British Register General's Report indicate that in the late nineteenth century, 2 % of the total deaths of the year in the country were due to whooping cough with 97 % of the cases occurring in children less than 5 years old (Lee 1879). Several studies suggest that in the early twentieth century, 75–80 % of all children experienced pertussis infection by age of 12 (Collins 1929; Sydenstricker 1932). A larger study combined the statistics of 24 countries during a 5-year period and determined that the average annual death rate from whooping cough reached 8.0/100,000 of the total population, varying from 66.5/100,000 in Chili to 8.2/100,000 in France and 1.9/100,000 in Ceylon (Crum 1915).

The introduction in most industrialized countries of the first whole-cell pertussis vaccines combined with diphtheria and tetanus toxoids in the 1940s and the 1950s resulted in a dramatic decrease of the incidence of whooping cough, with as much as 99 % reduction of reported cases in United States (US Public Health Services 1953). In low vaccine uptake countries, the disease burden remains high, with 12.5 million cases both for Africa and for Southeast Asia estimated in 1999 and 170,000 deaths due to pertussis in Africa alone each year (Crowcroft et al. 2003).

Pertussis is a cyclic infectious disease. In the vaccine era, the mean periodicity is approximately 3–4.5 years (Hethcote 1998; Rohani et al. 1999; Skowronski et al. 2002; Broutin et al. 2005a, 2010). A recent analysis of long-term epidemiological data from eight countries across five continents in the pre-vaccine era and 64 countries in the vaccination era showed that the pertussis periodicity increased by 1.27 years on average following the introduction of vaccination in a country (Broutin et al. 2010). This change in the interepidemic periodicity suggests the induction of herd immunity following vaccination and is consistent with previous studies (Rohani et al. 2000; Broutin et al. 2005b).

During the last two decades, a reemergence of pertussis in developed countries with high vaccination coverage rates has been observed, particularly in teenagers and adults (Baron et al. 1998; Güriş et al. 1999; Galanis et al. 2006). This was particularly striking in Massachusetts in 1997 where 46 % of reported pertussis cases occurred in adolescents and adults (Lee et al. 2004). This epidemiological shift toward the older population may have a strong impact on the transmission of pertussis to highly susceptible young infants. A study gathering data from France, Germany, USA, and Canada showed that adults are the source case for 76–82 % of transmission of pertussis in the household (Wendelboe et al. 2007). The transmission rate of pertussis is indicated by its basic reproduction numbers, R_0, which evaluates the average number of secondary cases of pertussis generated by a primary case in an unvaccinated population. The R_0 for pertussis has been calculated to be approximately 5.5 in five European countries and was found to be similar among the five studied countries (Kretschmar et al. 2010). An increase of infant deaths has therefore not surprisingly been reported during recent outbreaks in the USA (Tanaka et al. 2003; Roehr 2010) and in Canada (Mikelova et al. 2003), along with the epidemiological shift toward adolescent and adult populations.

Adaptation of *B. pertussis* strains to vaccine-induced immunity has been pointed out as a possible explanation for the resurgence of pertussis (Mooi et al. 2001; Godfroid et al. 2005; Kallonen and He 2009). Molecular epidemiology studies on circulating *B. pertussis* strains showed a polymorphism of genes coding for pertussis toxin (PTX) and pertactin (PRN), with sequences distinct from those of vaccine strains. This phenomenon has been observed in many countries, such as France (Weber et al. 2001), the Netherlands (Mooi et al. 1998) and the United States (Cassiday et al. 2000). The polymorphism in PTX is mainly found in the gene encoding the S1 subunit of PTX bearing the enzymatic activity of the toxin (Mooi et al. 1998). In addition, recent studies highlighted the rise of *B. pertussis* strains with a different allele for the PTX promoter called *ptx*P3 (Mooi et al. 2009; Lam et al. 2012). The mutation of the *ptx* promoter in *ptxP3* strains results in higher production of PTX, which may perhaps be a selective advantage over other *B. pertussis* strains and could also lead to more severe disease in infected hosts (Mooi et al. 2009).

The other predominant polymorphism occurred in the region near the arginine-glycine-aspartic acid (RGD) motif of PRN involved in adherence of *B. pertussis* to host cells (Leininger et al. 1991; Mooi et al. 2009). Three prevalent variants have been described. *B. pertussis* strains expressing PRN1 were predominant in the pre-vaccination era and were therefore used as vaccine seed strains. PRN2 and PRN3 variants were first detected in the 1980s, but the PRN2 type soon became the predominant circulating strains (Mooi et al. 1999). Recently, the emergence of PRN-deficient strains has been described, with a rate of approximately 30 % in Japan (Otsuka et al. 2012). Similar findings, but to a lesser extent (rate of 5.6 %), have also been reported in France (Bouchez et al. 2009).

Polymorphisms in genes encoding fimbriae involved in attachment of *B. pertussis* to the host respiratory epithelium have also been described. *B. pertussis* is able to produce two serotypes of fimbriae, Fim2 and Fim3, resulting in Fim2, Fim3, or Fim2,3 serotypes. The phase transition between a high and a low level of their expression has been shown to depend on the number of C residues in *fim* promoters (Willems et al. 1990; Chen et al. 2010). The Fim2 serotype is prevalent in unvaccinated populations, while the proportion of Fim3 strains increased with the introduction of pertussis vaccination (Preston and Carter 1992; Hallander et al. 2005).

Pathogenesis

In this chapter, we limit our description to a summary of the main virulence traits. *B. pertussis* is a gram-negative pathogen which exclusively colonizes the human respiratory tract and is

the main etiological agent of whooping cough. Other members of the *Bordetella* genus such as *B. parapertussis* and *B. bronchiseptica* have been associated with milder forms of human illness (Mooi et al. 2007). Infection of the host by *B. pertussis* occurs through inhalation of droplets containing bacteria produced during the cough of an infected individual. Once the bacteria have entered the upper respiratory tract, they produce a range of virulence factors, adhesins and toxins, that will promote their adherence to the ciliated epithelial cells of the nasopharynx and the trachea. A fine-tuned temporal regulation of the transcription of genes encoding these bacterial factors contributes to the optimal initial adherence of *B. pertussis* to host cells (Kinnear et al. 2001; Veal-Carr and Stibitz 2005).

Among the adhesins expressed by *B. pertussis*, filamentous haemagglutinin (FHA) and fimbriae play a major role in the interaction of the bacteria with the host epithelium (Jacob-Dubuisson and Locht 2007). Mature FHA is a large monomeric protein of approximately 232 kDa, which corresponds to the N-terminal two-thirds of an even larger 360 kDa precursor called FhaB (Domenighini et al. 1990; Lambert-Buisine et al. 1998). The complex biogenesis of FhaB involves its export across the cytoplasmic membrane in a sec-dependent manner, followed by its translocation across the outer membrane through a protein called FhaC (Chevalier et al. 2004; Jacob-Dubuisson et al. 1999; Hodak et al. 2006). FhaB is eventually processed to mature FHA at the bacterial surface by a specific protease of the subtilisin family, called SphB1, and is found both non-covalently bound to the surface of the bacteria and in a secreted form (Coutte et al. 2001).

Several domains involved in the binding activity of FHA have been identified. An Arg-Gly-Asp (RGD) domain was shown to interact with the integrin CR3 ($\alpha_M\beta_2$, CD11bCD18) present at the surface of the macrophages (Relman et al. 1990). FHA interacts also with the leukocyte response integrin/integrin associated protein complex (LR1 or $\alpha_v\beta_3$ integrin/IAP or CD47) on human monocytes, which leads to upregulation of CR3 binding activity (Ishibashi and Nishikawa 2002). The interaction between FHA and CR3 promotes the uptake of the bacteria by macrophages and was suggested to play a role in the persistence of *B. pertussis* in the lungs (Saukkonen et al. 1991; Friedman et al. 1992; Ishibashi et al. 2001). A heparin-binding domain has also been identified and is involved in the interaction of FHA with sulfated carbohydrates present at the surface of the respiratory epithelial cells and sulfated proteoglycans contained in the extracellular matrix (Menozzi et al. 1991; Hannah et al. 1994). The binding properties of FHA were shown to be dependent on the presence of cholesterol-containing lipid rafts in the membrane of respiratory epithelial cells (Lamberti et al. 2009).

Fimbriae, initially called agglutinogens, are polymeric adhesive structures extending from the outer bacterial membrane of the bacteria (Blom et al. 1983). These structures are constituted by a minor subunit called FimD and by two major subunits defining the serotypes of fimbriae, Fim2 and Fim3. Similarly to FHA, fimbriae were shown to be involved in binding of the bacteria to sulfated carbohydrates as well as to the very late antigen-5 (VLA-5) integrin on monocytes (Geuijen et al. 1998; Hazenbos et al. 1995).

Pertactin (PRN), or p.69, was also initially described for its role in attachment of *B. pertussis* to epithelial cells. PRN is exposed at the surface of the bacteria and belongs to the autotransporter family. It is constituted of a C-terminal part which promotes the translocation through the outer membrane of the N-terminal part containing an RGD domain potentially involved in adherence of *B. pertussis* (Leininger et al. 1991; Henderson and Nataro 2001). The binding property of PRN is controversial since other studies failed to show its role in vitro in adherence to human respiratory epithelial cells (Roberts et al. 1991; van den Berg et al. 1999). On the other hand, a PRN-deficient *B. bronchiseptica* strain was shown to be impaired in colonization of the mouse respiratory tract due to a reduced resistance to neutrophil-mediated clearance (Inatsuka et al. 2010). Therefore, PRN might not contribute significantly to *B. pertussis* adherence to epithelial cells but rather play a role in survival of the bacteria in the host respiratory tract.

Indirect contribution of toxins such as pertussis toxin (PTX), adenylate cyclase toxin (ACT), and tracheal cytotoxin (TCT) in the early steps of *B. pertussis* infection has also been established (Hewlett and Donato 2007). Thanks to their cytotoxic and immunomodulatory activities, *B. pertussis* toxins will create an optimal environment for successful bacterial colonization of the host's upper respiratory tract (de Gouw et al. 2011).

PTX is a complex multimeric protein belonging to the ADP-ribosylating toxins family (for a recent review, see Locht et al. 2011). This major toxin, exclusively produced by *B. pertussis*, is composed of 5 different subunits, named S1 to S5, with S1 being responsible for the enzymatic and toxic activities of the protein. PTX is actively secreted through the bacterial outer membrane using a type IV secretion system called the Ptl machinery (Craig-Mylius and Weiss 1999). Despite the presence of carbohydrate recognition domains on S2 and S3 oligomers (Saukkonen et al. 1992; Millen et al. 2010), there is no in vitro nor in vivo evidence for a role of PTX in bacterial adhesion to epithelial cells and in colonization of the respiratory tract in a mouse model (van den Berg et al. 1999; Alonso et al. 2001). Nevertheless, a strain deficient in both PTX and FHA loses its ability to efficiently colonize the lungs of mice suggesting a redundancy in the activity of these two virulence factors (Alonso et al. 2001). Rather than its binding activity, the early role of PTX in lung colonization seems to be linked to its enzymatic activity and to its well-described immunomodulatory properties (for a recent review, see de Gouw et al. 2011).

ACT (or CyaA) is a polypeptide of 177 kDa which belongs to the RTX (repeat in toxin) family (Ladant et al. 1986). It is composed of a catalytic N-terminal region and a C-terminal domain which mediates the binding of the toxin to the host cell membrane and translocation of the catalytic domain through the plasma membrane (Ladant and Ullmann 1999).

ACT interacts with the integrin CR3 ($\alpha_M\beta_2$, CD11bCD18) on macrophages, neutrophils, dendritic and natural killer (NK) cells, and with LFA-1 ($\alpha_L\beta_2$, CD11aCD18) present at the surface of lymphocytes (Guermonprez et al. 2001; Paccani et al. 2011). ACT induces a rapid increase of the cAMP concentration resulting in a variety of effects such as inhibition of monocytes' phagocytic and oxidative activities, inhibition of chemotaxis, and induction of cell apoptosis (Gueirard et al. 1997; Eby et al. 2012). ACT was also shown to modulate innate and adaptive immunity (Boyd et al. 2005; Paccani et al. 2008; Fedele et al. 2010). ACT could therefore prevent the induction of an efficient immune response and promote the survival of the bacteria. This is in agreement with the observation that an ACT-deficient *B. pertussis* strain was impaired in its ability to colonize efficiently the lungs of mice (Khelef et al. 1992; Carbonetti et al. 2005). In addition, ACT was shown to interact with FHA, and it has been suggested that this association may increase the FHA-mediated adherence of the bacteria to host cells (Zaretzky et al. 2002).

Successful attachment of *B. pertussis* to respiratory epithelial cells is also promoted by the cytotoxic activity of the tracheal cytotoxin (TCT). TCT is a peculiar toxin since it is a muramyl peptide released from the *B. pertussis* cell wall during bacterial growth (Rosenthal et al. 1987). TCT expresses highly potent toxic activity as highlighted by the loss of ciliary activity and ciliated cells extrusion from hamster tracheal rings exposed to purified TCT (Goldman et al. 1982; Cookson et al. 1989). These effects are mediated by nitric oxide (NO-) and by interleukin-1 (IL-1), and it has been shown that TCT and endotoxins from *B. pertussis* act synergistically to activate the inducible nitric oxide synthase (iNOS) and increase IL-1 production by epithelial cells (Heiss et al. 1993, 1994). The source of NO- was later shown to be the secretory epithelial cells in direct neighborhood of ciliated cells (Flak and Goldman 1999). Destruction of ciliated cells in the trachea is a key step in the infection process of the host by preventing *B. pertussis* mechanical clearance early after bacterial entrance. In addition, TCT might also play a key role in transmission of *B. pertussis* since accumulation of mucus, due to the destruction of ciliated cells, has been associated with the cough of infected individuals.

Animal Models

Whereas certain *B. parapertussis* strains have been isolated from sheep and *B. bronchiseptica* has a broad mammalian host spectrum, *B. pertussis* is a strictly human pathogen, although anecdotal reports of zoo outbreaks also suggest that chimpanzees can be naturally infected with *B. pertussis*.

Since the discovery of the whooping cough agent, many efforts have been devoted to the development of suitable laboratory animal models in order to experimentally address the mechanisms of pertussis pathogenesis and to evaluate vaccine candidates. The most convenient animal model used so far is the mouse model. However, mice do not cough and do not transmit the disease to other mice, and therefore, *B. pertussis* infection of mice does not reflect the typical human disease. Nevertheless, mice have been useful to study several aspects of whooping cough, as, similar to humans, infant mice are more susceptible to *B. pertussis* infection than adult mice and can die from the infection. Furthermore, the duration of *B. pertussis* colonization in the respiratory tract is similar between mice and humans, and finally, lymphocytosis, one of the hallmarks of human pertussis, also occurs in mice, as well as hypoglycemia and histamine sensitization (Pittman et al. 1980).

Two types of mouse models have been widely used to study pertussis, an aerosol or nasal infection model and an intracranial challenge model. The latter has been introduced by Kendrick et al. (1947) as a potency assay to evaluate the protective potential of vaccines. In this test, 200 LD_{50} of *B. pertussis* is injected intracranially into mice, and survival is recorded for 2 weeks postinfection. Efficacious whole-cell vaccines protect mice from death, and, based on a good correlation between protection against intracranial challenge in mice and efficacy in the Medical Research Council clinical trials (Medical Research Council 1959), it has been used as a standard potency model to evaluate vaccines throughout the world (WHO 1979).

However, the intracranial challenge model is not the most satisfactory animal model to study the pathogenesis of whooping cough, and the potency assay to test vaccines is essentially only predictive when the atypical *B. pertussis* strain 18323 is used. Furthermore, it performs rather poorly to predict the efficacy of the second-generation, acellular vaccines. Therefore, respiratory challenges have also been used. In the 1930s, Burnet and Timmins (1937) have developed a method for intranasal inoculation of mice. However, initially, this model showed a less strong correlation of potency with vaccine efficacy in humans than the intracranial model. Mice can be infected either by the application of nasal drops containing the bacterial suspension or by exposing them to a *B. pertussis*-containing mist in an aerosol chamber. By assessing the bacterial clearance over time, these newly adapted challenge models show a good correlation between vaccine potency in the mouse and protective efficacy in children, even with acellular pertussis vaccines (Mills et al. 1998). Furthermore, using various genetic knockout mice, these models have revealed that protection against pertussis relies on both on B cells and on T cell-mediated immunity.

The mouse respiratory infection models have also been useful to identify the virulence determinants of *B. pertussis* (Weiss and Hewlett 1986). By using transposon-mediated mutagenesis and testing the mutants in this model, several essential genes involved in virulence have been identified, including those coding for toxins and adhesins, as well as for the general regulation of *Bordetella* virulence.

The major drawback of mouse models for pertussis is that these animals do not cough and cannot transmit the disease to other mice. Therefore, these models cannot be used to determine the components responsible for the paroxysmal cough, the stage at which most complications and deaths occur in children. Unlike mice, rats do cough when inoculated with *B. pertussis* (Hornibrook and Ashburn 1939). This led to the coughing rat

model as a potentially useful system to study the full pathogenesis of pertussis. In this model, *B. pertussis* is embedded in agarose beads and intrabronchially administered under anesthesia to Sprague–Dawley rats, which induces coughing episodes at 9–14 days after infection that can be monitored using sound-activated tape recorders with microphones above the rat cages in sound-insulated booths. However, the administration of simple suspensions of *B. pertussis* without beads does not induce significant coughing (Hall et al. 1994). In addition to coughing, agarose-beads-embedded *B. pertussis* also induces leukocytosis and antibody responses to the major *B. pertussis* antigens.

This model has subsequently been used to evaluate the role of virulence factors previously identified in mouse models. It was instrumental to show that pertussis toxin is required for cough induction, as a pertussis toxin-deficient mutant strain was inactive as a cough inducer (Parton et al. 1994). In addition, the model has been used to assess pertussis vaccines. Either whole-cell or acellular pertussis vaccine administrations indeed strongly reduced the incidence of coughing of the rats upon infection with *B. pertussis*, as did administration of certain antigens, such as detoxified pertussis toxin, but not others, such as pertactin, FHA, or fimbriae, although vaccination with the latter two prevented leukocytosis (Hall et al. 1998).

The fact that this model requires encasing the *B. pertussis* organisms in agarose beads and delivering them intrabronchially via tracheotomy certainly represents an important limitation. In addition, Sprague–Dawley rats are much less susceptible to *B. pertussis* infection than mice, 10^8 cfu are needed to infect a rat, compared to 10^3–10^5 cfu for a mouse, and the time to bacterial clearance is much shorter in rats than in mice. As with mice, infected rats do not transmit the organism to other rats within the same room.

More recently, a newborn piglet model has been developed, in an effort to resemble human infants more closely than rodent models (Elahi et al. 2005), as the anatomy and immunology of the newborn piglet respiratory tract has many common features with those of human newborns. When 3-day-old piglets are infected with 5×10^9 cfu of virulent *B. pertussis*, clinical symptoms can be observed as early as 2 days after infection. These symptoms include respiratory symptoms, such as nasal discharge, breathing difficulties, and occasional nonparoxysmal cough. Although *B. pertussis* infection does not induce mortality in 3-day-old piglets, autopsy results showed pathological alterations, such as hemorrhagic and necrotizing pneumonia and fibrinous pleuritis. Similar to the rat, neonatal piglets rapidly clear the infection. Infected piglets also exhibit a significant reduction in blood glucose levels and circulating lymphocytosis, compared to noninfected animals, and active pertussis toxin can be detected in the bronchoalveolar lavage fluids and in the serum of infected animals.

In contrast to 3-day-old piglets, intrapulmonary challenge of older piglets does not result in clinical symptoms or pathological alterations in the lungs, and no bacteria can be isolated from the lung tissues or bronchoalveolar lavage fluids of infected older piglets. Even encasement of *B. pertussis* in agar beads does not help to induce clinical signs of infection or pathological changes. These observations show that older piglets are not susceptible to *B. pertussis* infection. This may be related to the ability of bronchoalveolar lavage fluids from older but not from neonatal piglets to kill *B. pertussis*. This bacterial killing has subsequently been shown to be mediated by beta-defensin 1 (Elahi et al. 2006b), stressing the importance of innate immunity in the protection against *B. pertussis*. In contrast to older mice, 3-day-old piglets infected with *B. pertussis* exhibit thus a range of clinical symptoms that are also seen in pertussis patients, although in infants, the symptoms are often more severe and combined with paroxysmal cough, which is not seen in the piglet model.

This model has been used to study the role of maternal immunity in the protection of newborns against pertussis (Elahi et al. 2006a). Pregnant sows immunized intramuscularly or orally with whole-cell vaccine preparations produce anti-*B. pertussis* serum and colostral antibodies that can be passed on to their offspring, which may result in significant protection against challenge in the piglets, both with respect to pathology and to bacterial load.

Other animal models that have been used to study pertussis include rabbits, guinea pigs, and puppies (Elahi et al. 2007). None of these animals develop paroxysmal cough and are able to transmit the disease. Paroxysmal cough and mucus induction has been reported in nonhuman primates (Huang et al. 1962) but has not always been reproducible. Most published primate studies have focused on two species, *Macaca mulatta* and *Macaca cyclopis*. However, very large inoculum sizes are needed to establish a *B. pertussis* infection in macaques, and infection is not always consistent. Although chimpanzees can be readily infected with *B. pertussis* and reproduce many of the human pertussis symptoms, including paroxysmal cough (Rich et al. 1932), for ethical reasons, these animals cannot be used for pertussis research.

A very recent reevaluation of the macaque model has shown that 43- to 64-week-old rhesus macaques can be infected with high doses of *B. pertussis* but fail for the most part to exhibit signs of disease (Warfel et al. 2012). This was ascribed to the elevated body temperature of rhesus macaques (38.7–39°8 C) compared to humans. Growth of *B. pertussis* is somewhat reduced at 39 °C, compared to 37 °C. However, most importantly, *B. pertussis* grown at 39 °C lacks hemolytic activity because of instability of the adenylate cyclase/hemolysin at elevated temperatures. These observations may explain the variable results of *B. pertussis* infection in rhesus macaques.

Compared to rhesus macaques, baboons have a lower body temperature of 37–39 °C and indeed provide a more reliable model of clinical pertussis. 28- to 39-week-old baboons infected with 10^9–10^{10} cfu of virulent *B. pertussis* develop classical symptoms of clinical pertussis, as evidenced by very high whole blood cell counts and severe paroxysmal cough that persists for over 2 weeks and mucus production (Warfel et al. 2012). Furthermore, *B. pertussis* infection of baboons induces long-lived (at least up to 7 months) antibody responses to pertussis toxin and protection against a second challenge. As promising as this model looks, it is not known yet whether Baboons are able

to transmit pertussis to naïve monkeys. If this could be established, the baboons would provide a unique model to identify *B. pertussis* virulence factors involved in the spread of this organism, which would represent a major breakthrough in the understanding of pertussis pathogenesis.

Tools to Control Whooping Cough

Diagnostics

In the pre-vaccination era, typical childhood pertussis could easily be recognized by the specific paroxysmal cough with the classic whoop, which is much less the case in vaccinated populations (Yaari et al. 1999). Although quite specific, diagnosing pertussis on the whoop alone is thus not sensitive enough. The marked lymphocytosis during pertussis is also a classical marker and can be a useful indicator of the disease, especially when combined with other diagnostic methods (Fung et al. 2004).

Traditional laboratory methods to diagnose pertussis were based on *B. pertussis* identification after culturing nasopharyngeal secretions. The secretions can be collected by swabbing or aspiration. The swabs or aspirations are plated onto selective media after an enrichment step, and after several days of incubation, *B. pertussis* can be identified with high specificity (Kerr and Matthews 2000). Transport media are often used, as specimens are usually taken by private practice pediatricians, who generally do not routinely culture *B. pertussis*. Different transport media may lead to different *B. pertussis* recovery yields (Hoppe et al. 1986). *B. pertussis* can then be grown on Bordet-Gengou blood-agar plates, a method developed more than 100 years ago (Bordet and Gengou 1906). However, this medium has a short shelf live due to the high percentage of blood. Other media have also been developed, including blood-free beef heart charcoal agar (Mishulow et al. 1953). Variations of these media are still used today. The sensitivity of these microbiological methods is only satisfactory at the early stages of the infection, before the paroxysmal cough. It declines rapidly with time between onset of the disease and culturing and is strongly influenced by antibiotic treatment (Friedman 1988). The results are also influenced by collection procedures, delays in transmission of the specimens, overgrowth of other microorganisms, even on selective media, and the lack of experience in the recognition of the organism. Even with the best-trained laboratory workers, the isolation rates rarely exceed 50 %.

As an alternative, direct fluorescent-antibody tests have been developed, using polyclonal fluorescein-labeled antibodies against *B. pertussis* to directly identify the organism in the nasopharyngeal specimens (Parker and Payne 1985). This method appears to be more sensitive and provides results more rapidly than culture. However, results may sometimes be difficult to interpret and are prone to a high percentage of false-positives (Holwerda and Elderling 1963).

Serology has also been extensively used to diagnose pertussis. Initially, the most widespread method was based on the ability of anti–*B. pertussis* antisera to agglutinate the bacteria (Evans and Maitland 1939). With the advent of enzyme-linked immunosorbent assays (ELISA), more sensitive tests were designed. A handful of antigens have been used to determine seroconversion by ELISA. They include pertussis toxin, FHA, pertactin, or whole-cell extracts (Trollfors et al. 2003). However, seroconversion may be influenced by vaccination prior to infection, as the antigens used for serology are also included in most pertussis vaccines. In addition, with the exception of pertussis toxin, the antigens used for serodiagnosis are not specific for *B. pertussis* and can cross-react with other bacterial antigens. Cutoff values for seroconversion are not always easy to determine (Teunis et al. 2002). A threshold level of above 50–100 IU/ml of anti-pertussis toxin IgG has been proposed as a positive value (Guiso et al. 2010). However, the magnitude of serum anti-pertussis toxin antibodies varies considerably between individuals. It is also not always possible to obtain blood samples at different phases of the disease in order to compare the antibody titers. Usually, only single samples at the convalescence phase are available, which makes it impossible to determine titer rise.

The evaluation of antibody isotypes other than IgG may be helpful to partially solve this problem, as the production of IgA is mainly induced by infection and not by vaccination (Nagel and Poot-Scholtens 1983). However, these antibodies appear relatively late after the onset of the infection. Anti-*B. pertussis* IgA can also be detected in nasopharyngeal secretions, where they appear somewhat earlier than in the serum (Goodman et al. 1981).

Perhaps today, the most sensitive methods to diagnose pertussis are based on polymerase chain reaction (PCR). Sensitivities of up to 100 % have been reported (Halperin 2007). More than 100 protocols have been described, which differ in DNA isolation techniques, PCR primer selection, reaction conditions, and amplicon detection methods (Woods and McIntyre 2008). A standard protocol has been proposed by Meade and Bollen (1994). The specificity obviously depends on the selection of the PCR primers but may also be affected by laboratory or sample contamination. PCR assays require specially equipped laboratory environments and well-trained personnel, as the risk of false-positives is high due to the high sensitivity of the technology. Owing to its specificity, PCR has become the test of choice for the diagnosis of pertussis (Zouari et al. 2012). It has been suggested that PCR should be used to support the diagnosis of pertussis when the patient experiences at least 2 weeks of cough associated with paroxysms, whoop, or vomiting. However, PCR can also detect *B. pertussis* infection with high sensitivity in patients with atypical whooping cough and is less sensitive to treatment than culture, most likely because PCR is also able to detect killed bacteria.

More recently, rapid and sensitive genotyping assays and microarrays have been developed to identify pathogens responsible for upper respiratory tract infections, including *B. pertussis* (Lodes et al. 2007). Electrochemical detection on a semiconductor-based oligonucleotide array of amplified bacterial DNA can be automated and used for the simultaneous detection of several pathogens. This technology has the potential to increase speed of diagnosis at relatively low cost.

Treatment

B. pertussis is sensitive to a variety of antibiotics, and treatment given during the catarrhal phase of the disease may decrease the symptom severity (Bergquist et al. 1987) but has little effect if given during the paroxysmal or convalescent phase. In addition, even though *B. pertussis* may be very sensitive to certain antibiotics in vitro, these drugs may not necessarily be effective in vivo. As an example, the minimal inhibitory and minimal bactericidal concentrations of ampicillin are 0.068–8 μg/ml and 0.5–32 μg/ml, respectively, (Bass et al. 1969a) yet even at a dosage of 100 mg/kg/day, this drug is ineffective in clearing the organism from the nasopharynx (Bass et al. 1969b). This is likely due to the difficulty of ampicillin to reach the bronchopulmonary space.

Erythromycin is the drug of choice for pertussis. Its minimal inhibitory and minimal bactericidal concentrations range from 0.02 to 2 μg/ml and 0.04 to 8 μg/ml, respectively (Bass et al. 1969a), and at a dosage of 50 mg/kg/day given for 7–8 days, it is able to clear *B. pertussis* from the nasopharynx (Bass et al. 1969b). Nevertheless, some patients may remain culture positive, even after 10 days of treatment, which led to the recommendation to extend treatment to 14 days (Hoppe and Haug 1988). Even with prolonged treatment, bacteriologic relapse can occur (Halsey et al. 1980), which may be related to the type of erythromycin preparation used. With ethylsuccinate ester or stearate salt preparations, more treatment failures were observed than with erythromycin estolate (Bass 1985), which is more resistant to acid inactivation than the other forms. However, erythromycin estolate may perhaps be associated with higher rates of hepatic side effects than the other preparations, although erythromycin hepatotoxicity is very rare in children.

The effect of erythromycin treatment on disease outcome and duration is nevertheless very limited, unless given very early after the onset of catarrhal symptoms. On the other hand, its ability to reduce the bacterial load and to render patients noncontagious has led to the proposal to use this antibiotic for prophylaxis (Hoppe and Haug 1988). There is evidence that erythromycin given to contacts within 2 weeks of the onset of cough in the index case can reduce the transmission rate (Sprauer et al. 1992). It has been recommended that pregnant women with pertussis should be offered erythromycin in order to reduce the risk of transmission to the infant, especially if the disease occurs within 3 weeks before delivery (Evans et al. 1996). However, the effectiveness of chemoprophylaxis with erythromycin has not yet been clearly established, and the evidence to support erythromycin prophylaxis is only weak so far, although there may be benefit to household contacts (Dodhia and Miller 1998).

Resistance to erythromycin is rare (Wilson et al. 2002). Because of its rarity, antimicrobial susceptibility testing is usually not routinely done in clinical laboratories, and standardized methods are lacking. The first erythromycin-resistant *B. pertussis* organism was isolated in Arizona, USA, in 1995 from a 2-month-old child that remained culture positive even after intravenous administration of erythromycin (Lewis et al. 1995). The isolate had a minimal inhibitory concentration of more than 64 μg/ml. More recently, 1,030 isolates circulating in the USA were tested by the disk diffusion assay for their resistance to erythromycin, and less than 1 % of the isolates were found to be resistant (Wilson et al. 2002). However, by using the disk diffusion assay, erythromycin-resistant isolates may potentially be missed, which can be picked up by using a 7-day-incubation period.

B. pertussis is also susceptible to other macrolide antimicrobials, such as azithromycin, which is better tolerated than erythromycin. A large randomized trial with children enrolled from 11 sites across Canada and the USA comparing safety and efficacy of azithromycin with those of erythromycin has shown that azithromycin is as effective as erythromycin estolate for the treatment of pertussis in children but much better tolerated. This resulted in a markedly better compliance for the azithromycin treatment (Langley et al. 2004). In addition, macrolides are more efficiently absorbed, display better acid stability, and have longer half-lives than erythromycin. Therefore, they may potentially be used in shorter treatment courses with less frequent dosing.

Prevention

Whooping cough continues to be a major public health issue despite the widespread use of efficacious vaccines, highlighting the limits of current pertussis vaccination programs. Pertussis vaccination usually begins at 2 or 3 months of age, and optimal protection requires at least three immunizations at 1- to 2-month intervals. A booster dose is commonly given 1–6 years later. This schedule implies that children are not optimally protected before the age of 6 months where they are the most vulnerable to the severe forms of the disease (Bisgard et al. 2005). Two types of vaccines, the whole-cell (wPv) and the acellular (aPv) pertussis vaccines, are currently available. They are combined with diphtheria (D) and tetanus (T) toxoids and often also include inactivated polio vaccine (IPV), Haemophilus influenza b (Hib), and hepatitis B (HepB) vaccines (Storsaeter et al. 2007).

Whole-Cell Pertussis Vaccines

First attempts to develop a vaccine against whooping cough began soon after discovery of *B. pertussis* bacteria in 1906 at Pasteur Institute in Brussels, Belgium, by J. Bordet and O. Gengou (Bordet and Gengou 1906). In 1913, C. Nicolle produced several batches of wPv but they produced inconsistent results (Nicolle and Conor 1913). In the 1920s, LW Sauer, an American pediatrician, visited the Pasteur Institute in Brussels and worked on the improvement of wPv when he came back to the USA (Sauer 1933). In parallel, in 1925, T. Madsen, a Danish physician, was the first to report positive results of clinical trial in children in the Faroe Islands using a new generation of wPv

(Baker and Katz 2004). By the 1930s, wPv were introduced in many countries and were rapidly combined with diphtheria and tetanus toxoids to produce the DTP vaccines still in use in routine vaccinations today. Production process of wPv may vary between manufacturers and explain the relative heterogeneity between the vaccines, especially the lipopolysaccharides content which contributes to the reactogenicity of the vaccines (Fine and Clarkson 1987). All the processes involved a step of inactivation by heat, formaldehyde treatment, or other chemical procedures, and final formulations contain aluminum salt as adjuvant. The effectiveness of wPv has been clearly demonstrated in numbers of clinical trials usually reaching 93 % or more (for review, see Storsaeter et al. 2007). Consequently, wPv have been administered to millions of young children worldwide and have therefore prevented hundreds of thousands of deaths. Immunization with wPv frequently induces minor adverse reactions such as pain, local redness, and mild to moderate swelling. Fever and agitation following vaccination can also be observed transiently. Seizures (<1 in 100) and hypotonic-hyporesponsive episodes (<1 in 1,000–2,000) are much less common (WHO 2005). The incidence of the local reactions increases with subsequent vaccine doses. Allegations that wPv might induce encephalopathy have been raised but never confirmed by thorough investigations (WHO 2005). wPv is one of the oldest vaccines still in use. Its proven efficacy associated with a very low cost of production by national manufacturers makes it still one of the cornerstones of pertussis vaccination worldwide.

Acellular Pertussis Vaccines

Efforts to develop a "better" pertussis vaccine began already in the late 1940s with studies aiming to identify non-reactogenic protective antigens from *B. pertussis* extracts. This strategy could be developed thanks to the finding of Kendrick et al. (1947) that results obtained with a potency test performed in a mouse model were correlated with efficacy of pertussis vaccines in clinical trials. The works of Sato et al. (1974) and Munoz et al. (1981) first demonstrated the protective effect of FHA and PTX in this mouse model. All currently commercially available aPv contain inactivated PTX usually in combination with FHA. Additional components such as PRN and fimbriae can also be part of the aPv (Storsaeter et al. 2007). Differences between current aP vaccines are mainly based on bacterial strains, numbers, and quantities of each antigen and adjuvant. It was shown during clinical trials that aPv has a better safety profile than wPv and that aPv needs to contain at least three components (detoxified PTX, FHA, and PRN) to have similar efficacy and be comparable to good wPv (Jefferson et al. 2003). Nevertheless, the frequency of local adverse events tends to increase in rate and severity with each successive aPv dose (WHO 2005). Transient large swelling reactions that may affect the entire injected limb have been reported in 2–6 % of individuals after the fourth and fifth booster doses of aPv (Rennels 2003). This adverse event has been associated with a general Th2 cytokine profile induced by aPv (Rowe et al. 2005). New generation of aPv containing reduced amounts of antigens has therefore been developed and can be used as booster for adolescents and adults (Grimprel et al. 2005). In many countries, aPv therefore progressively replaced wPv. Nevertheless, the considerable increased production cost of aPv compared to wPv prevents its global implementation especially in developing countries.

New Vaccination Strategies

The main goal of pertussis vaccination strategy should be to reduce incidence and severity in the most vulnerable group of age, the very young children. The currently available wPv and aPv confer optimal protection against *B. pertussis* infection after the third vaccine dose, usually not before the age of 6 months. Several strategies are currently being developed to protect against whooping cough earlier in life.

Maternal Vaccination

Indirect evidences suggest that maternal antibodies might provide passive protection against *B. pertussis* infection early in life (Van Rie et al. 2005). Moreover, the administration of wPv (Kendrick et al. 1945) or aPv (Lewis 2011) late in pregnancy was shown to be safe for the mother and the baby and resulted in high levels of *B. pertussis*-specific antibodies in the infants. Nevertheless, the protective effect which would be associated with maternal antibodies could only be provided for a short period of time, could have a very limited impact in preterm infants (Haberling et al. 2009), and may potentially interfere with priming vaccination in infants (Polewicz et al. 2011). New vaccine strategies for children might therefore need to be developed in parallel to overcome this interference by maternal antibodies.

The Cocoon Strategy

This strategy targets the parents as well as all persons in close contact with a newborn to prevent adult-to-child transmission (DeMaria and Lett 2010). A successful cocoon strategy implies therefore a very high number of individuals to be vaccinated in order to significantly reduce incidence and severity of infant pertussis. A recent study showed that such a number would to be too high in low-incidence countries and the strategy too resource intensive to be efficient for the prevention of severe pertussis in early infancy (Skowronski et al. 2012). In addition, the implementation of the cocoon strategy requires intense parental education and efforts to reduce practical and logistical barriers (Healy et al. 2011).

Neonatal Vaccination

Early vaccination, possibly at birth, might represent the most efficient alternative to prevent severe pertussis in the very young children. Neonatal vaccination with wPv has already been evaluated in the 1960s, but the results were disappointing

(Provenzano et al. 1965). Indeed, antibody responses to *B. pertussis* antigens upon both primary and booster vaccinations were reduced compared to those of infants vaccinated at an older age, suggesting the induction of "immune tolerance." In mouse models, similar reduced antibody levels were measured after neonatal vaccination, but the level of protection against a nasal challenge infection with virulent *B. pertussis* was equivalent to that obtained after vaccination of 3-week-old mice (Roduit et al. 2002). Vaccination of newborns with current DTaPv is generally well tolerated but induces poor antibody responses early after vaccination (Halasa et al. 2008). In addition, a significantly lower response to diphtheria and 3 of 4 pertussis antigens was measured at 6, 7, and/or 18 months in boosted children who received DTaPv at birth compared with boosted children non-vaccinated at birth. This is in contrast with an earlier study showing that neonatal DTaPv resulted in efficient priming as suggested by the significantly higher antibody levels against *B. pertussis* antigens after the second booster dose in children who received DTaPv at birth compared to non-primed children (Belloni et al. 2003). However, at 12 months, the anti-PTX antibody levels were lower in the first group than in the second group. Interestingly, when a stand-alone aPv vaccine was used for neonatal immunization, accelerated and increased antibody responses to *B. pertussis* antigens were measured after booster vaccine administrations, and no interference with subsequent vaccinations was observed (Knuf et al. 2008; Wood et al. 2010). Neonatal vaccination with current aPv is thus generally well tolerated and can accelerate antibody responses to *B. pertussis* antigens. Nevertheless, this accelerated antibody response still leaves an unprotected window for the two first months of life when newborns are the most vulnerable to severe and life-threatening pertussis (Haberling et al. 2009). Moreover, interference with diphtheria, Hib, or HepB vaccines was observed when DTaPv was given at birth (Knuf et al. 2008, 2010). Further studies are needed to evaluate the potential negative impact of such interference on protective efficacy of other recommended vaccines during childhood. Protective immunity against *B. pertussis* infection is mediated at least partially by a Th1-mediated cellular immune response (Redhead et al. 1993; Feunou et al. 2010a). However, a recent study suggests that neonatal immunization with aPv results in Th-memory profiles with a strong Th2 bias as suggested by the high IL-5 and IL-13 production at 8 months of age (White et al. 2010). Further information on T cell responses after neonatal immunization with aPv is needed.

New Pertussis Vaccines

New protective antigens

One line of research to overcome the limits of the current acellular vaccines is to identify additional protective antigens. ACT is one of the candidates since its protective efficacy against a challenge infection with *B. pertussis* has been demonstrated in a mouse model (Guiso et al. 1991). In addition, coadministration of inactivated ACT with aPv enhanced the protective efficacy of aPv (Cheung et al. 2006). The vaccine potential of the autotransporter BrkA was also evaluated since antibodies against BrkA augment serum killing of *B. pertussis* (Marr et al. 2008). While BrkA as the only antigen did not protect mice against challenge infection with *B. pertussis*, the addition of BrkA to PTX and FHA improved the protection over PTX and FHA alone to a level similar to the three components (PTX, FHA, PRN) of aPv. Interestingly, BrkA is ubiquitously expressed by prevalent clinical isolates suggesting that BrkA-containing vaccines may be broadly protective. A novel vaccine candidate called IRP1-3 was recently described (Alvarez Hayes et al. 2011). This surface-exposed protein is induced under iron starvation, an environmental condition the bacteria will encounter during colonization, and is strongly recognized by sera from *B. pertussis*-infected individuals. IRP1-3 induces a slight but significant protection in mice against challenge infection with iron-starved *B. pertussis*. The potential additional protective effect of IRP1-3 to current pertussis vaccines has not been assessed yet.

New formulations

Several groups focused their efforts on new formulations to improve the current vaccines.

As discussed previously, the protective efficacy of wPv to protect children against pertussis is without question, but the occurrence of adverse events has resulted in its reduced acceptance. Efforts were therefore devoted to decrease the reactogenicity of wPv while keeping its protective immunity. One of the main components responsible for this reactogenicity is lipopolysaccharide (LPS) which expresses endotoxic activity. A process to remove more than 95 % of the LPS contained in wPv was developed at the Instituto Butantan, Brazil. This wPv with low LPS content (wP_{low}) was evaluated in a phase 1 comparative trial involving 234 infants (Zorzeto et al. 2009). The safety profiles as well as the levels of *B. pertussis*-specific Th1 immune responses were similar in the groups vaccinated with wP_{low} or with wPv. A clinical development of wP_{low} is currently ongoing. In a similar effort to reduce reactogenicity, outer membrane vesicles (OMVs) with low endotoxic activity were engineered from *B. pertussis* (Asensio et al. 2011). These OMVs contain a large range of potentially protective antigens and have the advantage over purified proteins to be very easy to purify by ultracentrifugation. *B. pertussis* OMVs were shown to protect mice against a nasal challenge infection.

Th1-inducing adjuvants have also been explored to replace alum which biases immune responses to coadministered antigens toward a Th2-type of response. Indeed Yet, protective immunity against *B. pertussis* infection was shown to be mediated at least partially by a Th1-type cellular immune response (Redhead et al. 1993; Feunou et al. 2010). CpG oligodeoxynucleotides are potent inducers of Th1 responses and can overcome the interference of maternal antibodies in animal models when contained in vaccine formulations (Polewicz et al. 2011). Combining CpG oligodeoxynucleotides with additional immunostimulatory molecules, such as polyphosphazenes and cationic innate defense regulator peptides, further increases *B. pertussis*-specific

IgG2a responses, both in adult and in neonatal mice and was shown to induce long-lasting immunity (Gracia et al. 2011).

Mucosal administrations

B. pertussis is a mucosal pathogen which infects its host via the nasal route. It is therefore likely that mucosal immunity may play a role in protection against pertussis (Hellwig et al. 2001). Nevertheless, all current vaccines are administered parenterally.

Oral administration of wPv has been assessed in newborn babies on days 2, 3, 4, and 5 after birth and at 6 weeks (Baumann et al. 1985). Salivary anti-*B. pertussis* IgA, serum IgG, and cellular immune responses were induced. Importantly, oral vaccination provided significant protection against pertussis morbidity in infants. On the other hand, nasal spraying of non-adjuvanted wPv induced *B.pertussis*-specific antibodies in nasal secretions and to a lesser extent in saliva (Berstad et al. 2000a). Serum IgG and IgA against *B. pertussis* antigens as well as specific T cell responses were also detected (Berstad et al. 2000a, b). The antigen-specific T cell proliferation persisted for at least 9 weeks after nasal immunization.

Nasal administration of aPv would need the help of a powerful mucosal adjuvant. Protective efficacy of nasal immunization with pertussis vaccines using genetically detoxified *Escherichia coli* heat labile enterotoxin (LT) as adjuvant has been assessed (Hale et al. 2004). Nevertheless, potential severe adverse events (such as Bell's palsy) associated to the nasal use of LT (Mutch et al. 2004) prevent further clinical development. Chitosan or polyphosphazene can be used in alternative mucosal adjuvant formulation. Nasal administration of these vaccine formulations induced the production of systemic and mucosal immune responses and strong protection against nasal challenge infection when polyphosphazene was used as adjuvant (Jabbal-Gill et al. 1998; Shim et al. 2010).

Live attenuated vaccines

An alternative promising approach to vaccinate against pertussis is to use a live attenuated form of *B. pertussis* delivered nasally to mimic as closely as possible the natural route of infection without causing disease. As early as the 1960s, Huang et al. (1962) came to the following conclusion using primate model: "ultimate protection against whooping cough probably best follows a live *B. pertussis* inoculation." Natural infection is known to result in a long-lasting protection which has been estimated to nearly 30 years on average using mathematical modeling (Wearing and Rohani 2009). In contrast, immunity induced by aPv is relatively short-lived. Recent data from Norway showed that by age 14 over 90 % of pertussis cases occurred in aPv-vaccinated individuals (Lavine et al. 2012). Moreover, natural *B. pertussis* infection results in a strong and long-lasting Th1 response even in very young infants (Mascart et al. 2003). Interestingly and in contrast to current aPv vaccines, *B. pertussis* infection can also protect against *B. parapertussis*, responsible for a milder form of pertussis (Watanabe and Nagai 2001) and whose incidence has been increasing over the last decades.

The first attempts to genetically attenuate *B. pertussis* for use as a live vaccine date back to 1990 when Roberts et al. (1990) developed an *aroA* mutant. The resulting strain was highly attenuated, but its poor efficiency to colonize the respiratory tract of mice required multiple administrations of high doses to obtain significant protection against virulent *B. pertussis* infection.

A better understanding of the specific molecular mechanisms of pertussis pathogenesis (Locht et al. 2001) associated with the availability of the entire genome sequence of *Bordetella* (Parkhill et al. 2003) made it possible to develop a rational approach for genetic attenuation of *B. pertussis*. The vaccine strain called BPZE1 contains genetic alterations that abolish or inactivate three different toxins: PTX, dermonecrotic toxin (DNT), and TCT (Mielcarek et al. 2006). BPZE1 was shown to be highly attenuated, yet able to efficiently colonize the mouse respiratory tract. Importantly, a single intranasal administration of this strain induced full protection against challenge infection with virulent *B. pertussis* in mice, including infant mice. This protection was dose dependent (Mielcarek et al. 2010) and mediated by both antibodies and CD4+ T cells (Feunou et al. 2010a). The protective immunity is long lasting, providing full protection after a single nasal administration for up to at least 1 year (Feunou et al. 2010b; Skerry and Mahon 2011). BPZE1 induces also a very rapid protection which can be detected as early as a few days after vaccination (Debrie et al. in preparation). This early-induced protection might be particularly important to protect newborns during the window of vulnerability, before the administration of the current pertussis vaccines. In addition to *B. pertussis*, BPZE1 vaccination also protects mice against *B. parapertussis* (Mielcarek et al. 2006; Feunou et al. 2010a) and *B. bronchiseptica* infection (Kammoun et al. 2012).

The clinical development of BPZE1 raises important issues such as genetic stability of the mutations and safety in immunocompromised individuals. Genetic stability of BPZE1 was established upon continuous serial passages both in vitro and in vivo in mice for up to 1 year (Feunou et al. 2008). Safety issues in immunocompromised subjects have been studied using SCID and IFNγ-R deficient mice. Virulent *B. pertussis* is not more frequent in AIDS patients than in non-HIV individuals (Cohn et al. 1993). Nevertheless, in severely immunocompromised individuals, the organism can occasionally be isolated from blood (Troseid et al. 2006). This atypical disseminated infection can be mimicked using immunodeficient mice. While virulent *B. pertussis* disseminates to the liver of IFNγ-R-deficient mice resulting in atypical pathology, BPZE1 does not disseminate and does not induce atypical pathology in adult and neonatal mouse models (Skerry et al. 2009). BPZE1 was also shown to prevent allergic pulmonary pathology (Kavanagh et al. 2010) and to present anti-inflammatory properties. This was particularly striking with the evidence that BPZE1 administration protects mice against lethal challenge with influenza viruses by dampening the cytokine storm induced by the viral challenge thereby reducing lung inflammation and tissue damage (Li et al. 2010).

Based on the safety data obtained in preclinical models, BPZE1 was downgraded from a safety level 2 to a safety level 1 organism in several countries. A placebo-controlled,

double-blind, and dose-escalating safety trial was initiated at the end of 2010 (Clinicaltrials.gov ID: NCT01188512), and has now been successfully concluded (Thorstensson et al., in preparation)

Conclusion

Pertussis epidemiology has profoundly changed with the introduction of the different generations of vaccines. However, pertussis still remains a major public health issue especially in very young children, despite the availability of effective vaccines and high vaccine coverage worldwide. The limits of the current strategies could be overcome by developing new approaches, and it is likely that a combination of several innovative strategies will be needed to successfully fight against severe pertussis in the very young. Neonatal vaccination might be one of the key components of this new strategy and could be added to the current routine vaccination schedule as a priming immunization to be boosted later in life with current pertussis vaccines (Locht 2008). Further clinical studies will be needed to evaluate the efficacy of such strategies.

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14 Dysentery

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Abstract
Dysentery is bloody diarrhea caused by infection with certain bacteria or parasites. The most common bacterial causes are members of the Genus *Shigella*. *Shigella* are Gram-negative intracellular bacterial pathogens that cause diarrheal disease by infecting intestinal epithelial cells. Following invasion of intestinal cells, *Shigella* induce host cell cytoskeletal rearrangements and interfere with host cell signal transduction cascades. These effects are mediated by multiple different effector proteins that are translocated from the bacterial cell into the host cell through a type three secretion system. Translocated *Shigella* effector proteins modulate the host immune response, which contributes to inflammation during infection and to clearance of the organism. Antibiotics are available and effective against *Shigella* infection; however, isolates resistant to routine antibiotics are increasingly frequent in many areas of the world. Vaccine development is an ongoing area of research.

Introduction

Dysentery is bloody diarrhea caused by infection with certain bacteria or parasites. The most common bacterial causes are members of the genus *Shigella*, the biology of which is discussed in this chapter. The most common parasitic causes of dysentery are the amoebae.

Shigella spp. are nonmotile Gram-negative, nonspore forming, non-lactose fermenting, facultative anaerobic bacillus-shaped bacterium that are very closely related to *Escherichia coli*. Within the genus *Shigella* are four species (*S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*) and multiple serotypes of each species. *S. dysenteriae* is the most virulent pathogen within this genus; it was first discovered and isolated by the Japanese microbiologist Kiyoshi Shiga in 1898. The epidemiology and pathology of this microbe is of particular clinical significance as *Shigella* is associated with severe diarrheal disease and dysentery in humans. The organism is spread from person to person through contact with contaminated food and water products.

The complete genome of *Shigella* includes a single circular chromosome, one large virulence plasmid, and a variable number of small plasmids, which collectively encode genes for a type three secretion system and multiple virulence factors that enable this pathogen to invade epithelial cells, manipulate the host cytoskeleton, spread through tissue, and modulate the innate immune response.

Taxonomy

The genus *Shigella* is very closely related to the genus *Escherichia*; both belong to the family Enterobacteriaceae. In the current classification, within the genus *Shigella* are four species: *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D). The most virulent of the *Shigella* species, *S. dysenteriae*, was discovered by the Japanese microbiologist Kiyoshi Shiga during a severe outbreak in 1898. Serogroups A and C (*S. flexneri* and *S. sonnei*) are most commonly associated with clinical disease. Within each serogroup are multiple serotypes: serogroup A (*S. dysenteriae*), 12 serotypes; serogroup B (*S. flexneri*), 6 serotypes; serogroup C (*S. boydii*), 23 serotypes; and serogroup D (*S. sonnei*), 1 serotype (*Table 14.1*). The four species share several key

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Table 14.1
Classification of *Shigella* serotypes

Serogroup	Species	No. of serotypes	Phylogenetic cluster	Serotype designation
A	*S. dysenteriae*	13	C1	D3, D4, D5, D6, D9, D11, D12, D13
			C2	D2
			C3	None
			Outliers	D1, D8, D10
B	*S. flexneri*	6	C1	F6
			C2	none
			C3	F1a, F1b, F2a, F2b, F3, F4a, F4b, F5, Fx, & Fy
			Outliers	None
C	*S. boydii*	23	C1	B1, B2, B3, B4, B6, B8, B10, B14, B18
			C2	B5, B7, B9, B11, B15, B16, B17
			C3	B12
			Outliers	B13
D	*S. sonnei*	1	Outlier	SS

Peng et al. (2009), Yang et al. (2007)

features, including lack of motility, inability to form spores, and inability to ferment lactose, and all four species are facultative anaerobes. *Shigella* species are differentiated from one another using a method of serotyping, which is based on antigen type.

Habitat

Humans are the only natural host for *Shigella*. Monkeys and certain small animals can be infected in the laboratory, but are not natural hosts. In most cases, spread of disease from one individual to another occurs via the fecal-oral route, typically via contamination of the hand. However, with increasing frequency, spread involves ingestion of contaminated foodstuffs or contaminated water.

Epidemiology

Humans are the only natural reservoir of *Shigella*. Annually in the United States, *Shigella* are estimated to cause approximately 450,000 cases (Mead et al. 1999), with about four cases per 100,000 population (2009). Annually worldwide, they are estimated to cause 165 million infections (Kotloff et al. 1999). The species most commonly associated with sporadic infections and outbreaks are *S. flexneri* and *S. sonnei*. *S. flexneri* is overall the most common serogroup isolated from clinical infections worldwide. It is most prevalent in the developing world, whereas *S. sonnei* is the most prevalent serogroup in Europe and the United States. *S. flexneri* and *S. sonnei* are associated with endemic forms of the disease, while *S. dysenteriae* serotype 1 is responsible for most epidemics. *S. dysenteriae* infections in North America are most commonly due to serotype 1, whereas in other areas of the world, other serotypes of *S. dysenteriae* have largely replaced serotype 1. Infections due to *Shigella* typically occur in situations of overcrowding or poor hygiene and sanitation, such as day care centers, institutions for the mentally disabled, and cruise ships.

Evolution and Genomics

The genome of *Shigella* consists of a single circular chromosome, a virulence plasmid, and multiple smaller "cryptic" plasmids. The sequence of the entire *Shigella* genome, including the chromosome, the virulence plasmid, and the cryptic plasmids, was completed in the early 2000s by several independent groups of researchers (Jin et al. 2002; Nie et al. 2006; Wei et al. 2003; Yang et al. 2005, Venkatesan et al. 2001, Buchrieser et al. 2000). The genomes of five different strains, representing all four species of *Shigella*, *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*, are currently available (Jin et al. 2002; Nie et al. 2006; Wei et al. 2003; Yang et al. 2005). The circular chromosome of *S. flexneri* is 4.6 Mbp (4,599,354) with a G+C content of 50.9 % and 4,084 predicted genes (Wei et al. 2003). The large virulence plasmid, which is present in all isolates of *Shigella* and is required for virulence, is 0.220 Mbp (220 kbp), while the size and number of additional plasmids vary depending on the isolate.

Several studies have shown high sequence similarity between the genomes of *Shigella* spp. and *Escherichia coli*. Early studies using DNA hybridization revealed that these two microbes are taxonomically indistinguishable (Brenner et al. 1972). More recent studies using multilocus enzyme electrophoresis, comparative genomic hybridization, and multilocus sequence typing have confirmed early predictions of the high sequence similarity between *Shigella* spp. and *E. coli* (Lan and Reeves 2002; Pupo et al. 1997, 2000).

Sequence analysis of eight housekeeping genes in four different regions of the chromosome of multiple species of *Shigella*, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, revealed that *Shigella* spp. evolved from *E. coli* at least 35,000–270,000 years ago. Sequence variation of these eight genes and more extensive sequence analysis of housekeeping genes suggest multiple independent lines of evolution (Pupo et al. 2000; Yang et al. 2007). Based on these studies, it is now well accepted that *Shigella* belongs to the species *E. coli*, instead of belonging to its own separate genus (Schroeder and Hilbi 2008) and that pathogenic strains that are commonly known as *Shigella* spp. emerged from *E. coli* at least seven times during evolution (*Fig. 14.1*).

Comparative genomics studies further substantiate the genetic and taxonomic relationship between *Shigella* and

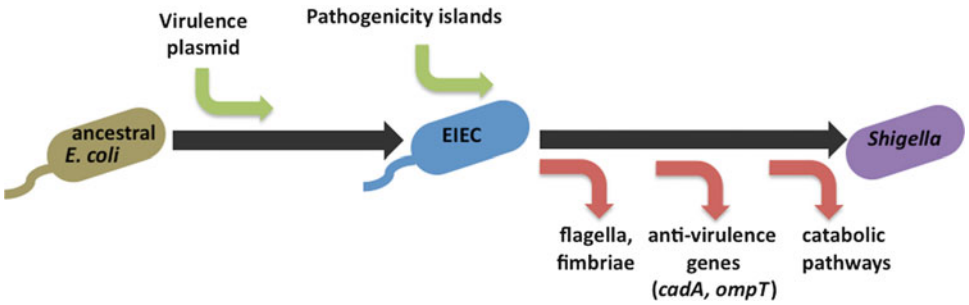

Fig. 14.1

Sequence of evolutionary events that result in the classification of *Shigella* as a distinct genus. Through a series of gene acquisition and gene loss events, *Shigella* acquired a virulence plasmid and multiple pathogenicity islands and lost genes required for flagella and fimbriae synthesis, genes encoding proteins whose activity inhibits virulence (e.g., CadA and OmpT), and genes for catabolic pathways (Adapted from Peng et al. (2009), Schroeder and Hilbi (2008)).

E. coli. The genomes of *Shigella* and *E. coli* are only 1.5 % divergent (Fukushima et al. 2002; Lan and Reeves 2002; Pupo et al. 1997, 2000). The chromosome of *S. flexneri* is slightly smaller (4,599,354 bp) than that of enterohemorrhagic *E. coli* (4,639,221 bp) (Perna et al. 2001; Wei et al. 2003). The overall organization of the two chromosomes is similar, consisting of large regions of backbone with islands. *S. flexneri* has a slighter larger amount of backbone (82 %) than enterohemorrhagic and uropathogenic *E. coli* (75 %) and 200 more pseudogenes than these *E. coli* (Wei et al. 2003).

Shigella spp. are most closely related to enteroinvasive *E. coli* (EIEC) as opposed to other strains of *E. coli* (Lan et al. 2004; Yang et al. 2007). *Shigella* spp. and EIEC evolved from other *E. coli* via convergent evolution (Lan et al. 2004; Pupo et al. 2000) involving multiple events of gene acquisitions, horizontal gene transfer, and genetic loss, through gene deletion (Yang et al. 2007). *Shigella* spp. acquired the large virulence plasmid and five chromosomal pathogenicity islands (SHI-1, SHI-2, SHI-3, SHI-O, and SRL) (Ingersoll et al. 2002; Luck et al. 2001; Ochman et al. 2000; Peng et al. 2009; Purdy and Payne 2001; Rajakumar et al. 1997; Schroeder and Hilbi 2008; Vokes et al. 1999). *Shigella* spp. lost genes for flagella synthesis, rendering the organism nonmotile, and for fimbriae synthesis (Al Mamun et al. 1996; Hacker et al. 1990; Tominaga et al. 2005). *Shigella* spp. also lost the gene encoding the outer membrane protein OmpT, a protease that can cleave the outer membrane protein IcsA (VirG) at the bacterial surface, and acquired on the virulence plasmid the gene encoding a similar yet more highly regulated protease (IcsP, SopA). IcsA, described below, is required for actin polymerization and intracellular spread (Bernardini et al. 1989; Lett et al. 1989). The genes involved in the biosynthesis of cadaverine, the small polyamine product of lysine decarboxylation, were lost during the evolution of both *Shigella* spp. and EIEC from *E. coli* (Casalino et al. 2003; Maurelli et al. 1998). The presence of cadaverine during *Shigella* infection leads to delayed lysis of the phagocytic vacuole by intracellular bacteria, decreased transmigration of polymorphonuclear leukocytes across the infected epithelium, and consequent attenuation of the infection (Fernandez et al. 2001; Maurelli et al. 1998; McCormick et al. 1999). *Shigella* spp. also lost genes for the L-aspartate-dihydroxyacetone and lactose fermentation pathways (Ito et al. 1991; Prunier et al. 2007a, b; Yang et al. 2005).

The chromosomal loci that have been acquired during evolution are designated chromosomal pathogenicity islands SHI-1, SHI-2, SHI-3, SHI-O, and the *Shigella* resistance locus (SRL) (Ingersoll et al. 2002). Pathogenicity islands are large genomic regions that encode virulence factors and are typically characterized by a G+C content and codon usage that are distinct from the chromosome. Acquired by horizontal gene transfer events, pathogenicity islands are often associated with mobile genetic elements and insertion sequences (Dobrindt et al. 2004). SHI-1 encodes the immunoglobulin A-like protease SigA (Al-Hasani et al. 2000), the serine protease Pic (Henderson et al. 1999), and the enterotoxin ShET1 (Fasano et al. 1995, 1997). SHI-2 encodes ShiD and ShiA, which has been shown to interfere with the T-cell immune response during infection (Ingersoll et al. 2003; Ingersoll and Zychlinsky 2006). SHI-2 and SHI-3 encode factors involved in iron acquisition, including the siderophore aerobactin and enterochelin receptors (Luck et al. 2001; Nassif et al. 1987; Purdy and Payne 2001; Vokes et al. 1999). SHI-O, which is present in a subset of strains, contains genes that modify the O-antigen of lipopolysaccharide in ways that contribute to virulence (Huan et al. 1997; Lindberg et al. 1991; Zhong 1999). SRL encodes genes for antibiotic resistance, including tetracycline, chloramphenicol, ampicillin, and streptomycin (Luck et al. 2001; Turner et al. 2001, 2003). Collectively, these genetic acquisition and loss events have lead to the evolution of *Shigella* spp. from *E. coli* as a discrete pathogen adapted to a distinct, predominantly intracellular, lifestyle.

Pathogenesis and Virulence Factors

A key distinguishing feature of *Shigella* is its ability to invade host intestinal epithelial cells. The factors required for invasion are encoded on a large plasmid, known as the "virulence plasmid" or "invasion plasmid," which is present in all virulent

strains (Parsot 2009; Sansonetti et al. 1982). Two adjacent loci on the virulence plasmid confer invasion capabilities: the *mxi-spa* locus, which encodes the structural components of the type three secretion system (T3SS), and the *ipa* (*i*nvasion-related *p*lasmid-encoded *a*ntigens) locus, which encodes multiple different factors, including those required for delivery of effector proteins into host cells, transcriptional regulators, chaperones, and effector proteins (Schroeder and Hilbi 2008). The T3SS is essential for *Shigella* invasion, as plasmid-cured strains and strains carrying disruptions or deletions of any of the T3SS structural genes are unable to invade (Sansonetti et al. 1982). Expression of the genes encoding the structural proteins and of many of the effectors is regulated by two virulence plasmid-encoded transcription activators, VirB and VirF (Le Gall et al. 2005; Schroeder and Hilbi 2008). VirF, a member of the AraC family of transcription activators, activates transcription of *virB* and *icsA* (*virG*) in response to increase in temperature to 37 °C (Hale 1991; Tobe et al. 1993). VirB activates transcription of the T3SS structural proteins and the type three secreted invasion proteins (Porter and Dorman 1997).

The *Shigella* spp. T3SS is a multi-protein apparatus in the bacterial cell envelope that allows for the transport of effector proteins from the bacterial cell cytoplasm across both the bacterial cell envelope and the host epithelial cell plasma membrane into the host epithelial cell cytoplasm (Blocker et al. 2001). The apparatus consists of a gated channel that traverses the inner membrane, the periplasm, and the outer membrane and extends in the form of a long needle into the extracellular space. Activation of secretion is initiated upon contact with host epithelial cells (Enninga et al. 2005). Upon activation, three translocators and 25 or more effector proteins are delivered through the T3SS apparatus into the host epithelial cells (*Table 14.2*) (Enninga et al. 2005; Parsot 2009).

Delivery of proteins through the T3SS occurs in an orderly fashion, with the proteins involved in the formation of a pore in the host cell membrane being delivered first, followed by the effector proteins involved in the entry process, and lastly by the effector proteins that modulate later stages of infection, including those that participate in bacterial intercellular spread and those that manipulate the innate immune response. The genes encoding the proteins that are secreted early, including IpaB, IpaC, and IpaD, which are involved in pore formation, IpaA and IpgB1, which participate in entry, and IcsB, which functions in avoidance of autophagy, along with their chaperones, are transcribed independent of MxiE (see below) (Le Gall et al. 2005; Parsot 2009). Consequently, they are preformed in the bacterial cell and, upon contact with the host cell, are ready to be secreted.

A second group of proteins secreted by the T3SS are those that modulate later stages of infection. As a rule, transcription of these effectors is dependent on MxiE, an AraC family transcription activator encoded within the T3SS locus, whose transcription is regulated by VirB. Transcription by MxiE is intricately co-regulated by IpgC, the chaperone for the translocases IpaB and IpaC. Prior to host cell contact, IpgC is bound to IpaB and IpaC, preventing their premature association (Menard et al. 1994). Upon contact, IpaB and IpaC are dissociated from IpgC in the bacterial cytoplasm and are secreted, whereupon they interact with each other to form a pore in the host plasma membrane (Menard et al. 1994). Concurrently, IpgC becomes available to serve as co-activator of MxiE-mediated transcription (Mavris et al. 2002a, b). Prior to MxiE-IpgC assembly, MxiE is in complex with OspD1 and the chaperone Spa15, which effectively inhibit the activity of MxiE (Page et al. 2002; Parsot et al. 2005). Four effectors secreted by the T3SS, VirA, OspB, OspC1, and OspF, are partially regulated by MxiE-mediated transcriptional activation (Parsot 2009).

In addition to the transcriptional regulation described above, Spa32 negatively regulates the length of the T3SS apparatus needle that extends from the bacterial surface and controls the selection of substrates for secretion, whereas Spa33 regulates Ipa protein secretion (Magdalena et al. 2002; Schuch and Maurelli 2001). Under anaerobic conditions, such as those present in the lumen of the human intestine, transcription of *spa32* and *spa33* are repressed by FNR, a conserved regulator of anaerobic metabolism, and the needles become long (Marteyn et al. 2010). Close to the epithelial surface, however, oxygen tension increases sufficiently to de-repress transcription of *spa32* and *spa33*, triggering molecular events necessary for cellular entry (Marteyn et al. 2010).

The best-described functions of IpaB, IpaC, and IpaD are as translocators that form a pore in the host plasma membrane (Menard et al. 1994). In addition, they are thought to possibly gate the pore and anchor the T3SS needle to the plasma membrane. IpaB and IpaC are positioned at the tip of the T3SS apparatus from where they integrate into cholesterol-rich domains of the plasma membrane (De Geyter et al. 1997; Harrington et al. 2006; Lafont et al. 2002), creating a pore and a conduit between the bacterial and host cells that allows for the subsequent delivery of effector proteins (Blocker et al. 1999; Espina et al. 2006; Menard et al. 1993, 1994; Veenendaal et al. 2007). IpgD is also positioned at the tip of the T3SS apparatus, where in addition to providing scaffolding, it also regulates secretion (Picking et al. 2005; Schroeder and Hilbi 2008). IpaD is anchored directly to MxiH, the protein that forms the needle of the T3SS apparatus (Zhang et al. 2007). The precise signals responsible for activating secretion and the molecular mechanisms by which these signals trigger the assembly of the T3SS apparatus tip upon epithelial cell contact are unknown and yet are active areas of research. In addition to their roles as translocators, IpaB and IpaC have also been shown to have effector-like activities.

The early effector IpaA causes localized actin depolymerization at the sites of bacterial invasion, leading to enhanced bacterial uptake (Bourdet-Sicard et al. 1999). The effects of IpaA activity are mediated by its direct interaction with the host actin cytoskeletal protein vinculin. The C-terminal domain of IpaA binds to the amino-terminal head domain of vinculin (Bourdet-Sicard et al. 1999; Demali et al. 2006; Ramarao et al. 2007; Tran Van Nhieu et al. 1997). Independent of its interaction with vinculin, IpaA induces weakening of cellular adhesion to the extracellular matrix (Demali et al. 2006). Spa15 serves as a chaperone for IpaA (Page et al. 2002).

Table 14.2
Known functions of *Shigella* virulence proteins

Effector	Expression	Function
IpaB	Early effector; not regulated by MxiE	Translocator; essential for pore formation and delivery of effectors; integrates into cholesterol-rich domains of the plasma membrane
IpaC	Early effector; not regulated by MxiE	Translocator; essential for pore formation and delivery of effectors; integrates into cholesterol-rich domains of the plasma membrane
IpaD	Early effector; not regulated by MxiE	Translocator; essential for pore formation and delivery of effectors; provides scaffolding and regulates secretion; anchored to MxiH
IpaA	Early effector; not regulated by MxiE	Involved in entry into host epithelial cells; causes localized actin depolymerization; interacts with vinculin; weakens integrin interactions with extracellular matrix
IpgB1	Early effector; not regulated by MxiE	Involved in entry into host epithelial cells; causes membrane ruffling; serves as a GTP exchange factor (GEF) for the Rho GTPase RhoG; interacts with ELMO-Dock180, which results in activation of Rac1 and Cdc42
IpgB2	Not regulated by MxiE	Homologue of IpgB1; serves as a GEF for the Rho GTPase RhoA
IcsA	Activated by VirF	Required for actin tail polymerization via N-WASP-Toca-1-Arp2/3; activates autophagy through Atg5
IcsB	Early effector; not regulated by MxiE	Evasion of autophagy through Atg5
MxiE		AraC family transcriptional activator; regulated by VirB and IpgC
IpgC		Transcriptional co-activator of MxiE; chaperone for IpaB and IpaC
Spa15		Chaperone for multiple effectors
OspD1	Not regulated by MxiE	MxiE anti-activator; function in host cell unknown
VirA	Partially regulated by MxiE	Function in host cell unknown
OspB	Partially regulated by MxiE	Function in host cell unknown
OspC1	Partially regulated by MxiE	Function in host cell unknown
Spa32		Negatively regulates the length of the T3SS apparatus needle Spa33
Spa33		Regulates Ipa protein secretion
MxiH		Constitutes the needle of the T3SS apparatus
IpgD	Early effector; not regulated by MxiE	An inositol phosphatase; mediates the dephosphorylation of PI-(4,5)P_2 into PI-(5)P, which leads to PI3-kinase activation of Akt that results in decreased lysosomal degradation and increased host cell survival
OspC2 OspC3 OspC4		Function in host cell unknown
OspE1		Interact with integrin-linked kinases (ILKs) to stabilize cell adhesion to the substratum
OpsE2		
OspD2	OspD2 is not regulated by MxiE	Function in host cell unknown
OspD3		
IpaH family		E3 ligases
OspF	Partially regulated by MxiE	Phosphothreonine lyase activity; dephosphorylates and inhibits MAPK signaling
OpsZ		Inhibits NF-kB activity
OspG		Protein kinase whose function leads to inhibition of NF-κB activation

The early effector protein IpgB1 activates a cellular pathway that induces formation of membrane ruffles, likely by serving as a GTP exchange factor (GEF) for the Rho GTPase RhoG. Like RhoG, IpgB1 interacts with the cellular protein complex ELMO-Dock180, which results in activation of the actin nucleation-promoting factors Rac1 and Cdc42 and actin-mediated formation of membrane ruffles (Handa et al. 2007; Ohya et al. 2005). IpgB2, a homologue of IpgB1, serves as a GEF for the Rho

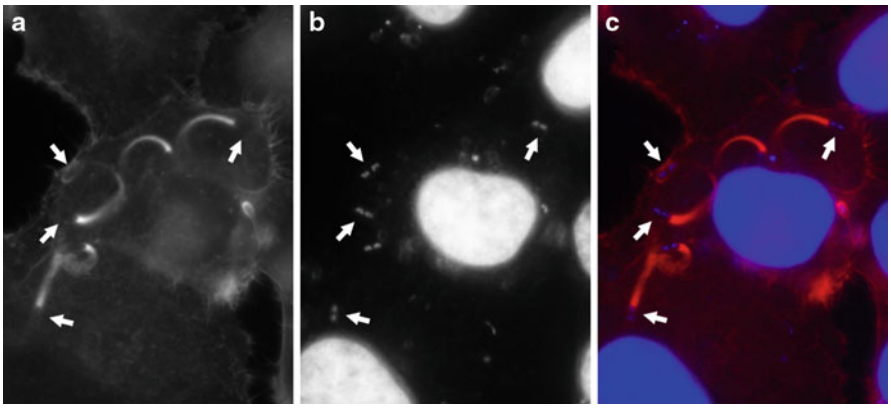

Fig. 14.2
Actin tail assembly by *Shigella* during infection. (a) Polymerized actin; (b) bacterial and cellular DNA; (c) overlay of polymerized actin (*red*) and DNA staining (*blue*). *Arrows*, bacteria at the tip of actin tails

GTPase RhoA (Klink et al. 2010) and activates the immune modulator NF-κB (Fukazawa et al. 2008), but its molecular function in *Shigella* pathogenesis remains uncertain. OspB also activates NF-κB by a mechanism that is unclear (Fukazawa et al. 2008).

IpgD, an early type three secreted effector, is an inositol phosphatase that promotes membrane ruffling during bacterial entry and alters cellular survival and lysosomal degradation pathways (Niebuhr et al. 2000; Pendaries et al. 2006; Ramel et al. 2011). IpgD specifically mediates the dephosphorylation of phosphatidylinositol 4,5-bisphosphate (PI-(4,5)P_2) to yield phosphatidylinositol 5-monophosphate (PI-(5)P) (Niebuhr et al. 2002). IpgD-induced formation of PI-(5)P results in activation of the PI3-kinase signaling pathway that leads to Akt phosphorylation in a manner that depends on the epidermal growth factor receptor (EGFR), which modulates endosomal trafficking (Pendaries et al. 2006; Ramel et al. 2011). Increased levels of PI-(5)P lead to decreased lysosomal degradation and increased host cell survival (Pendaries et al. 2006; Ramel et al. 2011).

Following *Shigella* uptake into host epithelial cells, a second wave of effectors is secreted via the T3SS into the *Shigella*-containing vacuole and, after vacuolar lysis, into the cell cytoplasm. Some of these effectors are important for pathogen survival, others are essential for *Shigella* dissemination through the epithelial cell layer, and others modulate the host immune response. Finally, the functions of other effectors are less well defined.

Shigella effectors that mediate lysis of the *Shigella*-containing vacuole are unknown. Early work suggested that IpaB is involved in this process (High et al. 1992), but given what has been learned since about the role of IpaB in secretion of other effectors, the mechanism of its involvement in vacuolar lysis is unclear. Lysis of the vacuole releases the bacterium into the cell cytoplasm, where it utilizes the cellular actin polymerization machinery to move. The bacterium polymerizes actin into a tail at one end of the bacterial body (*Fig. 14.2*). Recruitment of the actin polymerization machinery to the bacterium depends on the *Shigella* outer membrane protein IcsA (VirG) (Bernardini et al. 1989; Lett et al. 1989), which is a member of the autotransporter family of proteins and is not secreted by the T3SS. IcsA binds the cellular actin nucleation-promoting factor N-WASP, and N-WASP is activated by the cellular protein Toca-1, whereupon it recruits and activates the actin polymerizing complex Arp2/3 (Leung et al. 2008; Lommel et al. 2001; Snapper et al. 2001; Suzuki et al. 1998). Polymerization of the tail propels the bacterium to the cell periphery, whereupon through processes that are incompletely understood, it utilizes diaphanous formins to generate protrusions of the plasma membrane that enclose the bacterium (Heindl et al. 2010). Bacterium-containing protrusions are engulfed by adjacent cells, leading to spread of the bacterium into these cells.

In addition to recruiting actin polymerization machinery, IcsA can be recognized by the cellular autophagy protein Atg5. Atg5 recognition activates the autophagosome formation pathway. Autophagy is a cellular pathway that engulfs foreign objects present in the cytoplasm, such as intracellular bacteria, and kills and degrades them. The type three secreted effector IcsB shares the same binding region and has higher affinity than Atg5 for IcsA, such that IcsB binding to IcsA masks Atg5 recognition and allows the bacterium to escape detection and destruction via autophagy (Ogawa and Sasakawa 2006; Ogawa et al. 2005).

Shigella spp. encode two copies of the type three secreted effectors OspE, OspE1, and OspE2, which have nearly identical protein sequences. OspE proteins interact with integrin-linked kinase (ILK) within sites of cellular attachment to the extracellular matrix, causing stabilization of these attachment sites and preventing cell release from the substratum during infection (Kim et al. 2009).

The type three secreted effector VirA is homologous and structurally similar and can partially functionally complement EspG, a type three secreted effector of enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) (Davis et al. 2008; Elliott et al. 2001; Germane and Spiller 2011; Selyunin et al. 2011). EspG regulates endomembrane trafficking through interactions with ADP-ribosylation factor GTPases and

p21-activated kinases (Germane and Spiller 2011; Selyunin et al. 2011). However, the specific function of VirA remains uncertain, as a possible role in endomembrane trafficking has not been examined and data indicating a role in microtubule destabilization and protease activity are conflicting (Germane et al. 2008; Yoshida et al. 2006).

Among the type three secreted effectors of *Shigella* spp. is a family of 5–7 effector proteins designated IpaH, including some encoded on the *Shigella* chromosome (Ashida et al. 2007). IpaH proteins contain a conserved C-terminal domain and a variable N-terminal domain. The C-terminal domains function as E3 ligases (Rhode et al. 2007; Singer et al. 2008; Zhu et al. 2008), proteins that target specific substrates for degradation via the cellular ubiquitination pathway. The N-terminal domain is the site of a leucine-rich repeat domain, which is a classical pathogen-associated molecular pattern recognition site involved in the host epithelial cell immune response during pathogen infection (Bell et al. 2003; Hartman et al. 1990; Okuda et al. 2005; Parsot 2009; Venkatesan et al. 1991). The substrate specificity of the IpaH proteins is determined by the N-terminal domain and has been identified for only one, IpaH9.8, which targets NEMO/IKKγ, a host inflammatory response modulator, for degradation, thereby dampening the NF-κB regulated inflammatory response (Ashida et al. 2010). In the same pathway, OspG, a type three effector protein that is not a member of the IpaH family, binds ubiquitinated ubiquitin-conjugating enzymes, thereby preventing the degradation of the NF-kB inhibitor IκBα (Kim et al. 2005).

Two other type three secreted effector proteins involved in modulation of the host inflammatory response are OspF and OspZ. OspF possesses phosphothreonine lyase activity, an unusual enzymatic activity. It irreversibly dephosphorylates components of the mitogen-activated protein kinase (MAPK) signaling pathway, leading to inhibition of this pathway (Arbibe et al. 2007; Kramer et al. 2007). OspZ inhibits the nuclear translocation of NF-kB (Newton et al. 2010). As for the activity of IpaH9.8 and OspG, the activities of OspF and OspZ attenuate the host inflammatory response. The functions of several type three effectors, including OspC1, OspC2, OspC3, OspD2, and OspD3, are currently unknown (*Table 14.2*).

Shigella and the Immune Response

Shigella infection is generally restricted to the mucosal layer of the large intestine. The organism is able to survive the environment of the stomach due to acid resistance mechanisms (Gorden and Small 1993). Once at the epithelial lining of the large intestine, *Shigella* may be taken up by M-cells, which are specialized in gut-lumen sampling; uptake by M-cells leads to transcytosis of the bacteria across the epithelial layer (Sansonetti et al. 1996; Wassef et al. 1989). Transcytosis enables *Shigella* to enter the epithelial cell lining at the basolateral surface, instead of at the apical surface, and it also enables bacterial interactions with macrophages and dendritic cells within the mucosa (Mounier et al. 1992; Sansonetti et al. 1999). *Shigella* may also enter cells by disrupting epithelial intercellular junctions (Perdomo et al. 1994a; Sakaguchi et al. 2002). Whether organisms also enter cells from the apical side of the epithelium in vivo is uncertain.

When phagocytosed by macrophages, *Shigella* evades killing by triggering apoptosis, which is accompanied by the release of massive amounts of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (Islam et al. 1997; Sansonetti et al. 2000; Zychlinsky et al. 1992; Zychlinsky et al. 1996). Release of IL-1β triggers intestinal inflammation (Sansonetti et al. 1995). Release of IL-18 is associated with an antimicrobial response that involves NK (natural killer) cell activation and the production of interferon (IFN)-γ, which is critical for mounting an innate immune response against microbial infection (Hilbi et al. 1997; Le-Barillec et al. 2005; Sansonetti et al. 2000; Way et al. 1998).

Shigella enters the intestinal epithelium by the basolateral surface of cells (Sansonetti et al. 1986). Following entry, internalized bacteria escape the uptake vacuole, replicate within the cytoplasm, and utilize the host cytoskeleton to move to the cell periphery and into adjacent cells. All eukaryotic cells possess mechanisms for eliminating intracellular foreign bodies, including autophagy and activation of the innate immune response. *Shigella* has evolved mechanisms to evade each of these host responses.

Eukaryotic cells possess a lysosomal degradation pathway called autophagy that serves both to recover nutrients during periods of starvation and to rid the cell of undesirable particles, including invading pathogens. Upon entry into cells, *Shigella* is surrounded by a vacuolar membrane, which it rapidly lyses. The remnants of the vacuolar membrane are degraded by the autophagy pathway (Dupont et al. 2009). Then, a tug-of-war is staged between the intracytoplasmic bacteria and the innate immune response, in which some of the intracytoplasmic bacteria succumb to autophagy and some escape. Those that are destined to succumb are ubiquitinated and may be surrounded by a scaffold of the cytoskeletal protein septin, before being engulfed in cellular membranes and degraded (Mostowy et al. 2010; Ogawa et al. 2005). Escape from autophagy is mediated at least in part by the type three secreted effector protein IcsB, which blocks binding of the autophagy protein Atg5 to the surface of *Shigella* (Ogawa et al. 2005).

Peptidoglycan fragments from intracellular bacteria are sensed by the pattern recognition receptor Nod1, the activation of which results in NF-kB activation and subsequent release of the pro-inflammatory cytokine IL-8 (Girardin et al. 2003; Pedron et al. 2003; Philpott et al. 2000; Sansonetti et al. 1999). IL-8 is responsible for the recruitment of polymorphonuclear leukocytes (PMNs) to the sites of *Shigella* infection (Sansonetti et al. 2000; Singer and Sansonetti 2004). Infiltrating PMNs entrap and kill invading bacteria but also contribute to the destruction of the epithelial cell lining, which further enables the entry and invasion of more *Shigella* into intestinal epithelial cells (Perdomo et al. 1994a; Perdomo et al. 1994b). The ability of PMNs to destroy invading bacteria contributes to the resolution of infection (Brinkmann et al. 2004; Mandic-Mulec et al. 1997; Zhang et al. 2001).

The adaptive immune response to *Shigella* provides partial protection against subsequent infection (Taylor et al. 1989). Particularly surprising is the observation that although *Shigella* are intracellular pathogens, individuals who have been infected are protected in a serotype-specific manner (Ferreccio et al. 1991; Lerman et al. 1994; Mel et al. 1965, 1968, 1971), suggesting that protection is mediated by the humoral immune response and not the cellular immune response. In animal models, serotype-specific IgA can provide protection (Phalipon et al. 1995), yet is not required for protective immunity (Way et al. 1999), suggesting that IgG is the protective isotype. Whereas it is known that *Shigella* blocks aspects of the adaptive cellular immune response (Jehl et al. 2011), how it does so is unclear.

Clinical Disease Due to *Shigella* spp

Shigella causes diarrhea and dysentery, a diarrheal syndrome characterized by blood and white blood cells in the stool. *Shigella* is a human pathogen, with no reservoir in other animals. In the majority of cases, the organism is acquired from an infected individual by direct human-to-human spread. In other cases, the organism is acquired from food or water that has been contaminated by an infected individual. The incubation period averages 3 days, with a range of 1–7 days.

The infectious inoculum is as few as 10–100 bacteria (Dupont et al. 1989), in large part because the organism is relatively resistant to stomach acid, such that even when only a small number of organisms are ingested, a sufficient number gain access to the intestine, where they replicate and cause disease. As a consequence, outbreaks are common in day care centers, mental institutions, and other settings where housing is crowded or hygiene suboptimal, and secondary infection rates among family members are reported as high as 20 %.

Shigella infect intestinal epithelial cells of the sigmoid colon and rectum, the distal most segments of the colon. The organism is thought to enter the epithelium largely by transcytosis of microfold (M) cells, whose normal function is to sample antigens from the intestinal lumen. Following transcytosis to the subepithelium, *Shigella* enter into the epithelial cells using a type three secretion system apparatus (T3SS). Once within the cells, the organism spreads into adjacent cells. The release of pro-inflammatory cytokines leads to an acute inflammatory cell infiltrate. The combination of bacterial spread through the epithelium and the inflammatory response leads to local destruction of the epithelium with ulceration and abscess formation and, in many cases, blood and white blood cells in the stool. Symptoms characteristically include severe abdominal cramping, rectal urgency (tenesmus), frequent small loose stools, general malaise, and fever. In the absence of antibiotic therapy, the diarrhea is typically self-limited and resolves in 7 or fewer days.

Significant complications are uncommon. Approximately 4 % of infected individuals will have transient seeding of the bloodstream (bacteremia), 2.5 % will experience obstruction of the intestine, and a small percentage of children will develop rectal prolapse or seizures. In infection due to *S. dysenteriae* 1, 3 % of individuals will develop a severe dilatation of the colon, called toxic megacolon, which is treated with surgery.

Two uncommon, yet important, complications of *Shigella* infection are post-infectious arthritis (formerly Reiter syndrome) and hemolytic-uremic syndrome. Occurring in a small percentage of cases, post-infectious arthritis develops 1–2 weeks after the diarrhea and may be accompanied by conjunctivitis and painful urination (urethritis). Seventy percent of patients who develop this syndrome have the haplotype HLA-B27. Post-infectious arthritis can occur following infection with any of several enteric and urethral bacterial pathogens, including *Campylobacter*, *Salmonella*, and *Yersinia* spp.

Hemolytic-uremic syndrome is a potentially life-threatening complication that is characterized by the combination of anemia due to hemolysis of red blood cells, decreased platelets, and kidney failure due to injury of the renal glomeruli. Most commonly affected are children under the age of 5, with 5–25 % suffering from some degree of permanent kidney dysfunction. The damage is mediated by Stx toxin (formerly Shiga toxin), which among *Shigella* spp. is encoded only by *S. dysenteriae* 1.

Treatment with antibiotics is recommended for all individuals infected with *Shigella*. Prognosis is excellent, with nearly all individuals recovering fully. No *Shigella* vaccines are currently approved for use, although both live attenuated vaccines and subunit vaccines that combine purified protein and lipopolysaccharide (LPS) are under development. Prevention of spread of *Shigella* depends on meticulous hand hygiene.

Laboratory Identification, Isolation, and Clinical Diagnosis of *Shigella* Infection

The diagnosis of *Shigella* infection is made by culture of the organism from stool samples. *Shigella* can be isolated from stool of infected individuals. In approximately 4 % of infections, *Shigella* can also be isolated from the bloodstream. Organisms cannot be isolated from other body sites. Stool is plated both on nonselective indicator media and on selective media. The nonselective media is typically MacConkey agar, on which *Shigella* spp. grow as white colonies and *E. coli* grow as red colonies (Fig. 14.3a). Selective media include *Salmonella Shigella* agar, which, as the name suggests, is selective for growth of *Salmonella* and *Shigella* spp., and Hektoen enteric agar, which is both selective for growth of *Salmonella* and *Shigella* spp. and differentiates between the two on the basis of the appearance of the colonies. On Hektoen, *Salmonella* spp. grow as black colonies because they produce hydrogen sulfite, whereas *Shigella* spp. grow as green colonies because they do not (Fig. 14.3b). To maximize the likelihood of recovering the organism from stool, it is generally advised to initially plate it on MacConkey agar (nonselective media) and then re-streak lactose negative colonies onto selective media. Colonies are convex, with smooth edges, and translucent, with a typical diameter of 0.5–2.0 mm, and on MacConkey are white in color.

Confirmation of the genus as *Shigella* is performed using biochemical tests. The organism is oxidase negative, catalase

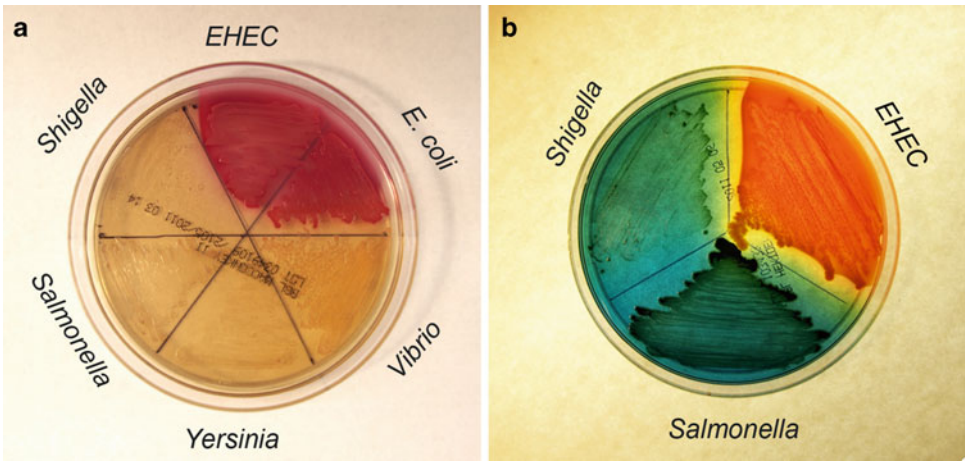

Fig. 14.3
Growth of enteric pathogens on selective agar. (a) Enteric pathogens were grown on MacConkey agar. Lactose non-fermentors, including *Shigella*, form white colonies, and lactose fermentors, such as *E. coli*, form pink colonies. EHEC, enterohemorrhagic *E. coli*. (b) On Hektoen enteric agar, *Salmonella* form *black* colonies due to production of hydrogen sulfite (H_2S), *Shigella* form green colonies, and lactose-fermenting bacteria, such as EHEC, form orange colonies

positive, Voges-Proskauer and Simmons citrate negative, lysine decarboxylase negative, arginine dihydrolase negative, and variable for indole production and ornithine reaction. It does not produce hydrogen sulfite, does not hydrolyze urea, does not utilize malonate, and does not grow on potassium cyanide (KCN) agar. *Shigella* spp. ferment glucose, but do not ferment lactose. *S. dysenteriae* can be discriminated from the other species of *Shigella* by its inability to ferment mannitol, and *S. sonnei* can be discriminated from the others by its ability to produce ornithine decarboxylase. Most clinical microbiology laboratories determine the species of a *Shigella* isolate by O-antigen typing (serotyping) using O-antigen specific antisera.

Treatment and Vaccine Development

All individuals infected with *Shigella* should receive a course of antibiotics. Agents that are recommended include the fluoroquinolones (e.g., ciprofloxacin), azithromycin, or trimethoprim-sulfamethoxazole. Resistance to ciprofloxacin, ampicillin, and trimethoprim-sulfamethoxazole is increasing worldwide, so whenever possible, the selection of an antibiotic should be based on laboratory susceptibility data. If left untreated, *Shigella* infection will resolve over 5–7 days. Antibiotic treatment has been shown to shorten the duration of illness by a couple of days (Christopher et al. 2010).

At present, no vaccine for *Shigella* is approved for use in the United States. Several distinct types of vaccines are being developed, including subunit vaccines, live attenuated vaccines, and outer membrane vesicle vaccines. The subunit vaccines under development consist of various combinations of purified IpaB and IpaC, translocases of the type three secretion system, purified IpaD, the type three secretion system needle tip, and purified lipopolysaccharide (LPS) (Martinez-Becerra et al. 2011; Riddle et al. 2011). The live attenuated vaccines under development carry deletions in genes involved in intercellular motility (*icsA*), the toxins ShET2-1 and ShET2-2 (*senA*, *senB*), acetylation of LPS (*msbB1*, *msbB2*), and guanine biosynthesis (*guaBA*) (Barnoy et al. 2010, 2011; Ranallo et al. 2010; Wu et al. 2011). Outer membrane vesicle vaccines consist of outer membrane vesicles purified from virulence strains (Camacho et al. 2011).

Conclusion

Shigella is a Gram-negative intracellular bacterial pathogen that causes diarrheal disease by infecting intestinal epithelial cells. Following invasion of intestinal cells, *Shigella* induces host cell cytoskeletal rearrangements and interferes with host cell signal transduction cascades. These effects are mediated by multiple different effector proteins that are translocated from the bacterial cell into the host cell through a type three secretion system. Translocated *Shigella* effector proteins modulate the host immune response, which contributes to inflammation during infection and to clearance of the organism. Antibiotics are available and effective against *Shigella* infection; however, isolates resistant to routine antibiotics are increasingly frequent in many areas of the world. Vaccine development is an ongoing area of research.

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15 Urinary Tract Infections

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Abstract
Urinary tract infections (UTIs) are among the most common bacterial infections and generally occur when uropathogens, normally quiescent residents of the gastrointestinal tract, enter the urinary tract. Cystitis, caused by uropathogens that colonize the lower urinary tract, is usually self-limiting and amenable to antibiotic therapy. In spite of this, uropathogens can gain access to the upper urinary tract, causing pyelonephritis, and may enter the bloodstream from this site, causing potentially fatal urosepsis. The vast majority of UTIs are caused by uropathogenic *Escherichia coli* (UPEC), a heterogenous group of *E. coli* strains. Other organisms that cause UTIs include *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Staphylococcus saprophyticus*. All of these organisms have a variety of virulence factors, often encoded on large, horizontally acquired pathogenicity islands, which promote growth in urine, colonization of the host urinary tract, and evasion of the host immune system. These virulence factors include a plethora of distinct fimbrial and non-fimbrial adhesins, flagella, ureases, osmolarity and pH homeostasis factors, nutrient transporters, extracellular polysaccharides, metal scavenging systems, an assortment of toxins, an array of bacteriocins, and envelope damage response systems. Uropathogens also have sophisticated genetic regulatory mechanisms to coordinate expression of these virulence factors. This is especially true between diverse fimbrial operons and between fimbrial and flagella operons. However, no one preeminent set of virulence factors exists among uropathogens, which instead use combinations of the aforementioned virulence factors to facilitate uropathogenesis. Thus, a broad understanding of these virulence factors is necessary to gain a comprehensive understanding of uropathogenesis and UTIs.

Introduction

The urinary tract consists of kidneys and ureters, referred to as the upper urinary tract, and bladder and urethra, referred to as the lower urinary tract. The kidneys filter blood and produce urine, which is transmitted via the ureters to the bladder. The bladder stores urine until it can be released from the body via the urethra (Moore et al. 2010). Urinary tract infections (UTIs) occur when the upper or lower urinary tract is colonized by pathogenic bacteria, typically when a reservoir of a bacterial pathogen residing within the gastrointestinal tract (Hooton 2001; Schilling et al. 2002; Czaja et al. 2009) gains access to the urethra and ascends to the bladder (Bacheller and Bernstein 1997). Lower UTI, also called cystitis, is usually self-limiting (Gupta et al. 2011) and rarely leads to severe complications. Upper UTI, also called pyelonephritis, attends greater severity and can lead to renal dysfunction, bacteremia, or potentially fatal sepsis (Ikaheimo et al. 1994; Faro and Fenner 1998). Access of bacteria to the bloodstream is facilitated because there are only two layers of cells between the kidney tubules and host blood supply.

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Clinical diagnosis of bacterial UTI is frequently based on signs, symptoms, and analysis of urine. Symptoms of lower UTI may include urinary urgency and pain associated with voiding. However, UTI can be asymptomatic in certain patients, either due to impaired sensory apparatus or due to infection with a strain of bacteria that causes asymptomatic bacteriuria (ABU). Signs of lower UTI may include frequent urination and altered urine characteristics, such as odor, color, or visible texture due to presence of blood or high concentrations of bacteria (Bent et al. 2002). The signs and symptoms of UTI can vary; therefore, diagnosis is often heavily dependent on urinalysis or urine culture, and a diagnosis of UTI can be made based on the presence of $\geq 10^3$ bacteria/ml in a midstream clean-catch urine sample from a patient (Faro and Fenner 1998; Warren et al. 1999). The most common cause of upper UTI is lower UTI (Bacheller and Bernstein 1997). Upper UTI can present with all of the above signs, symptoms, and lab values but additionally presents with flank pain or costovertebral tenderness, fever, chills, nausea, and vomiting (Fairley et al. 1971) as well as renal function impairment.

Microscopically, urine from a patient with UTI may reveal leukocytes and bacteria, and renal tubular casts consisting of cellular debris and leukocytes are diagnostic for upper urinary tract involvement (Stamm 1983). Other laboratory values may indicate reduced ability of the kidneys to concentrate urine and general systemic inflammatory markers, including elevated leukocytes in the bloodstream, elevated erythrocyte sedimentation rate, and elevated C-reactive protein (Kunin 1997). Histologic examination of the urinary tract during pyelonephritis may reveal polymorphonuclear leukocytes (PMNs, also called neutrophils) extending from the renal papillae to the cortex, tubules filled with PMNs, and necrosis of proximal tubular epithelial cells. Glomeruli are frequently spared, even when renal inflammation is intense (Heptinstall 1983). The infection may spread beyond the kidneys, and bacteremia develops secondarily in 12 % of cases of pyelonephritis (Ikaheimo et al. 1994; Faro and Fenner 1998).

There are two broad categories of UTI, complicated and uncomplicated. Uncomplicated UTI occurs in the setting of

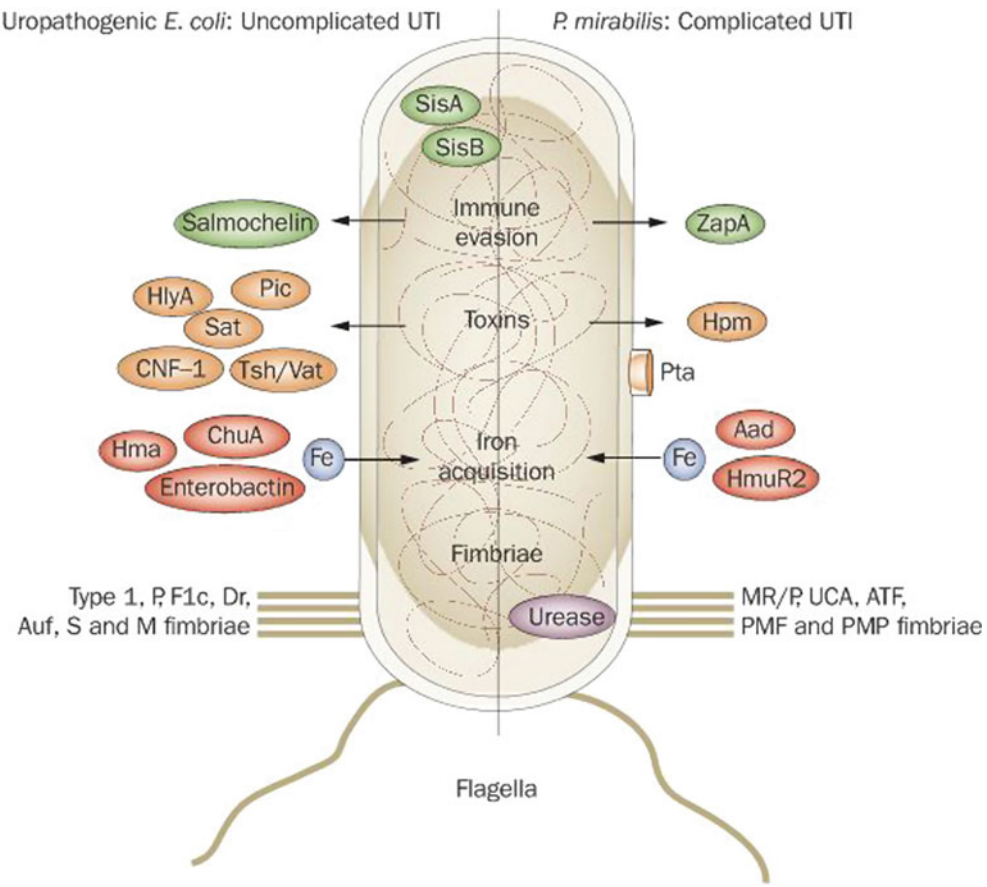

Fig. 15.1

***Overview of uropathogen virulence factors*. The *left-hand side* of this schematic bacterial cell represents uropathogenic *Escherichia coli*, and the right-hand side represents *Proteus mirabilis*. Proteins that contribute to each mechanism of pathogenesis—immune evasion, toxin production, iron acquisition, adherence, and motility—are highlighted for each pathogen. Some strains of uropathogenic *E. coli* strains encode the proteins SisA and SisB, which suppress the host inflammatory response during early stages of infection. *P. mirabilis* produces urease, which plays a role in the formation of urinary stones (Reprinted with permission from Nielubowicz and Mobley (2010))**

a healthy, nonpregnant adult woman with no urinary tract abnormalities, such as anatomical defects, presence of devices, or obstructions (Bacheller and Bernstein 1997). The etiology of complicated urinary tract infection is diverse and expansively broad and beyond the scope of a single review. Uncomplicated urinary tract infections are most commonly caused by four bacterial species. *Escherichia coli*, specifically uropathogenic *E. coli* (UPEC), cause up to 75–95 % of all uncomplicated UTI. *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Staphylococcus saprophyticus* represent the plurality of the remaining etiologic agents of UTI. After *E. coli*, *S. saprophyticus* is the leading cause of urinary tract infections in young sexually active women (Gillespie et al. 1978; Wallmark et al. 1978; Jordan et al. 1980; Podschun and Ullmann 1998; Echols et al. 1999; Gupta et al. 1999; Ronald 2003; Raz et al. 2005; Czaja et al. 2007; Lin et al. 2010). Other species cause sporadic infections.

UTI-causing bacteria are studied using a combination of basic microbial techniques, murine, primate, and canid animal models (Johnson and Russell 1996), and epidemiological and molecular analysis of samples derived from patients. Uropathogenic bacteria all make use of specialized virulence traits to cause UTI, employing adherence organelles to resist expulsion from the host, motility apparatuses to ascend the urinary tract, specialized nutrient scavenging systems and osmolarity regulatory systems to support growth in urine, toxins to destroy, invade, or liberate nutrients from host cells, enzymes to modify the environment of the urinary tract, and immune-evasion properties such as the encoding of capsule to resist phagocytosis and host killing (Norinder et al. 2012) (*Fig. 15.1*).

Adherence

UPEC strains can encode up to 15 epidemiologically and genetically distinct clusters of fimbriae, organelles primarily implicated in adherence; individual strains have been observed to encode up to 11 distinct fimbriae. Fimbriae project from the surface of bacteria and can be directly observed by transmission electron microscopy (*Fig. 15.2b*). Recent studies of fimbriae in UPEC support the model that adherence is an important component in UTI pathogenesis (Spurbeck et al. 2011). In this study, the genomes of 303 *E. coli* were analyzed by multiplex PCR to assay for the presence of fimbrial operons. A bimodal distribution of fimbriae is encoded by uropathogenic and nonpathogenic *E. coli*: UPEC strains carry a high number and diverse array of fimbrial operons, while nonpathogenic *E. coli* tend to carry only a few. This suggests that acquiring or encoding a diverse array of fimbriae is an important characteristic of UPEC. Adherence is known to be important to prevent the flushing mechanism of voiding, and specific adherence organelles have been implicated in eliciting protective immune responses and are part of vaccine development (Langermann et al. 1997, 2000), highlighting their importance in the pathogenesis and study of uropathogenic bacteria.

P Fimbriae

The earliest virulence trait established for UPEC was P fimbria. In 1976, Svanborg-Eden et al. (Eden et al. 1976) discovered that pyelonephritis isolates of UPEC adhered to uroepithelial cells in

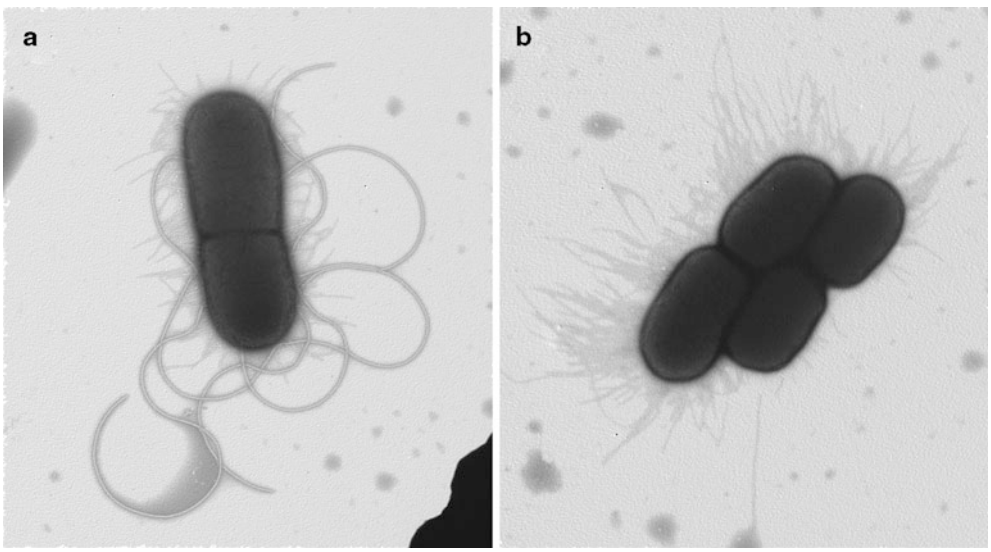

Fig. 15.2
***Transmission electron micrographs of uropathogenic E. coli expressing both flagella and fimbriae*. Transmission electron microscopy was used to capture 34,000x magnified images of *E. coli* strain CFT073 expressing either (a) flagella, an organelle that mediates motility or (b) fimbriae, an organelle that mediates adherence**

greater numbers than did cystitis or fecal isolates. The adherence phenotype of those isolates was subsequently correlated with expression of fimbriae as assessed by transmission electron microscopy and hemagglutination (Eden and Hansson 1978). These structures were designated P fimbriae because they adhered to the P blood group antigen, common among most humans. Preparations of purified P fimbriae selectively adhered to uroepithelial cells (Korhonen et al. 1986) and mediated mannose-resistant hemagglutination (MRHA) of human type O erythrocytes (Vaisanen et al. 1981). Using erythrocytes, Korhonen et al. (1982) identified the receptor for P fimbria as the $\alpha - \mathrm{Gal}(1 \rightarrow 4)\beta - \mathrm{Gal}$ moiety of the glycosphingolipid component of the P blood group antigen, a glycosphingolipid anchored in the cell membrane of erythrocytes and uroepithelial cells. The presence of P1 and P2 phenotypes of the P blood group antigen, which bind pyelonephritogenic *E. coli* (Kallenius et al. 1981b), is elevated in women prone to UTI (Lomberg et al. 1983; Mulholland et al. 1984) compared to non-UTI prone women, suggesting a causal link between carrier state of P blood group antigen and development of UTI.

Assembled by the chaperone-usher pathway (Kline et al. 2010a), fimbriae generally consist of a composite fiber coupled to a tip adhesin. P fimbriae fibers, for example, are primarily composed of PapA (*pap*, *p*yelonephritis-*a*ssociated *p*ilus) subunits, and the adhesive tip fibrillum is composed of repeated PapE subunits that terminate in PapG, the component of P fimbria responsible for specific binding to its receptor (Kuehn et al. 1992). P fimbriae bind the α-D-Gal-(1 → 4)-β-D-Gal digalactoside moiety of the P blood group antigen. Indeed, the free disaccharide is capable of inhibiting P fimbria from binding to its receptor on both erythrocytes (Kallenius et al. 1980) and uroepithelial cells (Kallenius et al. 1981b). Binding studies of PapG identified *p*-methoxyphenyl galabioside (Larsson et al. 2003) and heavily modified galactopyranoside derivatives (Ohlsson et al. 2002) as inhibitors of substrate binding for P fimbria. Structural studies of PapG in complex with receptor homologues have refined and supported these findings (Dodson et al. 2001).

Strains that carry P fimbriae can be categorized by which of three PapG adhesins are expressed at the tip of the fimbria. Each adhesin recognizes a slightly different portion of the Gal-Gal disaccharide-containing glycosphingolipid (Stromberg et al. 1991). Of these alleles, *papGI* was cloned first and is encoded by the fewest isolates, but *papGII* is the most common among pyelonephritis and complicated UTI UPEC isolates; *papGIII* is found in only some cystitis and ABU isolates (Spurbeck et al. 2011). The related fimbria, Pix, identified in UPEC strain X2194 and also found in UPEC strain 536, has no known substrate for binding (Lugering et al. 2003), and its contribution to virulence has not been well studied. Another fimbria, encoded by the *prs* (*pap* related sequence) operon, is serologically identical to P fimbriae, but the two do not share virulence characteristics as the tip adhesin, PrsG, is antigenically unique from PapG and binds the Forssman antigen at GalNAc-α-(1–3)-GalNAc moieties (Lund et al. 1988).

In 1981, Hull et al. (1981) isolated a cosmid clone carrying the genes that encode P fimbria. Subcloning revealed that a minimal operon of 11 genes was necessary and sufficient to generate functional P fimbriae. The *pap* operon was crucial for discovering and characterizing the chaperone-usher pathway for assembly of fimbria by Normark and Hultgren, which is a pathway of fimbrial biogenesis that is broadly applicable to a wide range of enterobacterial fimbriae (Normark et al. 1983; Norgren et al. 1984; Uhlin et al. 1985; Lund et al. 1987; Kuehn et al. 1991, 1992; Dodson et al. 1993; Hultgren et al. 1993). Thus, P fimbriae represented not only a UPEC-specific virulence determinant but also served as the cornerstone for understanding adherence in the *Enterobacteriaceae*.

Expression of the *pap* operon is phase variable and regulated by a Dam-methylation system (Baga et al. 1985; Braaten et al. 1994). PapB and PapI act in concert with Lrp to control the methylation state and binding of two GATC sites, GATC1128 and GATC1130, located in the *pap* regulator region located between *papI* and *papB* (Peterson and Reich 2008). When GATC1128 is methylated, the operon is in the OFF state, while methylation of GATC1130 is required for the ON state.

Since its initial discovery, the association of P fimbria with virulent uropathogenic strains has been confirmed in many studies (Hagberg et al. 1981; Kallenius et al. 1981a; Vaisanen et al. 1981; Latham and Stamm 1984; Vaisanen-Rhen et al. 1984; Jacobsen et al. 1985; O'Hanley et al. 1985; Dowling et al. 1987; Johnson et al. 1987; Sandberg et al. 1988; Johnson et al. 1991; Ulleryd et al. 1994; Spurbeck et al. 2011). In a meta-analysis of prevalence studies, Welch and Donnenberg reported that UPEC isolates from otherwise healthy patients with pyelonephritis have a sixfold elevated relative risk of encoding P fimbriae compared to *E. coli* fecal isolates, which translates to roughly 80 % of pyelonephritogenic UPEC strains. In humans, expression of P fimbriae by UPEC in the urine (Kisielius et al. 1989) and antibodies to P fimbriae in the serum (de Ree and van den Bosch 1987) suggests that *E. coli* produce and display P fimbria in vivo in infected patients.

In animal models, demonstrating the direct contribution of P fimbria to infection has proven elusive. Two P-fimbrial isogenic deletion mutants of clinical isolates have been tested for virulence, in CBA mice (Mobley et al. 1993) and in cynomolgus monkeys (Roberts et al. 1984, 1994). In work done by Mobley et al. (1993) CBA mice were challenged with strain CFT073 or its P fimbria-negative mutant (both *pap* operons were disrupted by allelic exchange). At the end of a week-long infection using a wide range of infectious doses, no significant difference in pathogen concentration or histological findings were found between the parent and mutant strains in urine, bladder, or kidney. Due to technical limitations at the time of the experiment, only discrete, cross-sectional data are available for those infections. A recent study by Melican et al. (2011) used GFP-expressing *E. coli* and multiphoton-based live-animal imaging during ascending and descending UTI in a mouse model of infection. When scrutinizing the behavior of a *pap* knockout strain of CFT073, rates of tubular infection in the kidney were one-third that of the parental strain. They also

observed that *E. coli* strain K12 transformed with a plasmid encoding the *pap* operon achieved visible growth more rapidly in the kidney tubules than did a strain containing vector (Melican et al. 2011). Together, these studies suggest that P fimbriae play a subtle role in the pathogenesis of pyelonephritis in the CBA mouse model of UTI. This has been supported in later work in which P fimbrial knockouts did not affect urovirulence in a Type 1 fimbria-negative background in UPEC (Snyder et al. 2005). It was found, however, that synthesis of the P fimbria-related F1C fimbriae was upregulated in P fimbrial knockouts, hinting that some complementation or functional compensation between fimbrial organelles may be possible. Roberts et al. (1984, 1994) studied the effects of a *papG* mutation on colonization of the bladder and development of pyelonephritis in a primate model. No difference was observed by analysis of the primate urine (which gauged leukocyte esterase and analysis of bacteria/ml, by culture, in the urine of infected monkeys) between parent and mutant strain after bladder inoculation, although it was found that the parent strain persisted longer than the mutant strain in the urinary tract, and only animals infected with the parent strain showed evidence of impaired renal function and decreased renal mass. However, in both studies, the mutant strain was still capable of infecting the kidneys. Together with epidemiological data, these studies suggest that while P fimbriae are associated with and probably contribute to virulence in the urinary tract, the murine model may not be the optimal setting in which to test the role of P fimbria in urovirulence.

Immune Response to P Fimbria

An asymptomatic bacteriuria (ABU) strain of *E. coli* capable of infecting humans, but which normally produces no P fimbriae, was transformed with a plasmid encoding P fimbria. When volunteers were infected with the recombinant strain, urinary IL-6, IL-8, and neutrophils were elevated, compared to infections caused by the parental strain (Wullt et al. 2001). When a Δ*papG* construct was introduced into the parent ABU strain, the resulting nonadhesive strain failed to trigger these responses (Bergsten et al. 2004), suggesting that specific adherence mediated by the tip adhesin is critical for the normal response to infection. This supports the hypothesis that PapG is crucial for the contribution to virulence of P fimbriae.

Proteus mirabilis Adherence Factors

Like uropathogenic *E. coli, P. mirabilis* also expresses a host of adherence factors during uropathogenesis. Genomic analysis of the uropathogenic *P. mirabilis* isolate HI4320 reveals that it encodes 17 complete fimbrial operons and another 13 incomplete fimbrial operons (Pearson et al. 2008). As early as 1958, Duguid and Gillies demonstrated fimbriae-dependent hemagglutination of *Proteus* spp. (Duguid and Gillies 1958). Subsequent studies employing electron microscopy elaborated the binding characteristics of *Proteus* spp. fimbriae, characterizing types of hemagglutination employed by different fimbriae of *Proteus* spp. and uroepithelial specificity (Old and Adegbola 1982; Adegbola et al. 1983; Wray et al. 1986; Yakubu et al. 1989). As for *E. coli*, only a subset of the adherence factors of *P. mirabilis* have been studied in a lab setting and found to be related to the pathogenesis of UTI.

Perhaps the best studied *Proteus* adherence factor is mannose-resistant *Proteus*-like (MR/P) fimbria. MR/P fimbriae are typical chaperone-usher fimbriae that are similar to P fimbria of *E. coli*. Indeed, MrpA has some homology to PapA, the major structural subunit of P fimbria (Bahrani et al. 1991). Phenotypically, MR/P fimbriae also agglutinate erythrocytes in a mannose-resistant manner (Bahrani et al. 1991; Li et al. 2002a; Rocha et al. 2007), although the precise receptor has not been identified. Epidemiologically, *P. mirabilis* strains that cause pyelonephritis tend to be enriched for MR/P fimbriae compared to cystitis and fecal isolates (Mobley and Chippendale 1990). Using genetic studies, MR/P fimbriae have been shown to contribute to virulence in a murine model of UTI, but are not required to cause UTI (Bahrani et al. 1994; Li et al. 2002a; Zunino et al. 2007). Like P fimbriae, MR/P fimbriae induce a strong immune response (Bahrani et al. 1991), and recent vaccine trials indicate that vaccination with either MrpH or MrpA is highly protective against UTI in the mouse model of infection (Li et al. 2004; Scavone et al. 2009, 2011).

MR/P fimbriae are phase variable and, similar to the regulation displayed by the Type 1 fimbrial operon, are regulated by an invertible element containing an orientation-specific promoter. Orientation of the invertible element is controlled by a single recombinase, MrpI, and the promoter can be forced to remain constitutively ON or OFF by modifying direct flanking DNA repeats (Li et al. 2002a). Locking the promoter ON or OFF changes the pattern of adherence of *P. mirabilis* in the bladder in the mouse model of UTI, but it does not change the total number of adherent *P. mirabilis* bacteria during infection (Jansen et al. 2004).

Several other fimbriae of *Proteus* have been studied to varying degrees. Uroepithelial cell adhesins (UCA, also known as nonagglutinating fimbriae) do not agglutinate erythrocytes. They appear to bind to galectin-3-containing and GalNac-β-1-4Gal moieties of canine uroepithelial cells, and antibodies to these substrates inhibit *P. mirabilis* adherence in the mouse model of UTI (Lee et al. 2000; Altman et al. 2001). However, evidence for relevance of UCA to pathogenesis in UTI has not been established. Another fimbria, *Proteus mirabilis* fimbriae (PMF), also do not mediate hemagglutination, and no difference in adherence to uroepithelial cells was observed between isogenic mutants and parent strains (Massad et al. 1994). However, molecular pathogenesis studies have shown that PMF fimbriae contribute to virulence in the mouse model of UTI via an unknown mechanism (Massad et al. 1994; Zunino et al. 2003). Like MR/P fimbriae, components of *P. mirabilis* P-like fimbriae (PMP) have some homology to PapA from *E. coli* (Bijlsma et al. 1995), but the mechanism of action and importance in UTI of PMP fimbria is not established.

Reciprocal Repression of Motility Mediated by Nonstructural Genes of the MR/P and P Fimbrial Operons

The last gene of the MR/P fimbrial operon is *mrpJ*, which encodes a transcription factor predicted to employ a winged-helix DNA-binding motif. MrpJ represses motility by binding to the *flhD* promoter and repressing transcription of the flagellar master regulator, as shown by expression studies and electrophoretic mobility shift assays (Pearson and Mobley 2008). Similarly, most *pap* operons in *E. coli* encode PapX, a functional homolog of MrpJ, at the end of the operon (Reiss and Mobley 2011). Like MrpJ, PapX represses motility by binding to the *flhD* promoter (Simms and Mobley 2008; Reiss and Mobley 2011) at a sequence present in a degenerate form in the *P. mirabilis flhD* promoter. Interestingly, there is up to 45 % amino acid identity between PapX and MrpJ in some of their domains, and *papX* functionally complements isogenic *mrpJ* deletion mutants of *P. mirabilis* (Li et al. 2001). Both *papX* and *mrpJ* encode nonstructural components of their respective fimbriae and are not required for fimbrial synthesis. *P. mirabilis* HI4320 has 14 paralogues of *mrpJ*, 12 of which also repress motility and 10 of which are encoded at the end of fimbrial-encoding operons (Pearson and Mobley 2008). Both PapX and MrpJ make use of specific motifs within their respective predicted helix-turn-helix domains to bind the *flhD* promoter as shown by site-directed mutagenesis of each of these regions (Pearson and Mobley 2008; Reiss and Mobley 2011). It appears that both *E. coli* and *P. mirabilis* make use of fimbrial operon-associated helix-wing transcription factors to repress motility via *flhD* and *flhC*. Thus, when these adherence-mediating fimbriae are synthesized, motility is reciprocally repressed.

Type 1 Fimbria

Nearly all *E. coli* strains, including UPEC strains, express the mannose-sensitive adhesin Type 1 fimbria (Duguid et al. 1979; Orndorff and Bloch 1990). Within the urinary tract, Type 1 fimbriae bind to the six inner domains of uroplakin Ia, which is found in plaque-forming particles that line the interface between the luminal urothelium and urine (Zhou et al. 2001; Min et al. 2002, 2003). Structurally, Type 1 fimbriae stalks are 0.5–2 μm in length and 7 nm wide (Brinton 1965). At the distal end, they have a fibrillar adhesive tip, encoded by *fimH*, which extends another 16 nm from the stalk. FimH is responsible for the substrate specificity of type 1 fimbriae, binding to glycolipids and glycoproteins containing mannosides, such as the aforementioned uroplakin Ia (Abraham and Beachey 1987; Abraham et al. 1988; Hacker et al. 1990; Krogfelt et al. 1990; Wu et al. 1996; Hung et al. 2002). In addition to expression, it appears that shear forces, such as those generated in high-flow states in the urinary tract, conformationally enhance binding of Type 1 fimbria to its substrate in a so-called "catch bond" fashion (Thomas et al. 2004; Nilsson et al. 2007).

Type 1 fimbria has been well established as a virulence factor in the mouse model of UTI (Connell et al. 1996; Bahrani-Mougeot et al. 2002; Gunther et al. 2002; Snyder et al. 2006). However, Type 1 fimbriae are encoded by nearly all strains of *E. coli* with equal prevalence (Orndorff and Bloch 1990), which suggests that while Type 1 fimbriae may contribute to virulence, they almost certainly are not alone sufficient to cause virulence and cannot be invoked to explain the specific urovirulence of UPEC as compared to other strains of *E. coli*. Interestingly, FimH point mutants selected for stronger binding to mono-mannose residues than parental FimH also conferred greater urovirulence to select UPEC strains. However, the same variations caused FimH to be more sensitive to soluble inhibitors of adherence found in the oropharyngeal mucosa (Sokurenko et al. 1998), indicating reduced adaptive fitness at other mucosal sites in the body. These studies suggest that FimH may be under selective pressure at commensal sites within the body, the putative reservoir for UPEC strains, which may explain why Type 1 fimbriae are highly prevalent among *E. coli* strains and also suggests that the prevalence of Type 1 fimbriae is not primarily driven by selection for greater urovirulence.

Type 1 fimbriae are encoded by a cluster of nine genes, *fimBEAICDFGH*, which are expressed from at least three transcripts (Hull et al. 1981; Hultgren et al. 1991; Schilling et al. 2001a). One transcript controls the main structural component of Type 1 fimbria, *fimA*. Two transcripts encode FimH, the tip adhesin for Type 1 fimbria, and accessory proteins for assembly of the fimbrial stalk (Johnson 1991). Assembly of Type 1 fimbriae proceeds via the chaperone-usher pathway. FimC, a periplasmic chaperone, makes use of an immunoglobulin-like fold that stabilizes and contributes to the transport of fimbrial components as they are trafficked from the cytoplasm to the outer membrane (Jones et al. 1993). FimC also prevents fimbrial subunits from interacting until they are properly localized for fimbrial assembly (Choudhury et al. 1999). FimD, the usher, recognizes FimC complexes containing unincorporated subunits of Type 1 fimbria and serves to localize fimbrial subunit complexes to the appropriate location on the outer membrane (Klemm 1986). FimA protein forms the principal component of the shaft (Brinton 1965) serving as the chief structural subunit of Type 1 fimbriae. FimG and FimF form a complex with FimH to produce the tip adhesin, with FimH providing receptor-binding specificity (Jones et al. 1995).

Type 1 fimbrial expression is under the control of an invertible element, upstream of *fimA*, which contains an orientation-dependent promoter. When the element is in the "ON" position, the promoter functions to drive expression of the structural genes of Type 1 fimbriae. When the element is in the reverse orientation, the "OFF" position, the Type 1 fimbrial genes are not transcribed and no fimbriae are produced (Eisenstein 1981). During infection in mice, cystitis and pyelonephritis isolates have been shown to differ in their control of the invertible element orientation (Struve and Krogfelt 1999). The ON orientation is favored in cystitis isolates and is less common in pyelonephritis isolates (Gunther et al. 2001).

Orientation of the invertible element, and thus the orientation of the promoter, is controlled by FimB and FimE. FimB and FimE are recombinases that act on 9 bp inverted DNA repeats at the flanking ends of the invertible element to switch orientation of the invertible element (Abraham et al. 1985). FimB causes the element to switch between orientations, while FimE drives the element into the "OFF" orientation. Promoter activity can be engineered to remain "ON" or "OFF" constitutively ("locked") by manipulating the flanking 9 bp DNA repeats. IpuA and IpbA (FimX), encoded elsewhere in the genome, also appear to mediate recombinase activity at the site of the invertible element (Bryan et al. 2006; Hannan et al. 2008).

Expression of Type 1 fimbriae appears to be influenced by a host of environmental factors, presumably by regulating the orientation of the promoter via the invertible element. Low pH and high osmolarity tend to enhance the activity of FimE and inhibit FimB, which results in the "OFF" orientation of the promoter. Temperature, sialic acid derivatives, and branched-chain amino acids also influence FimB and FimE recombinase activity to affect phase variability (Gally et al. 1993; El-Labany et al. 2003). Additionally, culture conditions, such as static growth in broth or growth on agar plates, tend to favor ON and OFF orientations of the invertible element, respectively (Klemm 1985; Hultgren et al. 1986). Leucine-responsive protein (LRP), integration host factor (IHF), and histone-like nucleoid protein (H-NS) all affect orientation and sensitivity of recombinase activity of the invertible element by binding within or near sequences related to the element as well (Gally et al. 1994). Status of the invertible element also varies by strain type, where fecal and cystitis isolates tend to favor the "ON" orientation but pyelonephritis isolates tend to favor the "OFF" orientation (Lim et al. 1998).

As mentioned, orientation of the invertible element can be constitutively set by manipulating the 9 bp DNA flanking sequences. The pyelonephritis isolate CFT073 shows a colonization disadvantage in the mouse model of UTI at 24 h post inoculation when the element is in the constitutively "ON" position and a defect when constitutively "OFF," both compared to wild type (Gunther et al. 2002). However, when the same experiment was done with the *E. coli* cystitis isolate F11, while the constitutively "OFF" mutant was attenuated both at 24 and 168 h post infection, the constitutively "ON" strain showed no difference in colonization as compared to wild type (Snyder et al. 2006).

When *E. coli* colonize the murine bladder, Hultgren et al. noted apoptosis and exfoliation of umbrella cells lining the walls of the urinary tract; however, bacteria evaded this shedding by invading underlying cells to form intracellular bacterial communities (IBCs) (Mulvey et al. 1998) via a mechanism dependent on Type 1 fimbria (Martinez et al. 2000). Mast cells, which are necessary for clearance of Type 1 fimbriated bacteria during UTI (Malaviya et al. 1996), also experience Type 1 fimbria-dependent intracellular invasion by UPEC strains via a caveolae-dependent mechanism (Shin et al. 2000). Intracellular invasion could provide protection from some immune functions in the urinary tract and provide a reservoir for recurrent infection. This model is supported by vaccine studies using FimH as the primary antigen, which demonstrated protection against UTI in mice (Langermann et al. 1997) as well as in cynomolgus monkeys (Langermann et al. 2000). Acutely relapsing UTIs (recurrence within a month) are often caused by a single UPEC strain. However, UTIs that recur within six months are often caused by different UPEC strains (Pigrau-Serrallach 2005). This led to the suggested model that UPEC use Type 1 fimbriae to attach to, cause apoptosis of, and facilitate invasion of host cells in acutely relapsing UTI.

Klebsiella pneumoniae also expresses Type 1 fimbriae and the FimH mannose-binding tip (Fader and Davis 1980; Fader et al. 1982; Gerlach et al. 1989). At least 90 % of *K. pneumoniae* isolates encode *fimH*, which has little nucleotide diversity among these isolates (Stahlhut et al. 2009). The *K. pneumoniae* Type 1 fimbrial operon is regulated in a manner similar to the UPEC Type 1 fimbrial operon; *fimB* and *fimE* homologs control the orientation of *fimS*, the invertible promoter. However, this bacterium encodes an additional gene, *fimK*, which also downregulates the expression of Type I fimbriae (Klemm 1986; Rosen et al. 2008a). FimK has a putative EAL domain, which may couple c-di-GMP metabolism with inversion of *fimS* into the OFF orientation (Rosen et al. 2008a; Clegg et al. 2011).

K. pneumoniae Type 1 fimbriae are important for the colonization of the mouse urinary tract, adhering to cultured bladder epithelial cells, hemagglutination, and the formation of intracellular bacterial communities (Fader et al. 1979; Maayan et al. 1985; Gerlach et al. 1989; Rosen et al. 2008a, b; Struve et al. 2008). However, a *K. pneumoniae* mutant that does not express Type 1 fimbriae is not outcompeted in the kidney by a *K. pneumoniae* strain that expresses Type 1 fimbriae, which suggests that Type 1 fimbriae do not have an obvious function in promoting *K. pneumoniae* murine kidney infection (Maayan et al. 1985).

K. pneumoniae also expresses Type 3 fimbriae with the MrkD adhesive tip (Gerlach et al. 1989). These fimbriae mediate *K. pneumoniae* adherence to host cells derived from the respiratory tract (Hornick et al. 1992), to host bladder cells (Tarkkanen et al. 1997), and to sites within the kidney (Tarkkanen et al. 1990). While the importance of Type 3 fimbriae during UTI is not entirely clear (Struve et al. 2009), they do have a function in biofilm formation (Langstraat et al. 2001; Struve et al. 2009; Wilksch et al. 2011).

Intriguingly, *K. pneumoniae* capsule (see below) obstructs fimbrial adherence (Sahly et al. 2000; Schembri et al. 2005; Hennequin and Forestier 2007), but at the same time, capsule promotes *K. pneumoniae* adherence to mucus-producing cells (Favre-Bonte et al. 1999). In *E. coli*, Type 1 fimbriae adherence activity results in a downregulation of *E. coli* capsule expression (Schwan et al. 2005). However, similar regulation in *K. pneumoniae* has not been explored in detail. It is also unclear whether or not capsule and fimbrial operon expression is coordinated through random events or through specific signals.

Dr Adhesins

Dr adhesins represent a mix of fimbrial and afimbrial structures on the surface of *E. coli* that, like P fimbriae, also mediate

mannose-resistant hemagglutination (Swanson et al. 1991). Dr adhesins bind to the Dr blood group antigen (Nowicki et al. 1990). Within the urinary tract, Dr adhesins bind to bladder epithelial cells and type IV collagen in the basement membrane (Westerlund et al. 1989). Although Dr adhesins are encoded in only a low percentage of UPEC isolates, they are more prevalent among cystitis and pyelonephritis isolates than in fecal isolates (Donnenberg and Welch 1996; Spurbeck et al. 2011). Additionally, DraD and DraE have been shown to be essential for invasion of host epithelial cells (Zalewska et al. 2001, 2005; Das et al. 2005). Evidence also suggests that Dr adhesins play a role in infection of the upper urinary tract because an isogenic Dr-negative mutant was unable to recapitulate chronic renal disease in a mouse model of ascending UTI caused by its parental strain. This deficiency was reversed by complementation (Goluszko et al. 1997).

F1C and S Fimbriae

S and F1C fimbriae are nearly identical in their biogenesis, structure, and sequence but possess distinct adhesive tips and thus differ in their substrate specificity. Pooled data suggest that F1C fimbriae are more common among pyelonephritis and cystitis isolates than among fecal strains of *E. coli* (Donnenberg and Welch 1996). The presence of F1C fimbriae is especially common in O6-antigen serotypes of UPEC, which themselves are associated with pyelonephritis isolates and UPEC strains that encode P fimbriae and hemolysin (Pere et al. 1985; Zingler et al. 1992, 1993). Interestingly, FocB, a regulatory member of the operon encoding F1C fimbriae, appears to be capable of regulating both Pap and Type 1 fimbriae, indicating that F1C fimbriae are involved in a complex genetic regulatory network with other fimbriae directly tied to urovirulence (Lindberg et al. 2008). F1C fimbrial expression has also been observed to be elevated when Type 1 fimbriae and P fimbriae encoding genes are deleted from the chromosome in UPEC, supporting the notion of coordinated regulation (Snyder et al. 2005). Mechanistically, F1C fimbriae could contribute to urovirulence by facilitating adherence to human distal tubular and collecting duct epithelial cells and vascular endothelial cells, phenotypes that have been demonstrated in the analysis of kidney cross sections by histology (Korhonen et al. 1990). Additionally, expression of F1C fimbriae in vivo has been demonstrated using monoclonal antibodies to F1C in the urine of patients with UPEC UTI (Pere et al. 1987). Using force-measuring optical tweezers, F1C fimbriae were shown microscopically to have a similar Young's modulus (stretch-to-strain ratio) as P fimbriae and Type 1 fimbriae, a range which has been proposed to be important in the urinary tract to suitably resist flushing by urine flow (Castelain et al. 2011). Though F1C fimbriae do not agglutinate erythrocytes, it has been shown that they bind to galactosylceramide and globotriaosylceramide (Backhed et al. 2002) and that this binding can be inhibited by GalNAcβ1 → 4Gal and GalNAcb1 → 4β1 → 4Glc (Autar et al. 2003). This may offer a clue to the mechanism by which F1C fimbriae bind to human epithelial and endothelial cells.

Other Mechanisms of Adherence

S. saprophyticus expresses a variety of proteins that mediate cell adherence. Much of the focus on *Staphylococcus* spp. adhesins has centered on sortase-dependent anchoring via an LPXTG signal (Mazmanian et al. 1999; Kuroda et al. 2005). Uro-adherence factor A and B (UafA and UafB) are both among these adhesins (Kuroda et al. 2005; King et al. 2011). UafA mediates hemagglutination of erythrocytes and adherence to a bladder cell line (Kuroda et al. 2005). Indeed, adherence to cultured cells and the hemagglutination phenotype of many strains of *S. saprophyticus* are well documented (Gatermann et al. 1988). UafB is an adhesin that allows *S. saprophyticus* to bind to fibronectin, fibrinogen, and human bladder epithelial cells. However, intriguingly, this protein does not contribute to adherence and colonization of the mouse bladder (King et al. 2011).

The cell wall-anchored serine-aspartate repeat protein I (SdrI) also serves a role in *S. saprophyticus* adherence (Sakinc et al. 2006, 2009). SdrI mediates binding to collagen (Sakinc et al. 2006) and fibronectin (Sakinc et al. 2009), which are both components of the extracellular matrix. Another cell wall-associated protein, autolysin/adhesin of *S. saprophyticus* (Aas), helps mediate *S. saprophyticus* binding to fibronectin, promotes hemagglutination, and functions as an autolysin (Gatermann and Meyer 1994; Hell et al. 1998). *S. saprophyticus* surface-associated protein (Ssp) is a cell-associated protein that was predicted to be an adhesin but was subsequently found to be a lipase and did not have an obvious role in promoting adherence (Sakinc et al. 2005). Elucidating the role of Ssp in virulence also remains an area of ongoing research. However, *S. saprophyticus* has a preference for infecting the kidneys of challenged animals, and both *S. saprophyticus sdrI* and *ssp* mutants have a defect in persisting in the kidneys and bladders of infected animals during later time points of infection (Kline et al. 2010b). These results suggest that SdrI and Ssp carry out an important function during *S. saprophyticus* pathogenesis.

Pathogenicity Islands

Pathogenicity islands (PAIs) were first defined as genomic regions spanning 30 kilobases or more, associated with pathogens, and encoding virulence genes not found in nonpathogens (Hacker et al. 1990). PAIs tend to insert near tRNA-encoding regions of the chromosome, contain different G + C content compared to non-island DNA, and tend to include intact or degenerate mobility genes such as exogenases, transposon sequences, insertion elements, or bacteriophage-like transfer genes. PAIs can carry essential pathogenesis genes in UPEC, encoding specific fimbriae and other adhesins, secreted toxins (Blum et al. 1995), and iron acquisition systems (Lloyd et al. 2007). It is therefore not surprising that deletion of PAIs can attenuate virulence of certain strains in the mouse model of UTI, for example, when a PAI containing iron-scavenging systems and certain toxins are deleted in CFT073 (Lloyd et al. 2009).

Some PAIs are unstable and can be deleted or excised from a population under certain environmental conditions or with artificially induced site-specific recombination (Middendorf et al. 2004). PAIs from different strains tend to be similar in structure and content (Dobrindt et al. 2002), suggesting that they may all have been acquired via lateral transfer and modified over time in different UPEC strains (Hacker et al. 1990; Dobrindt et al. 2003; Bidet et al. 2005). Numerous examples of similarly structured PAIs in UPEC (Knapp et al. 1986; Hacker et al. 1990; Blum et al. 1994; Swenson et al. 1996; Kao et al. 1997; Guyer et al. 1998; Dobrindt et al. 2002; Oelschlaeger et al. 2002; Schneider et al. 2004; Brzuszkiewicz et al. 2006) and other *E. coli* strains (Rasko et al. 2001; Parham et al. 2005) support this claim and suggest the importance of PAIs in defining virulence characteristics of UPEC strains.

While genomic differences between commensal strains and uropathogenic strains may include hundreds of unique or additional genes, the active transcriptional profile of some strains, such as the commensal strain Nissle 1917 and the uropathogenic strain CFT073 is quite similar. The similarity in organisms is underscored by the fact that strain Nissle 1917 is able to outcompete CFT073 during planktonic growth in urine and strain Nissle 1917 expresses many prototypically UPEC-associated genes, including a shared biofilm biogenesis pathway (Hancock et al. 2010). This suggests that acquiring PAIs could be sufficient to generate pathogenic strains derived from certain nonpathogenic strains of *E. coli*. In addition, comparative analysis of the complete genomes of enterohemorrhagic *E. coli* (EHEC) strain EDL933, UPEC strain CFT073, and commensal K12 strain MG1655 reveals that each strain is equally distantly related from the others (Welch et al. 2002). This suggests that multiple lineages branched from an initial precursor strain, with each lineage acquiring genetic characteristics to best patho-adapt to its unique environment. Repeated lateral acquisition of pathogenicity elements could explain this development, a model supported by the ubiquity of PAIs in pathogenic *E. coli* strains.

Integrative and conjugative elements (ICEs) represent another mechanism by which pathogens may acquire virulence characteristics. ICEs are similar to PAIs but are self-transmissible by a type IV secretion system. ICEs also contain different G + C content relative to the chromosome, are often inserted near tRNA loci, are flanked by repeats, and encode mobility elements and mobility regulatory elements such as integrases, excisionases, and conjugative machinery. However, whereas PAI mobility is implied, the ability to transfer is an explicit feature of ICEs (Wozniak and Waldor 2010). ICEs have been shown to be active in *E. coli* and, recently, in the uropathogenic *P. mirabilis* strain HI4320. This strain, representative of typical uropathogenic strains, contains an ICE encoding *Proteus* toxic agglutinin (Pta) and the high pathogenicity island (HPI) of *Yersinia* spp. that encodes yersiniabactin (Flannery et al. 2009). In *P. mirabilis*, ICEPm1 transfers itself between *P. mirabilis* isolates at a frequency of 1.35×10^{-5} transconjugants/donor, a rate easily great enough to affect the representation of encoded traits within populations of *P. mirabilis* (Flannery et al. 2011).

Motility

The pathogenesis of most UTIs proceeds via an ascending route (Bacheller and Bernstein 1997). Bacteria gain access to the urethra and ascend to the bladder and kidneys. In *E. coli* and *P. mirabilis*, motility is primarily dependent on flagella (Smith and Hoover 2009) (*Fig. 15.2a*). *Klebsiella pneumoniae* and *Staphylococcus saprophyticus* are nonmotile (Shaw et al. 1951; Podschun and Ullmann 1998). Flagella are complex membrane-bound extracellular organelles that consist of a basal body, a hook, and a filament. Synthesis proceeds in an "inside-out" manner so that the basal body is constructed first, then the hook, and finally the filament (Harshey and Toguchi 1996). Synthesis of flagella proceeds in a highly ordered hierarchical manner and is dependent on proper coordination of three classes of genes: class 1, class 2, and class 3 (Komeda 1982, 1986; Kutsukake et al. 1990; Macnab 1996; Chilcott and Hughes 2000; Kalir et al. 2001). Class 1 genes include primarily transcription of *flhD* and *flhC*, whose gene products form the $FlhD_4C_2$ complex that acts as a transcription factor to activate transcription of class 2 genes. *flhD* and *flhC* are located on a single operon, and their expression is highly regulated by numerous factors (such as OmpR, cAMP-CAP, DnaK, DnaJ, GrpE, H-NS, HU, DnaA, HdfR, SdiA, LrhA and UvrY (Soutourina and Bertin 2003)). Thus, *flhD* and *flhC* act as a central point of regulation of motility for many systems and pathways, leading to their designation as the master regulators of motility. Class 2 gene products are responsible for assembly of the basal body and hook and expression of FliA (sigma 28) and FlgM (anti-sigma 28) (Kalir et al. 2001). Once the basal body and hook structures are assembled, FlgM diffuses out of the bacterium (Hughes et al. 1993), allowing activation of FliA. Active FliA is required for the transcription of the class 3 genes (Ohnishi et al. 1990; Kutsukake and Iino 1994). Class 3 genes encode hook-associated proteins including FliC (flagellin), required to complete construction of functional flagella, and motility-associated genes *motA*, *motB*, *cheW*, and *cheY* (Kalir et al. 2001).

Experimentally, bacterial dependence on flagella-mediated motility in the pathogenesis of UTI has been illustrated in multiple studies. UPEC mutants defective in motility were able to cause UTI in independent challenge in mice, but the *fliC* mutants were outcompeted by motile parental strains in competition assays (Lane et al. 2005; Wright et al. 2005). In another study, nonmotile mutants were highly attenuated for colonization of the kidneys even in independent challenge (Lane et al. 2007). Interestingly, transcriptomic data from UPEC collected from the urine of mice with experimental UTI showed low levels of flagellar expression (Snyder et al. 2004), suggesting that overall flagellar expression is repressed in the general population of *E. coli* infecting the urinary tract. This may be because flagellar expression is highly coordinated during UTI depending on location within the urinary tract, as has been shown by biophotonic imaging studies (Lane et al. 2007), or because UPEC shed in urine are not highly flagellated. Flagella are highly immunogenic in pathogenic *E. coli* and are recognized by toll-like receptor 5 and induce IL-8 expression

(Donnelly and Steiner 2002). This may help to explain why UPEC tightly regulates expression of flagella, to prevent excessive immune activation.

P. mirabilis, while capable of swimming motility via flagella, also undergo swarming motility, which occurs when flagellar motility is coupled to morphological changes resulting in highly elongated, heavily flagellated swarmer cells, 50–100 times the length of *P. mirabilis* swimmer cells (Rather 2005) (*Fig. 15.3e*). Although swarmer cells are in the minority during UTI in the mouse model at 2 and 4 days post infection (Jansen et al. 2003), they have been shown to swarm across inorganic surfaces such as catheters and to facilitate urothelial cell invasion (Allison et al. 1992; Jones et al. 2004). Both swimmer and swarmer cells are present during UTI in mice, indicating that both forms of motility may play a role in uropathogenesis. In addition, *P. mirabilis* strains that are unable to produce flagella have 100-fold attenuated colonization in the mouse model of UTI (Mobley et al. 1996), although the presence of flagella is not strictly necessary as, rarely, uropathogenic strains of *P. mirabilis* have been found without the ability to synthesize flagella (Zunino et al. 1994).

Growth in Urine

Urine is the product of kidneys filtering blood plasma, and in a normal, healthy urinary tract, urine is relatively nutrient-poor as kidneys restrict loss of metabolically valuable compounds from the body (Putnam et al. 1971; Brooks and Keevil 1997). Urine can have a pH as low as 5.3, has high osmolarity, and contains concentrated urea. Since urine constantly refreshes, these conditions remain relatively constant, potentially inhibiting bacterial growth (Kaye 1968). In spite of this, some studies indicate that UPEC strains grow more rapidly in urine in vitro than fecal commensal strains of *E. coli*, suggesting that UPEC are patho-adapted to grow in urine; thus, the ability to grow in urine may contribute to virulence during UTI (Gordon and Riley 1992). However, other studies have indicated that only a subset of fecal commensal *E. coli* strains have reduced growth kinetics in urine as compared to UPEC strains (Alteri and Mobley 2007).

The contribution of osmotolerance in UPEC to UTI pathogenesis is a complex topic. Some studies suggest that to allow growth in the high-osmolarity environment of urine, UPEC has

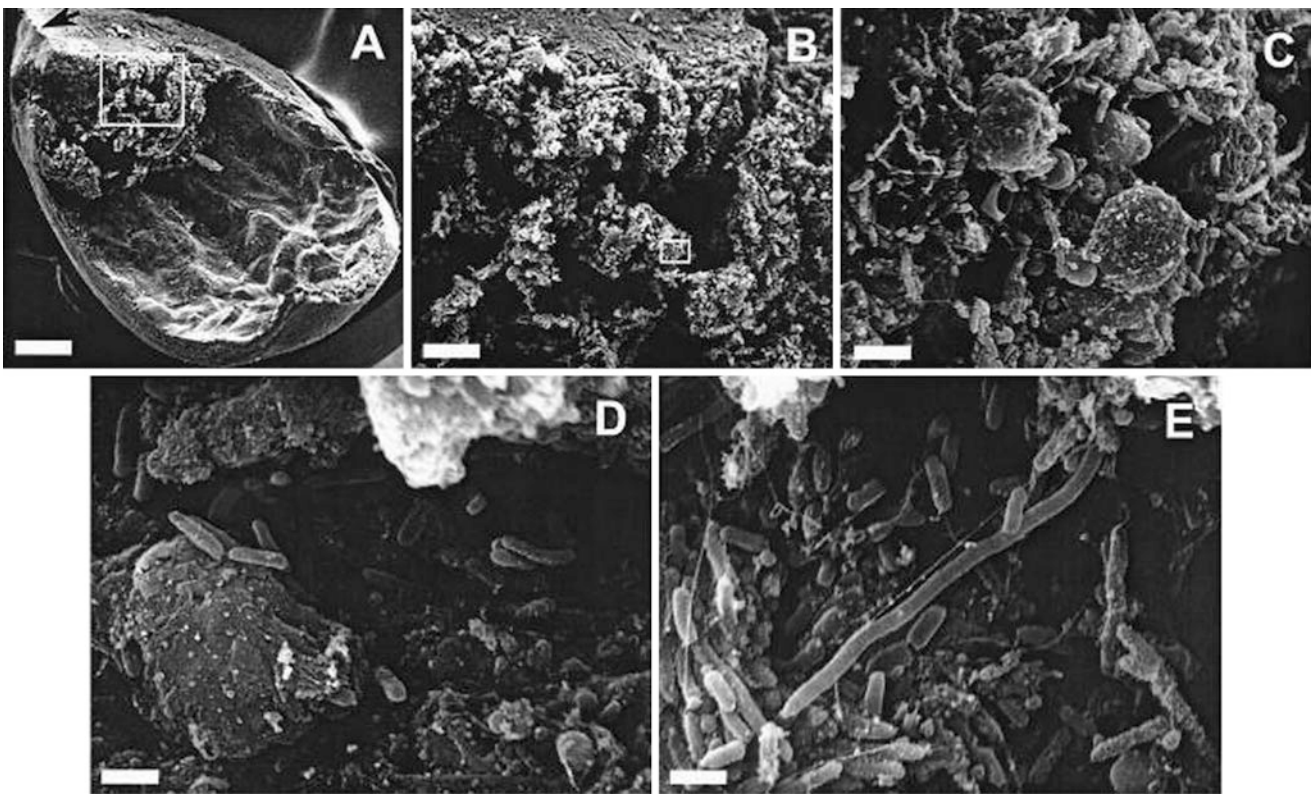

Fig. 15.3

Scanning electron micrographs of P. mirabilis urease-induced bladder stone. **(a) One-quarter of the bladder viewed at a low magnification (bar, 500 μm). The orientation of the bladder is indicated by an *arrow* pointing to the inferior end of the bladder (the end leading to the urethra). (b) Higher magnification (bar, 100 μm) of the area enclosed in a *box* in panel a. (c) Higher magnification (bar, 5 μm) of the area enclosed in a *box* in panel b. (d, e) Representative views of the bladder stone (bars, 2 μm) (Reprinted with permission from Li et al. (2002b))**

increased its capacity to transport osmotically active compounds. In one study, *E. coli* pyelonephritis isolates had threefold greater capacity for transporting proline betaine than the commensal *E. coli* strain K12. Mutating the locus containing the proline betaine transporter (*proP*) in UPEC strain HU734 reduced bladder colonization compared to the parental strain by 100-fold in a mouse model of UTI (Culham et al. 1998). This may be due to the fact that the bladder acts as a reservoir for urine, and thus, selective pressure for relative fitness in urine may be greater in the bladder than in the kidneys. In the pyelonephritis isolate CFT073, however, deletion of both the *proP* and *proU* loci together, responsible for glycine betaine transport, did not affect growth in urine or the ability of the mutant to colonize the mouse urinary tract (Culham et al. 2001). This may be due to compensatory osmoregulatory systems present in CFT073, such as OmpA and OmpR (Nicholson et al. 2009; Schwan 2009).

The ability to generate higher order metabolic compounds is essential for bacteria to grow in nutrient-poor environments. Disruption of either gluconeogenesis or the TCA cycle results in attenuation of urovirulence in *E. coli* (Alteri et al. 2009b). The ability to synthesize specific amino acids and nucleotides is also essential for full urovirulence. In a study of uropathogenic *E. coli* by Hull and Hall, it was found that guanine, arginine, and glutamine auxotrophs were unable to grow in human urine in vitro, and there was substantially reduced growth, compared to fully heterotrophic strains, in serine, proline, leucine, methionine, and phenylalanine auxotrophs (Hull and Hull 1997). However, in another study of 20 auxotrophs studied in a mouse model of UTI, only a pyrimidine auxotroph was attenuated for survival (Burns and Hull 1999). In another study, arginine and serine auxotrophs of UPEC strain CFT073 were shown to have no fitness defect in urovirulence by competition assay in the mouse model of UTI, though the arginine mutant exited logarithmic growth in vitro in urine at an OD_{600} of 0.45, compared with 0.59 for the parent strain (Alteri et al. 2009b). This suggests that amino acid synthesis genes may play roles in other pathways. Work by Russo et al. (1996b) screened a transposon mutant library of a blood isolate of UPEC for diminished growth in urine in vitro. Additionally, a *lacZ*-fusion library of the same isolate was examined for *lacZ* upregulation in urine compared to laboratory medium (Russo et al. 1996b). This study found that disrupted purine synthesis via disruption of *guaA* resulted in diminished growth in urine and resulted in colonization defects of urine, bladder, and kidneys in a mouse model of UTI. It was also found that the *argC* locus, required for arginine synthesis, was induced in urine in vitro, and *argC* mutants had diminished growth in urine and could not colonize kidneys in a mouse model of UTI. Together, this may suggest that urine is not the sole source of nutrients for bacteria in the host and that the relationship between metabolism and virulence in UPEC is complex. One possibility is that toxins liberate intracellular nutrients during UTI (for a more detailed discussion about cell lysis, see the "Toxins" section in this chapter). The pathways involved in synthesis of metabolic compounds do appear to be related to urovirulence, but the precise mechanisms by which they contribute remain incompletely understood.

Interestingly, D-serine is present in high concentrations in human urine (3.0–40 μg/ml), and UPEC isolates can make use of this amino acid (Roesch et al. 2003; Anfora and Welch 2006). *E. coli* strain CFT073 contains D-serine deaminase (DsdA), and mutation of *dsdA* results in an unusual, pleomorphic cell shape and a prolonged lag phase when cultured in urine as compared to its parent strain. Surprisingly, a *dsdA* mutant of CFT073 outcompetes its parent strain in coinfection experiments by 300-fold (Roesch et al. 2003). However, deleting the L-serine deaminase from CFT073, either in wild-type or in *dsdA* knockout backgrounds, attenuated CFT073 in a mouse model of UTI, suggesting that serine metabolism is not the determinant of high colonization observed in the *dsdA* knockout (Anfora et al. 2007). Microarray data indicates that the *dsdA* mutant of CFT073 has global differential effects on gene regulation compared to the parent strain, suggesting that accumulation of D-serine may be a signal for many other processes in CFT073 (Haugen et al. 2007). Interestingly, D-serine is also gluconeogenic, as it can be degraded into oxaloacetate or pyruvate to be used in gluconeogenesis. This is consistent with previous data indicating that urovirulence in UPEC strain CFT073 is dependent on gluconeogenesis in the mouse model of UTI (Alteri et al. 2009b; Alteri and Mobley 2011).

In the nutrient-scarce urinary tract, the ability to navigate toward areas of higher concentrations of nutrients should provide an advantage in UTI pathogenesis. This notion is supported by the distribution of chemotaxis systems in *E. coli*, which has four methyl-accepting chemotaxis protein receptors for sensing amino acids (encoded by *tar* and *tsr*), saccharides (encoded by *trg*), and dipeptides (encoded by *tap*) (Silverman and Simon 1977; Kondoh et al. 1979; Hedblom and Adler 1980; Manson et al. 1986). *E. coli* strains, including diarrheagenic and fecal strains, encode *tsr, tar, trg*, and *tap*. However, *trg* and *tap* are more commonly absent or mutated in uropathogenic strains (Lane et al. 2006). This suggests that specific nutrient sensing is modified depending on the trophism of specific *E. coli* strains.

To grow in the often acidic environment of the urinary tract, UPEC expresses several acid-resistance genes regulated by two-component systems PhoP-PhoQ and EvgS/EvgA, as well as SafA (Eguchi et al. 2011). Isogenic deletions of *phoP* result in dramatic attenuation of UPEC virulence in the mouse model of UTI (Alteri et al. 2011). PhoP also appears to be involved in resistance to antimicrobial peptides and represses motility of UPEC, so the deficiency in urovirulence may be due to pleiotropic effects. However, all *phoP* deletion phenotypes are linked to modulation of membrane potential and proton-motive force, once again suggesting a link between metabolism and survival in urine (Alteri et al. 2011).

P. mirabilis produces urease, an enzyme that hydrolyzes urea into carbon dioxide and ammonia (Mobley 2001; Nielubowicz and Mobley 2010; Pearson et al. 2011). Without urease, *P. mirabilis* has significantly diminished urovirulence in a mouse model of UTI (Jones et al. 1990; Johnson et al. 1993). Hydrolyzing the high levels of urea into ammonia rapidly alkalinizes urine. Wild-type *P. mirabilis* strain HI4320 urease activity is so strong in vitro that bacteria inhibit their own growth by

over-alkalinizing their environment (Mobley, unpublished observation). In the urinary tract, where urine is constantly refreshed, alkalinization should be somewhat stabilized. However, *P. mirabilis* urease activity is associated with the formation of struvite and other stones (*Fig. 15.3a–e*), a process termed urolithiasis; *P. mirabilis* strains without urease are unable to initiate the precipitation of magnesium ammonium phosphate necessary to generate struvite (Li et al. 2002b). Urolithiasis is itself associated with persistent urinary tract infection (Milliner and Murphy 1993), so the formation of stones by *P. mirabilis* may place the bacterium at an advantage within the urinary tract. Urease activity in *P. mirabilis* may explain why *P. mirabilis* and UPEC coinfection synergistically prolong persistence and bacterial load compared to infection with a single bacterial species in a mouse model of UTI (Alteri and Mobley, unpublished observation).

Both *K. pneumoniae* and *S. saprophyticus* also produce urease, but the contributions made by urease to *K. pneumoniae* urovirulence are less clear, compared to what is known about urease and *P. mirabilis* urovirulence. *K. pneumoniae* harbors a *ure* gene cluster, which is transcribed in response to elevated nitrogen levels (Collins et al. 1993). These urease genes are found in 98 % of cystitis and 100 % of pyelonephritis isolates, respectively (Podschun et al. 1993), and *K. pneumoniae* infection is also seen in some patients developing struvite urinary stones (McCartney et al. 1985; Bichler et al. 2002).

In contrast, the *S. saprophyticus* urease has a clearer function, compared to the urease expressed by *K. pneumoniae* (Gatermann et al. 1989; Gatermann and Marre 1989; Kuroda et al. 2005). Animals treated with *S. saprophyticus* that produced urease exhibited significant bladder lesions, increased bladder weights, and enhanced colonization in this organ (Gatermann et al. 1989; Gatermann and Marre 1989). A urease-deficient *S. saprophyticus* mutant also does not colonize the spleens of infected animals, which also suggests that the urease produced by this organism has a function in bladder and distal site invasive infection (Gatermann et al. 1989). In addition, urinary stones are associated with *S. saprophyticus* infection, similar to other urease producing bacteria (Fowler 1985). However, *S. saprophyticus* urease does not have an obvious function in promoting severe kidney pathology in infected animals (Gatermann et al. 1989; Gatermann and Marre 1989).

Extracellular Polysaccharides

E. coli produce several polysaccharides that coat the surface of the bacterium, including O antigen, LPS, colanic acid, group I and II capsule, and eubacterial common antigen. Polysaccharides may convey several advantages to *E. coli*, such as mediating serum resistance, resistance to phagocytosis (Horwitz and Silverstein 1980; Burns and Hull 1999), and increased adherence to certain cell types. Meta-analysis of the epidemiology of polysaccharides suggests no specific capsular types that are more prevalent in UPEC as compared to commensal *E. coli*, and capsule does not appear to play a role in resistance to human serum in UPEC (Donnenberg and Welch 1996). However, ongoing studies of capsule's role in uropathogenesis are complex and do not all converge. Recent studies implicate specific capsular types in certain components of uropathogenesis. In studies of UTI89, a mutant incapable of producing K1 capsule was deficient in forming IBCs (Anderson et al. 2010), though no differences in colonization in the bladder or kidneys between mutant and parental strain were reported. This work led to the development of a small-molecule screen for inhibitors of capsule formation as alternatives to classic antibiotics (Goller and Seed 2010).

In *E. coli* strain CP9, a K54 capsular mutant showed no difference in uropathogenesis from its parental strain in a mouse model of UTI, although double O4/K54 and single O4 antigen knockouts both showed fitness defects relative to the parental strain (Russo et al. 1995, 1996a). These results suggest that O antigen plays a role in uropathogenesis. In support of this, Cross et al. (1986) demonstrated that O antigen can play a role in serum resistance of *E. coli*, and studies by Burns and Hall support these findings by demonstrating that both LPS and O antigen play crucial roles in serum resistance (Burns and Hull 1998). In addition, C3H/HeJ mice, which produce a dominant negative form of TLR4 and thus have impaired response to LPS, had increased IBC formation compared to the parental strain during UTI with UTI89 (Schilling et al. 2001b).

A signature-tagged mutagenesis screen found attenuated strains of UPEC had insertions in extracellular polysaccharide biogenesis genes. Three such mutants were deficient in capsular production (Bahrani-Mougeot et al. 2002). Those same genes were up regulated in vivo in UPEC strain CFT073 in the mouse urinary tract compared to growth in LB (Snyder et al. 2004). Deleting the cluster II enzymes for producing K2 capsule in CFT073 caused a fitness defect compared to the parental strain in a mouse model of UTI, a defect which was reversed with complementation (Buckles et al. 2009). The same K2 capsular mutant demonstrated increased serum sensitivity compared to the parental strain, which was also reversible with complementation. In addition, a K2 capsular mutant of *E. coli* strain CFT073 was outcompeted by the wild-type parental strain in bacteremia model of disseminated infection in mice (Smith et al. 2010). Together, these results indicate that the role of capsule in *E. coli* UTI is complex and highly dependent on the specific makeup of capsule being studied. The work of determining the exact role of capsule and its contribution to urovirulence are ongoing.

There are at least 78 *K. pneumoniae* capsule serotypes (Pan et al. 2008). The *K. pneumoniae* capsule has a variety of functions associated with virulence including mediating resistance to antimicrobial peptides by limiting the ability of these peptides to bind to the outer membrane (Campos et al. 2004; Llobet et al. 2008). Capsule serotypes also have a function in preventing killing by serum (Simoons-Smit et al. 1986; Nagano et al. 2008). However, the O-antigen serotype of *K. pneumoniae* LPS, not necessarily capsule, may have a more important function in complement resistance (Tomas et al. 1986). Thus, despite

shielding *K. pneumoniae* from antimicrobial peptides, the precise role of *K. pneumoniae* capsule in inhibiting serum-mediated killing remains to be determined.

In addition to antimicrobial peptide and complement resistance, the *K. pneumoniae* capsule (particularly the K1 and K2 serotypes) inhibits phagocytosis by host phagocytes, which is exacerbated by conditions such as diabetes mellitus (Simoons-Smit et al. 1986; Podschun et al. 1992; Lin et al. 2006; Fung et al. 2011). However, *K. pneumoniae* resistance to phagocytosis was not observed uniformly for all capsule serotypes (Simoons-Smit et al. 1986; Podschun et al. 1992; Fung et al. 2011). Nonetheless, it follows that the *K. pneumoniae* capsule suppresses the host immune system, promoting pneumonia and septicemia (Yoshida et al. 2000).

K. pneumoniae infection of the rat urinary tract does not require capsule, but at the same time the O antigen of LPS is important for promoting *K. pneumoniae* UTIs (Camprubi et al. 1993). It is also apparent that the K1 and K2 capsule serotypes are not as prevalent among uropathogenic *K. pneumoniae*, compared with significant infections at other sites (Lin et al. 2010). Thus, taken together, these findings suggest that *K. pneumoniae* capsule may have a more important function during urosepsis.

One study (Lin et al. 2010) showed that an additional virulence plasmid-encoded gene, *rmpA*, is associated with strains of *K. pneumoniae* that cause urinary tract and other significant infections (Nassif et al. 1989a). On the contrary, others showed that *rmpA* was not necessarily associated with strains of *K. pneumoniae* causing UTIs (Yu et al. 2006). Nonetheless, *K. pneumoniae* isolates also encode a homolog of *rmpA*, referred to as *rmpA2*, which share 81 % nucleotide sequence identity, and RmpA2 is also plasmid encoded (Chen et al. 2004). RmpA and RmpA2 function as transcription factors to enhance capsule synthesis (Nassif et al. 1989b; Wacharotayankun et al. 1993; Lai et al. 2003; Cheng et al. 2010). It was also proposed that another gene, *rmpB*, controlled expression of *rmpA* (Nassif et al. 1989b), but it was subsequently suggested that *rmpA* expression was independent of *rmpB* expression (Wacharotayankun et al. 1993). Instead, RmpA and RcsB interact with each other to stimulate capsule gene expression in *K. pneumoniae*, and Fur, instead, has a function in repressing *rmpA* expression (Cheng et al. 2010).

Capsule genes were explored in depth for at least one *S. saprophyticus* serotype known to cause UTI (Park et al. 2010). This *S. saprophyticus* capsular gene locus carries at least 13 open reading frames, including known homologs of other *Staphylococcus* spp. capsule genes (Park et al. 2010). However, as expected, other strains of *S. saprophyticus* encode different capsular genes (Park et al. 2010). As highlighted in other capsule sections of this chapter, and as observed with other species of *Staphylococcus* (Xu et al. 1992; Nilsson et al. 1997; Thakker et al. 1998; Flahaut et al. 2008), the *S. saprophyticus* capsule has a function in obstructing phagocytosis (Park et al. 2010).

S. saprophyticus capsule also inhibits adherence to host cells. Indeed, loss of capsule increases adherence to T24 bladder carcinoma cells (Kuroda et al. 2005). However, capsule expression is not uniform in cultured *Staphylococcus aureus* cells (Poutrel et al. 1997). Therefore, *S. aureus* and *S. saprophyticus* resistance to phagocytosis while at the same time maintaining the ability to adhere to host cells could be achieved if a population of Staphylococci is made up of some bacteria that express high levels of capsule and others that express lower levels of capsule (Poutrel et al. 1997; Park et al. 2010). Nevertheless, how capsule expression in *S. aureus* and *S. saprophyticus* is coordinated is unknown.

Metal Acquisition

Iron

In humans and mice, free iron is scarce. Most iron is complexed in hemoglobin and iron-carrying proteins such as ferritin. During infection, the host further restricts bioavailable circulating iron by reducing the production of the iron-transporting glycoprotein transferrin, which is present in the blood and at mucosal surfaces (Barisani and Conte 2002), increasing the production of haptoglobin and hemopexin, which sequester free hemoglobin and heme (Boretti et al. 2009), and increasing production of hepcidin, which reduces absorption of iron in the gut (Ganz 2006). All uropathogenic bacteria require iron to live, but iron is not freely available in the urinary tract, and urine contains only trace amounts of iron (Dlugaszek et al. 2011). On the other hand, the gut, thought to be the reservoir for most bacteria that cause uncomplicated UTI, is relatively iron replete (Hahn et al. 1939). An overabundance of iron import can be damaging to an organism via the formation of oxidative free radicals. Thus, it is not surprising that UPEC encodes numerous iron receptors and has developed multiple strategies for mediating iron acquisition (Wandersman and Delepelaire 2004).

UPEC carry three general classes of iron-uptake systems: siderophores (aerobactin, enterobactin, salmochelin (Welch et al. 2002), and yersiniabactin (Garcia et al. 2011)), hemophores (or heme-binding and uptake systems, such as Hma or ChuA (Hagan and Mobley 2009)), and direct ferrous (FeII) or ferric (FeIII) iron uptake systems. Siderophores are small organic molecules that are secreted into the environment, have very high affinity for ferric iron, and can strip the metal ion from other complexes within the host or bind rare free ferric iron, which is normally present only in trace quantities. Once a siderophore binds iron, the ferrisiderophore complex may be bound by specific outer membrane receptors. Hemophores in UPEC, on the other hand, such as Hma and ChuA (Hagan and Mobley 2009), are outer membrane proteins that bind heme with high affinity and import it back into the bacterium (Wandersman and Delepelaire 2004). Finally, *E. coli* encodes a conserved ABC transporter on the *feo* operon, capable of directly importing ferrous iron (Kammler et al. 1993).

Both siderophore- and hemophore- mediated iron uptake depend on ferric iron. TonB, an inner membrane protein, is required for the function of all ferric iron uptake receptors in *E. coli*. The TonB-ExbBD complex transmits proton-motive force generated at the inner membrane to the outer membrane

receptors (Higgs et al. 1998). TonB mutants are defective for colonizing kidneys in coinfection in a mouse model of UTI, a defect that is complementable (Torres et al. 2001). Additionally, in independent challenge, the *tonB* mutant strain caused reduced kidney colonization compared to the parental and complemented strains at 48 h post inoculation. The prototype UPEC strain CFT073 contains at least ten ferric uptake systems and several putative systems (Welch et al. 2002). Mutations that disrupt salmochelin, enterobactin, heme, and other siderophores are outcompeted by wild-type UPEC in coinfection (Russo et al. 2001, 2002; Johnson et al. 2005; Hagan and Mobley 2009; Watts et al. 2012).

Iron acquisition appears to be especially important during UTI. The iron-chelating hydroxamate siderophore aerobactin is more common among UPEC strains than in fecal/commensal *E. coli* (Carbonetti et al. 1986; Johnson et al. 1988), and the presence of at least one siderophore synthesis system appears to be a common feature of UPEC strains (Vigil et al. 2011b). Microarray gene expression studies of urine from patients suffering from bacterial UTI suggest that bacterial iron acquisition systems are highly upregulated during infection (Hagan et al. 2010). Isotope dilution studies of siderophore activity, which can be more sensitive than transcript/expression analysis, confirm that siderophore activity is increased in UPEC compared to commensal strains of *E. coli* (Henderson et al. 2009). Highlighting their importance, there is substantial notable redundancy in iron acquisition systems in UPEC. Aerobactin-, enterobactin-, and heme-mediated iron uptake can each complement the activity of one another. Isogenic mutants of aerobactin or enterobactin were shown to be no different from wild type in kidney colonization in a mouse model of UTI (Torres et al. 2001). Vaccine studies using siderophore receptors (IutA, IreA) or heme-binding proteins (Hma) as antigens and a mucosal route of delivery show protection from transurethral challenge in the mouse model of UTI (Alteri et al. 2009a), offering further support that iron systems are requisite virulence factors during the course of a UTI.

In *P. mirabilis*, iron acquisition systems have also been implicated in fitness during UTI. A recent analysis of the *P. mirabilis* strain HI4320 revealed a vast network of systems implicated in iron acquisition, including at least eight loci devoted to five different mechanisms of iron acquisition, three other iron-related ABC transporters, and ten additional TonB-dependent receptors. In addition, two previously unidentified siderophores were discovered (Pearson et al. 2008), which have been shown to be functional. Mutants of *P. mirabilis* lacking yersiniabactin-related siderophores have reduced fitness during UTI (Himpsl et al. 2010), and several studies implicate an amino acid deaminase (Aad) in *P. mirabilis* iron acquisition as well (Evanylo et al. 1984; Drechsel et al. 1993; Massad et al. 1995). In addition, *P. mirabilis* can acquire iron in the forms of heme and hemin using HmuR2. Disruption of HmuR2 leads to a colonization defect during experimental UTI (Lima et al. 2007).

K. pneumoniae also makes use of iron acquisition systems and encodes many of the same iron-scavenging systems found in UPEC. Many of these iron-scavenging genes are induced by serum (Hsieh et al. 2008), and as above, the transcription factor Fur has a function in regulating iron-scavenging gene transcription (Lin et al. 2011). Likewise, as in UPEC, the inner membrane protein TonB (Moeck and Coulton 1998; Torres et al. 2001) is an important contributor to *K. pneumoniae* virulence, as *tonB* mutants show severe attenuation in vivo (Hsieh et al. 2008). This highlights the importance of iron acquisition during *K. pneumoniae* UTIs.

Zinc

In addition to iron, transport of the cation zinc (Zn^{2+}) has been implicated in fitness of bacteria during UTI. In UPEC strain CFT073, deletion of the zinc uptake regulatory operons *znuACB* or *znuACB* together with *zupT*, but not *zupT* alone, resulted in a comparative disadvantage in both bladder and kidney colonization in the mouse model of UTI compared to the parental strain (Sabri et al. 2009). Deletion of the zinc importer operon *znuACB* in *P. mirabilis* HI4320 leads to a comparative disadvantage in uropathogenesis in the mouse model of UTI compared to the parental strain, as well as impairing normal motility (Nielubowicz et al. 2010). This indicates that zinc uptake and regulation contributes to, but is not required for, uropathogenesis of *P. mirabilis* during UTI.

Toxins

Hemolysin (HlyA)

The hemolytic activity of some extraintestinal *E. coli* isolates is associated with virulence and is mediated by α-hemolysin production and secretion (Welch et al. 1981, 1983). The sequence of the *hlyCABD* operon suggests that these genes were likely acquired via a recent horizontal gene transfer event, as it neither reflects codon usage nor G + C content of the *E. coli* backbone (Felmlee et al. 1985b).

Studies on transcriptional organization suggested that at least two transcripts of the *hly* operon are expressed: *hlyCA* and a full-length *hlyCABD* transcript (Welch and Pellett 1988). Transcriptional antitermination accounts for this transcript differential. The amount of full-length transcript depends on the stage of growth of an *E. coli* culture. Maximal antitermination is observed during the early exponential phase, and moderate to low levels of antitermination are observed at the middle through late exponential and stationary phase, respectively (Koronakis et al. 1989a). An additional promoter may be located upstream of *hlyD*, which is logical when one considers HlyD stoichiometry necessary for HlyA secretion (Felmlee et al. 1985b; Mackman et al. 1985; Thanabalu et al. 1998; Holland et al. 2005) (see secretion section below). A weaker promoter may also exist upstream of *hlyB* (Felmlee et al. 1985b).

hlyA encodes the α-hemolysin protein, which is the prototype for a family of proteins known as the repeats-in-toxin

(RTX) family (Welch 1991). The RTX family harbors members that have a wide range of activities but are unified by their glycine- and aspartate-rich repeat consensus sequences near their C-termini and their secretion into the extracellular milieu via a type 1 secretion system (T1SS) (Welch 1991; Linhartova et al. 2010; Satchell 2011). From various sources, the RTX consensus sequence is GGXG(N/D)D (Welch 1991; Linhartova et al. 2010; Satchell 2011). These repeat sequences are hypothesized to coordinate binding of divalent metal cations, particularly Ca^{2+} (Linhartova et al. 2010; Satchell 2011), which facilitates extracellular protein folding (Linhartova et al. 2010). HlyA was predicted to have at least 13 tandem repeats of the RTX consensus sequence near its C-terminus (Felmlee and Welch 1988).

HlyA is a well-characterized pore-forming cytolysin (Bhakdi et al. 1986; Braun and Focareta 1991). Pores have been suggested to form by insertion of protein monomers into target membranes (Bhakdi et al. 1986). However, others have suggested that HlyA may form pores through oligomerization (Benz et al. 1992; Herlax et al. 2009). As the name suggests, α-hemolysin binds to and lyses erythrocytes (*Fig. 15.4*), and this activity is dependent on extracellular Ca^{2+} (Ludwig et al. 1988; Boehm et al. 1990). However, other reports have challenged this result by suggesting that Ca^{2+} only enhances pore formation (Bhakdi et al. 1986). This protein is also cytotoxic to granulocytes (Gadeberg et al. 1983; Gadeberg and Orskov 1984), monocytes and lymphocytes (to a small extent, compared to the other three cell types) (Gadeberg et al. 1983). Lysis was also found in cultured renal proximal tubular epithelial cells, as indicated by an enhanced release of lactate dehydrogenase (LDH) from these cells, following treatment with a hemolysin-producing strain of *E. coli* (Mobley et al. 1990). In vivo studies revealed that HlyA contributes to enhanced damage and hemorrhaging in the uroepithelium and bladder, suggesting a significant role in virulence (Smith et al. 2008). However, this damage to the uroepithelium is most apparent early in infection (*Fig. 15.5b, h*) (i.e., within the first day), and loss of hemolysin did not preclude UPEC from colonizing the mouse bladder and kidneys (Smith et al. 2008).

In addition to the above activities, HlyA, as one might predict from the membrane-associated activity of this protein, is implicated in host cell Akt (protein kinase B) perturbations at concentrations not causing significant lysis. HlyA was found to stimulate the dephosphorylation of Akt that in turn resulted in reduced phosphorylation of Akt targets GSK-3β and FOXO1, an effect that has been predicted to impair the inflammatory response and cell survival (Wiles et al. 2008). Insertion of HlyA into a host cell membrane may also lead to indirect activation of host cell caspases, mesotrypsin, and likely other host cell tosyl-L-lysine-chloromethyl ketone-sensitive trypsin-like serine proteases. Stimulation of these host cell proteases mediates the proteolytic degradation of a variety of proteins including paxillin, β-catenin, IκBα, and RelA. This suggests that HlyA may also function to disrupt the host cell cytoskeleton, cell survival, and the host inflammatory response (Dhakal and Mulvey 2012).

Acylation of HlyA is required for membrane binding. However, the acyl chains on HlyA may also serve a function in mediating HlyA oligomerization (Benz et al. 1992; Herlax et al. 2009). HlyA acylation is mediated through the function of HlyC and the acyl carrier protein (ACP) (Hardie et al. 1991; Issartel et al. 1991; Hughes et al. 1992; Ludwig et al. 1996; Stanley et al. 1998). Acyl chains are added to HlyA at Lys564 and Lys690, and these events may depend on the peptide sequences close to these modification sites (Stanley et al. 1994; Ludwig et al. 1996).

Secretion of mature HlyA has several unique features that resulted in early characterization of T1S. HlyA is secreted from *E. coli* without an obvious cleavage of an N- or C-terminal signal (Felmlee et al. 1985a; Koronakis et al. 1989b; Holland et al. 2005). A signal localized to the C-terminus of HlyA is important to mediate secretion (Gray et al. 1986; Mackman et al. 1987; Koronakis et al. 1989b; Holland et al. 2005), and production of a chimeric protein containing peptide sequences of OmpF and LacZ fused to a portion of the C-terminus of HlyA resulted in secretion of this construct into the extracellular environment (Gray et al. 1986; Mackman et al. 1987). However, the precise nature of the C-terminal signal remains unclear. Nevertheless, hemolysin secretion does not proceed through a periplasmic intermediate and does not accumulate in this compartment, even when the toxin is incapable of being secreted (Gray et al. 1986; Koronakis et al. 1989b; Holland et al. 2005).

HlyA secretion requires the activity of HlyB, HlyD (Wagner et al. 1983; Koronakis et al. 1992; Holland et al. 2005), and the outer membrane protein TolC (Wandersman and Delepelaire 1990; Holland et al. 2005). HlyB has sequence homology to other known ATP-binding proteins (Gerlach et al. 1986; Higgins et al. 1986). Additional work verified that HlyB was an ATPase and that ATP-binding resulted in conformational changes of the HlyB ATPase domain; ATP hydrolysis is independent of the presence of a protein that is capable of being secreted and plays an important role during HlyA secretion (Koronakis et al. 1993; Thanabalu et al. 1998; Holland et al. 2005). In addition, HlyB dimers also bind the HlyA C-terminal signal (Koronakis et al. 1993; Benabdelhak et al. 2003; Holland et al. 2005).

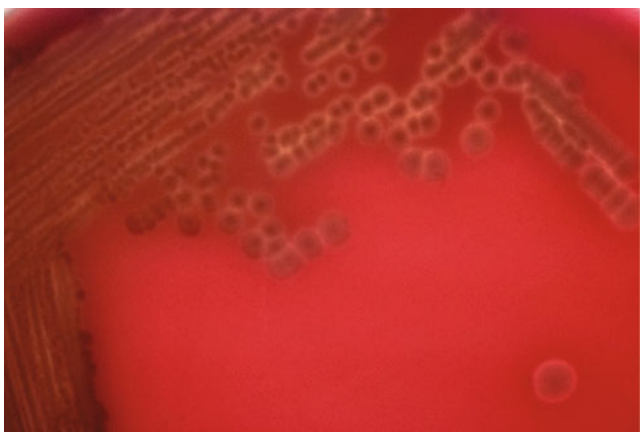

Fig. 15.4
Uropathogenic *E. coli* strain CFT073 is β-hemolytic on blood agar as a result of HlyA secretion

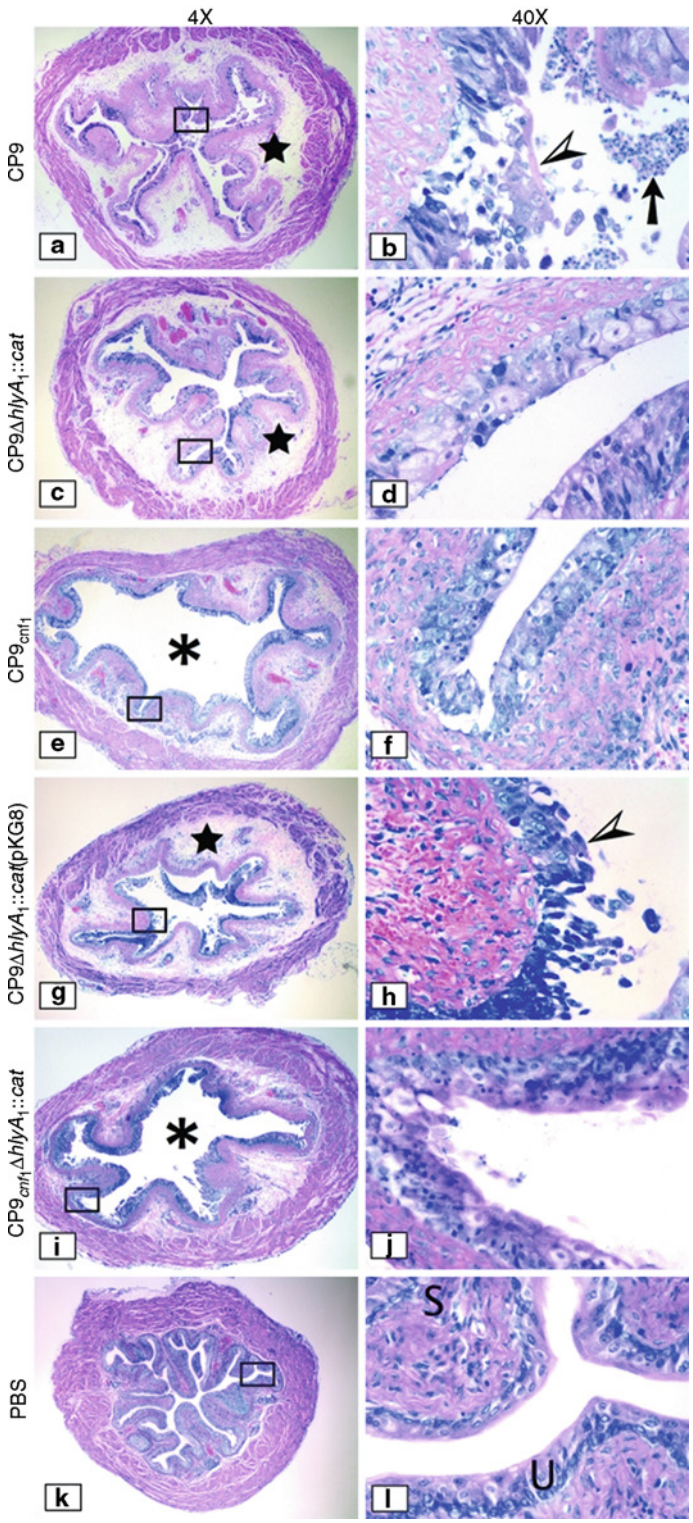

Fig. 15.5

The toxins HlyA and CNF1 cause significant pathology in vivo. **Bladder histopathology of female C3H/HeOuJ mice transurethrally inoculated with *E. coli* strain CP9 (a and b), the indicated CP9 mutants (c–j), a complemented CP9 mutant (CP9Δ*hlyA1::cat*(pKG8)) (g and h), or PBS (k and l) at 1 day post inoculation. *Rectangles* show the regions that were magnified at 40X to the *right* of the 4X image. A *star* in (a, c, and g) shows regions with significant edema, and an *asterisk* (e and i) indicates luminal space. The *arrow* in (b) points to a large number of inflammatory cells, and the *arrowheads* in (b) and (h) point to regions where the urothelium is damaged. *U* and *S* in (l) indicate the regions corresponding to the murine urothelium and submucosa, respectively (Reprinted with permission from Smith et al. (2008))**

HlyD forms the channel through which HlyA is conducted (Thanabalu et al. 1998; Holland et al. 2005). Trimers of HlyD also form a stable complex with TolC trimers, which is mediated through the HlyB-HlyD complex recognition of HlyA (Thanabalu et al. 1998; Holland et al. 2005). HlyD also helps facilitate the folding of the transported HlyA. This protein-folding function depends on residues located within the periplasmic domain of HlyD (Pimenta et al. 2005).

Thus, a general model for T1S includes recognition of the C-terminal signal sequence (and ATP binding) by HlyB and at some point HlyA recognition by HlyD. Stable association of HlyD with the outer membrane TolC pore follows these events. Following ATP binding and TolC-HlyD complex formation, HlyA is translocated through the cytoplasmic membrane and into the HlyD channel (now continuous with the pore produced by TolC), and following ATP hydrolysis, the system is reset for another round of protein secretion (Holland et al. 2005). Proton-motive force (PMF) is also important for early events in this process (Koronakis et al. 1992; Holland et al. 2005), but the exact contribution of PMF remains unclear.

Hemolytic activity in *Proteus mirabilis* and *P. vulgaris* is mediated by the Ca^{2+}-independent HpmA hemolysin, which is the primary hemolysin of *Proteus* spp. (Welch 1987; Swihart and Welch 1990b). HpmA has cytotoxic activity for a variety of cultured cells, including human B-cell lymphomas, human monocytes, kidney cells derived from African green monkeys, bladder epithelial cells, and cultured human renal cells; HpmA also mediates erythrocyte lysis (Swihart and Welch 1990a, b; Mobley et al. 1991; Alamuri et al. 2009). However, infection of the murine urinary tract with an *hpmA* mutant resulted in only a moderate, but statistically insignificant, reduction in the severity of the pathology in the bladders and kidneys of the infected mice and CFUs in these organs (Alamuri et al. 2009). Nevertheless, statistically significant reduction in bladder and kidney pathology was only achieved once *hpmA* and *pta* (see below) were both mutated together, arguing that HpmA must be playing some role in pathogenesis (Alamuri et al. 2009).

Based on sequence homology to ShlA and ShlB of *Serratia marcescens* and functional characterization, it was suggested that another protein, HpmB, is required for secretion of HpmA, while HpmA is required for hemolytic activity (Uphoff and Welch 1990). This pair of proteins constitutes a subtype of type 5 secretion (T5S), known as a two-partner system (reviewed in (Jacob-Dubuisson et al. 2001; Henderson et al. 2004; Newman and Stathopoulos 2004)). In agreement with this model of protein secretion, the N-termini of HpmA and HpmB both contain a leader sequence (Uphoff and Welch 1990). Thus, a general model for a two-partner secretion system involves HpmA and HpmB both utilizing the Sec secretion system for entry into the periplasm. After this translocation event, HpmB forms a pore in the outer membrane, which is exploited by HpmA for secretion into the extracellular milieu (Jacob-Dubuisson et al. 2001; Henderson et al. 2004; Newman and Stathopoulos 2004).

TosA

Approximately one-fourth of UPEC strains also encode an additional RTX protein, TosA (named for its putative mechanism of export, *t*ype *o*ne *s*ecretion), which was identified during an in vivo-induced antigen technology (IVIAT) screen (Vigil et al. 2011a, b). A putative T1SS is associated with TosA (Vigil et al. 2011c), but the *tos* locus secretion system genes precede *tosA*, which is the opposite of what is observed in the *hly* operon (Welch et al. 2002). However, as one may predict from having identified TosA in an IVIAT screen, *tosA* and its associated T1SS are highly expressed only in vivo (Vigil et al. 2011a, c).

The presence of the *tosA* gene in a specific UPEC strain is a predictor of urovirulence (Vigil et al. 2011b). In a survey of a large *E. coli* strain collection, including UPEC clinical isolates, encoding *tosA* on average predicted the presence of 11.2 of the 15 virulence and fitness genes used in the screen (Vigil et al. 2011b). UPEC strains not encoding *tosA* encoded on average only 5.4 of the 15 virulence and fitness genes used in the same screen (Vigil et al. 2011b).

Additional work verified that TosA is an important virulence factor, but not as a toxin. TosA mediates adherence of UPEC to host uroepithelial cells, especially those derived from the upper urinary tract. These results were corroborated by membrane fractionation, immunohistochemistry, proteinase K digestion assays, and immunogold labeling, which suggested that TosA localized to the outer membrane and was exposed to the extracellular milieu, which is different compared to HlyA secretion (Vigil et al. 2011c). TosA expression resulted in a more efficient colonization of the bladder and kidneys of mice transurethrally challenged with UPEC (Vigil et al. 2011a, b). It was also found that this protein served a role during bacteremia, as a *tosA* mutant was outcompeted by the parental wild-type strain in the liver and spleen of mice inoculated with these strains via tail vein injection. Vaccination with purified TosA resulted in protection against colonization of the spleen during transurethral challenge with virulent UPEC (Vigil et al. 2011c). However, the same vaccination trial revealed little protection against colonization of the bladder and kidneys, confounding the interpretation of the functions that TosA is playing in the urinary tract (Vigil et al. 2011c). Nevertheless, cell adherence and biofilm formation are noted roles for other RTX proteins (Linhartova et al. 2010; Satchell 2011).

CNF1

The toxin, cytotoxic necrotizing factor 1 (CNF1), was identified in *E. coli* and found to induce morphological changes in a variety of cultured cells as well as necrotic activity in vivo (Caprioli et al. 1983). It was also recognized that *E. coli* encoded two CNF toxins (CNF1 and CNF2), both having similar activities and sharing homology (evidenced by serum cross-neutralization) (De Rycke et al. 1990). This toxin is found in 37 % of UPEC strains causing cystitis and 30 % of UPEC strains causing pyelonephritis (Landraud et al. 2000), which suggests a function in urovirulence.

CNF1 function during a urinary tract infection has been extensively studied. It was predicted that CNF1 stimulates production of reactive oxygen species in PMNs, which could cause damage to the uroepithelium (Hofman et al. 2000). Indeed, CNF1 was found to cause cytopathic effects in a variety of cells derived from the upper and lower urinary tract and cause cell death in at least one bladder cell line (Mills et al. 2000). These results were corroborated in vivo by observing reduced virulence in the urinary tract during infection with a CNF1 mutant (Rippere-Lampe et al. 2001). CNF1 also mediates changes in the cytoskeleton of neutrophils (Hofman et al. 2000) and may cause reduced chemotaxis and antimicrobial activity in host phagocytes (Hofman et al. 2000; Davis et al. 2006). Therefore, another function of CNF1 during UTI is to reduce UPEC killing by neutrophils, thereby enhancing colonization of the urinary tract. The CNF1 produced by UPEC also promotes bladder inflammation and submucosal edema (*Fig. 15.5a, c, g*) (Rippere-Lampe et al. 2001; Smith et al. 2008). In addition, CNF1 allows *E. coli* to access underlying tissues and may contribute to UPEC persistence in the lower urinary tract (Mills et al. 2000; Rippere-Lampe et al. 2001). However, others have suggested that the function of CNF1 may be provided by another factor or may not be required by UPEC, as one study showed that CNF1 might not be essential for UPEC virulence (Johnson et al. 2000).

Much of the work describing CNF1 activity has focused on the morphological changes induced in eukaryotic cells by this protein, which is accomplished through actin reorganization (Fiorentini et al. 1995; Hofman et al. 2000). These changes in morphology are mediated through activation of Rho GTPases, which regulate remodeling of the actin cytoskeleton in host cells (Flatau et al. 1997; Schmidt et al. 1997; Doye et al. 2002). A similar phenotype is observed for CNF2 (Oswald et al. 1994). CNF1 deamidates Gln63 of Rho GTPase, which leads to constitutive Rho activity and actin remodeling (Fiorentini et al. 1995; Flatau et al. 1997; Schmidt et al. 1997). Similar modifications were proposed for other Rho GTPases, particularly Rac and Cdc42 (Lerm et al. 1999). Others have suggested that while the activity of Rho GTPases may be constitutive after this modification, this elevated activity may only be transient, as ubiquitination and subsequent degradation of these active GTPases are also observed (Doye et al. 2002).

CNF1 may also mediate internalization of *E. coli* into susceptible cells. Purified CNF1 caused membrane ruffling and internalization of bacteria into epithelial cells, likely induced by perturbations to Rac and Cdc42 signaling (Falzano et al. 1993; Lerm et al. 1999). Indeed, enhanced activity of Rac and subsequent downregulation of this protein resulted in an increased internalization of UPEC into uroepithelial cells (Doye et al. 2002). It has also been shown that internalization of UPEC mediated through Rac1 also depends on an associated partner, Tollip, which associates with Rac1 and ubiquitinated Rac1 (Visvikis et al. 2011). Therefore, it remains unclear whether or not this internalization is a passive process owing to UPEC adhering to these epithelial cells and being internalized during the downregulation of active Rac.

Although the activity of CNF1 requires secretion from *E. coli*, the mechanism of CNF1 secretion from UPEC remains unclear. However, active CNF1 has been isolated from outer membrane vesicles released from *E. coli* (Davis et al. 2006; Kouokam et al. 2006). In addition, translocation of CNF1 from the cytoplasm into the periplasm (a step that one would predict to be a prerequisite for outer membrane vesicle secretion) requires the activity of ferredoxin, but the precise role of this protein in mediating CNF1 translocation is unclear (Mills et al. 2000; Yu and Kim 2010). Likewise, alternative secretion mechanisms of CNF1 have also been proposed including secretion through lysis of a bacterial cell or secretion requiring UPEC contact with host epithelial cells (Falzano et al. 1993).

Autotransporter Toxins

UPEC encode a number of autotransporter toxins. Secreted autotransporter toxin (Sat) is a serine protease autotransporter of *Enterobacteriaceae* (SPATE) family member. Sat has a consensus sequence of known serine proteases and degrades factor V and spectrin in host cells and can cause cytopathic effects in HEp-2 cells (Guyer et al. 2000; Dutta et al. 2002). In addition to these activities, Sat was also found to induce the formation of vacuoles in cultured bladder and kidney cells (Guyer et al. 2000, 2002). Interestingly, vacuolation by Sat was observed more readily in kidney cells, suggesting an enhanced susceptibility of the kidney epithelium to this toxin. Recently, Sat was also shown to stimulate autophagy and cell detachment in HeLa cells and mouse embryonic fibroblast (MEF) cells (Lievin-Le Moal et al. 2011).

Another SPATE autotransporter of UPEC, Pic, displays mucinolytic activity (Dutta et al. 2002; Parham et al. 2004; Navarro-Garcia et al. 2010) and can also cleave factor V in vitro (Dutta et al. 2002). RT-PCR showed that the gene encoding Pic was expressed during an experimental UTI (Heimer et al. 2004). While the precise role for Pic during UTI is unknown, around 15 % and 31 % of cystitis and pyelonephritis strains, respectively, encode Pic (Heimer et al. 2004) suggesting it may be an important virulence factor.

An additional SPATE protein of UPEC is Tsh/Vat (temperature-sensitive hemagglutinin/vacuolating autotransporter toxin) (Parham et al. 2004). Tsh has mucinolytic activity and proteolytic activity against factor V (Dutta et al. 2002), and 61 % and 65 % of cystitis and pyelonephritis strains encode this gene, respectively (Heimer et al. 2004). Although this protein has sequence homology to other serine proteases, this type of activity against the substrate casein is controversial (Stathopoulos et al. 1999; Heimer et al. 2004; Kostakioti and Stathopoulos 2004). However, it is generally accepted that Tsh has a role in adherence, which is evidenced by mediating hemagglutination and hemoglobin binding (Provence and Curtiss 1994; Stathopoulos et al. 1999; Kostakioti and Stathopoulos 2004). The vacuolating activity of the Tsh homolog, Vat, has also been well described in cells (Parreira and Gyles 2003).

Proteus Toxic Agglutinin (Pta)

P. mirabilis also encodes at least two autotransporter toxins, HpmA and Pta (*Proteus* toxic agglutinin). This latter protein was identified as a serine protease, containing a characteristic catalytic triad composed of serine, histidine, and aspartate at residue positions 366, 147, and 533, respectively. Pta protease activity was confirmed by the observation that Pta could digest chymotrypsin-specific substrates. However, contrary to what one might expect for many proteases, Pta was found localized in the outer membrane fraction of fractionated *P. mirabilis* cells, and a predicted domain of this protein rich in beta sheets was important for this localization. Pta also promotes cell adherence, and cells lacking *pta* had a reduced degree of autoaggregation compared to cells expressing a functional Pta. The separate adherence and proteolytic activities of Pta are associated with the passenger domain (Alamuri and Mobley 2008).

Pta is cytotoxic to a variety of cell lines, causing lysis in bladder epithelial cells, human kidney cells, and monkey kidney cells. Pta also causes changes in the actin cytoskeleton by depolymerizing F-actin, and it damages the nucleus of a target cell. Experimental mouse UTI also confirmed that Pta is a virulence factor, as during co-challenge with the parental HI4320 strain of *P. mirabilis*, a *pta* mutant was outcompeted by the wild-type strain in the bladder, kidney, and spleen (Alamuri and Mobley 2008). Mice challenged with individual *P. mirabilis* strains observed a less severe pathology in the kidney when treated with a *pta* mutant. Reduced colonization by a *pta P. mirabilis* mutant was also observed in the bladder, kidney, and spleen of infected animals. Mice intranasally vaccinated with inactive Pta or the passenger domain alone also resulted in reduced *P. mirabilis* colonization in the kidneys and spleen of vaccinated animals (Alamuri et al. 2009).

Urease

In addition to its function in stone formation (urolithiasis), the urease of *P. mirabilis* may generate cytotoxic effects toward the uroepithelium. Urease cytotoxicity, as determined by elevated LDH released from cultured cells, is observed in human renal proximal tubular epithelial cells (HRPTECs). LDH release from HRPTECs, during coculture with a urease producing *P. mirabilis* strain or urease alone, is also dependent on the presence of extracellular urea (Mobley et al. 1991). Cell death, renal damage, and urothelium disruption promoting tissue invasion are suspected to be due to urease-catalyzed ammonia production and subsequent alkalization of the urinary tract (Braude and Siemienski 1960; Musher et al. 1975; Jones et al. 1990; Johnson et al. 1993). However, unlike the other toxins described above, urease is found in the *P. mirabilis* cytoplasm (Jones and Mobley 1988).

Emerging Virulence Factors

PhoU

In an unbiased mutagenesis screen of over 2,000 CFT073 transposon insertion mutants, several novel virulence factors involved in uropathogenesis were identified (Bahrani-Mougeot et al. 2002) in addition to those described previously. Among the virulence factors identified was *phoU*, the last gene in the *pst-phoU* operon (Bahrani-Mougeot et al. 2002). *phoU* encodes either a repressor or corepressor of the phosphate transport system, itself encoded by the *pstSCAB-phoU* operon (Surin et al. 1985). UPEC that contains an isogenic deletion of *phoU* is outcompeted by the wild-type parental strain in coinfection in the mouse model of UTI. This defect in uropathogenesis is reversible with complementation of the *phoU* gene (Buckles et al. 2006). In *P. mirabilis* strain HI4320, transposon mutants that disrupted *pstS* or *pstA* genes also displayed reduced fitness in the mouse urinary tract compared to wild type, a defect that was reversible with complementation (Jacobsen et al. 2008). The impaired phosphate transporter system did not affect growth of *P. mirabilis* HI4320 in vitro under phosphate-limited conditions, leading the authors to conclude that the *pst* system provides a selectively in vivo fitness advantage to *P. mirabilis* in the mouse model of UTI. Together, these data suggest that regulation of phosphate transport is important in both UPEC and *P. mirabilis* pathogenesis in the murine urinary tract. The precise mechanism of that role is not yet well understood, however.

CdiA and CdiB

Aoki et al. (2005, 2009) identified contact-dependent inhibition genes, *cdiA* along with *cdiB*, among various strains of *E. coli* including UPEC strains (Aoki et al. 2005). The *cdi*-system involves secretion of CdiA and CdiB that bind to BamA on target cells, repressing their growth. This repression acts via downregulation of numerous metabolic processes and is reversible if target cells both express an immunity protein, CdiI, and are exposed to sufficiently rich medium (Aoki et al. 2009). The role that this system plays in urovirulence is currently speculative as the contribution of contact-dependent inhibition to UTI pathogenesis has not been directly demonstrated.

USP and Microcins

Uropathogenic-specific protein (USP) is encoded more frequently in UPEC strains than in human fecal (Nakano et al. 2001; Bauer et al. 2002; Kanamaru et al. 2003) or animal fecal (Kurazono et al. 2003) strains of *E. coli*. Further evidence for the role of USP in virulence derives from a study that showed that USP is encoded on a pathogenicity island. The same work demonstrated that addition of the USP gene on a plasmid to UPEC strains that normally do not encode USP enhanced their virulence in the mouse model of UTI compared to wild type

(Yamamoto et al. 2001). Amino acid homology suggests that USP is a bacteriocin (Parret and De Mot 2002; Sharma et al. 2002) with C-terminal endonuclease activity and a nearby ORF encoding the immunity factor. Bacteriocins are a class of bacterial-encoded secreted factors capable of killing bacteria that do not encode the appropriate immunity factor, both within and across bacterial species. The mechanism by which USP conveys a fitness advantage to UPEC during UTI in mice is not known, but given the possibility that USP is a bacteriocin, it is sensible speculation that USP inhibits the growth of competing bacteria within the urinary tract.

In addition to USP, microcins H47 and M and their conjugate immunity proteins are found in the genomic sequences of UPEC strains CFT073, 536, and UTI89. Microcin production in *E. coli* is Fur-regulated, and microcin-producing strains, but not isogenic *mchFDEC* deletion strains, outcompete strains sensitive to microcin-induced killing in broth culture in the presence of an iron chelator (Patzer et al. 2003). This supports the model that environmental iron can affect the efficiency of microcin-dependent killing. It has been proposed that microcin uptake may be mediated by catecholate siderophore receptors such as FepA, Cir, or Fiu, as mutants in those systems were resistant to microcin-induced killing. As UPEC strains often carry multiple siderophore receptors, microcins could take advantage of these conserved import pathways to induce interstrain killing under iron-limited conditions, such as those found within the urinary tract (Patzer et al. 2003).

DegS

DegS is an inner membrane protease required to relieve transcriptional repression of the sigma E regulon, which itself encodes a system of proteins required for maintenance and repair of the *E. coli* cell envelope. In independent challenge, isogenic *degS* deletions of UPEC strain CFT0733 had a fitness disadvantage during UTI or peritonitis in mice, compared to the wild-type parental strain. The virulence defect was reversible with complementation (Redford et al. 2003). Isogenic deletions of two sigma E-dependent genes, *degP* and *skp*, in UPEC strain CFT073, resulted in fitness defects in co-challenge in a murine model of UTI (Redford and Welch 2006). The mechanism by which *degP* and *skp* contribute to virulence in murine UTI remains unknown, although both are periplasmic chaperones. Together, these data suggest a model in which the DegS-sigma E-*degP/skp* pathway contributes to urovirulence of UPEC strains.

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16 Burn Infections

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Burns are a common and devastating form of trauma. Worldwide, the incidence of burns severe enough to require medical attention in 2004 was nearly 11 million people and ranked fourth in all injuries (Peck 2011). Recent data from the United States estimate that approximately 450,000 upward to 1.1 million burn injuries occur per annum based on visits to hospital emergency departments (ABA 2011; CDC 2011). Moderate to severe burn injuries requiring hospitalization account for approximately 45,000 of these cases, of which 20,000 are major burns involving ≥25 % of the total body surface area. Based on selected statistics for admissions to burn centers in the United States, the overall survival rate from burn injury was 94.8 % in the past decade (2000–2009), and improved survival is attributed to medical advances in fluid resuscitation, nutritional support, pulmonary care, burn wound care particularly early excision and wound closure, and infection control practices. However, although approximately 3,500–4,500 patients currently die each year as a direct result of their burn injury, up to 10,000 patients die from burn-related infections, particularly sepsis from burn wound infection, or other sources often associated with inhalation injury (Roth and Hughes 2004; Church et al. 2006; ABA 2011; CDC 2011). This chapter reviews our current understanding of the mechanism, pathogenesis, immune response, diagnosis, management, and prevention of burn wound infections.

Burn Injury

The skin is the largest organ in the human body, measuring 0.2–0.3 m^2 in the adult (*Fig. 16.1*, Roth and Hughes 2004). It consists of two layers, the epidermis and the dermis, both of which vary in thickness in different areas of the body, the epidermis being thickest in the soles of the feet, while the maximal dermal thickness is present in the back. The dermis is several times thicker than the epidermis in all locations. Skin is thin in newborns, increases until mature adulthood, before gradually becoming thinner with age, potentially affecting the severity of burn injury in the very young and the elderly. The epidermis consists of the prevalent epithelial cell, the keratinocyte, the melanocyte that is responsible for pigment production, Langerhans cells which are of the immune system, and Merkel cells which function as mechanoreceptors.

The basal layer of keratinocytes is where cell division occurs, the cells migrating outward, passing through various phases and layers until they form the outer stratum corneum, composed of the protein keratin and cell debris, which is a relatively impervious barrier. Epidermal appendages—hair follicles, sebaceous glands, and sweat glands—extend down into the dermis. The dermis is relatively thick and consists of connective tissue—collagen and elastin produced by the fibroblast and ground substance, an intercellular matrix—that is largely responsible for the physical characteristics of skin. Vascular plexuses, nerves, and lymphatics are found in the dermis. The junction of epidermis and dermis is a specialized structure consisting of a basement membrane to which cells of the basal epidermal layer are attached and to which the dermis is anchored.

Functions of the skin include the following: action as a protective barrier—both to ingress and egress; immunologic; thermoregulation; neurosensory; aesthetics; metabolic—vitamin D.

Serious burns result from thermal, chemical, radiation, or electrical injury that causes loss of the skin surface over large areas of the body (Hunt et al. 1973; Mozingo et al. 1988;

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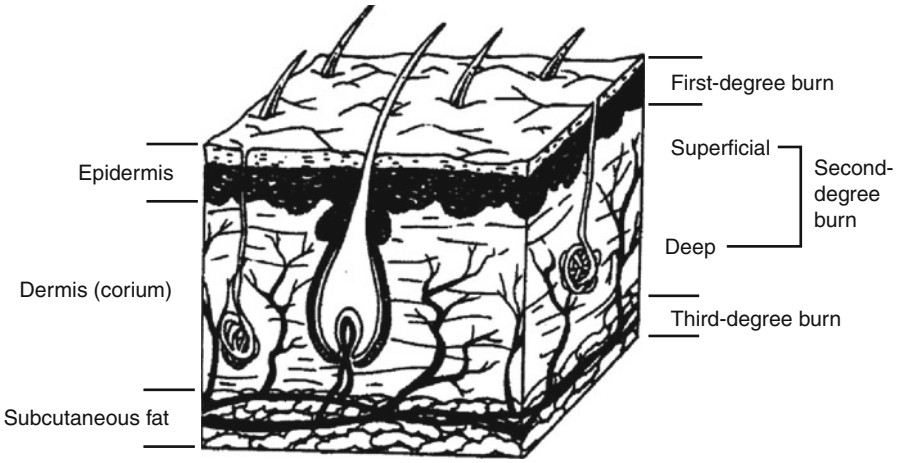

Fig. 16.1
Basic skin anatomy showing the depth of injury for first-, second-, and third-degree burns (Adapted from Roth and Hughes (2004))

Lee and Capelli-Schellpfeffer 1998; Herndon and Spies 2001; Roth and Hughes 2004; Palao and Mange 2010). Three distinct zones within the burn wound have been described by Jackson: (1) the zone of coagulation in the center where cell death is complete and protein architecture disrupted; (2) the zone of stasis lies adjacent, where cells are initially viable but perfusion is progressively reduced and tissue viability is threatened by ischemia although recovery is possible; (3) the zone of hyperemia lies peripherally and is characterized by vasodilatation and increased blood flow with minimal cellular injury (*Fig. 16.2*, Jackson 1953; Roth and Hughes 2004; Evers et al. 2010).

Burn wound edema is a critical feature of the pathophysiology of the injury both locally where it impairs tissue oxygenation, nutrient delivery, and removal of by-products and systemically with the development of hypovolemia. All factors in the Starling-Landis equation are altered in the direction of fluid flux in to the interstitium in the early period after the insult. Major influences are increased capillary and venular permeability, increased hydrostatic pressure within vessels, increased oncotic pressure and particularly a dramatic decrease in interstitium hydrostatic pressure thought to be the result of heat-induced collagen disruption (Arturson and Mellander 1964; Lund et al. 1988; Lund 1999). Edema is rapid in onset, biphasic, reaching its peak in approximately 24 h after injury.

The initial clinical assessment of a burn victim is that of any trauma patient. Specific features relating to the burn that require consideration include the mechanism of injury, the potential for accompanying inhalation injury, and assessment of the extent of the total body surface area involved and the depth of the burn.

Chemical burns are relatively uncommon but may be associated with systemic toxicity and may be locally destructive such as with anhydrous ammonia absorption (White et al. 2007), with alkali burns to the eye (Rozenbaum et al. 1991), and with hydrofluoric acid that is both a destructive acid and a chemical poison as the fluoride ion chelates calcium and magnesium (Dunser et al. 2004).

Electrical injury accounted for 4 % of admissions to burn centers in the USA in the years 2000–2009 (ABA 2011). The pathophysiology of high-voltage electrical injury is complex. Characterized by contact points (entrance and exit wounds) on the surface and extensive deep tissue destruction under what may be intact skin, the obvious skin injury represents only "the tip of the iceberg." Tissue damage is both by heat generated as current flows through tissue according to Joule's law and by direct effect of ion flow in the disruption of cell membranes (Hunt et al. 1976; Lee and Capelli-Schellpfeffer 1998). Cardiac arrest or dysrhythmia may be produced, and direct damage to the central nervous system may occur as well as to any organ next to a contact point.

The large majority of fire-related deaths occur from the inhalation of products of combustion. The outcome of those who survive the fire scene with thermal injury is adversely affected by an associated inhalation injury (Thompson et al. 1986). Pulmonary complications account for the majority of deaths in those with the combined injuries (Darling et al. 1996; Tredget et al. 1990; Colohan 2010). The upper airway may become obstructed by heat injury and swelling. Toxins such as carbon monoxide and hydrogen cyanide may have acute effects (Ernst and Zibrak 1998; Barrillo 2009), while respiratory failure may ensue later.

The severity of a burn injury is related to the area of skin involved. This may be estimated using "the rule of nines" and more accurately for children with the Lund and Browder chart (*Figs. 16.3*, *16.4*) (Lund and Browder 1944). An accurate estimate of %TBSA can also be obtained using a Berkow's percentage chart (Berkow 1924).

Burn injury is also classified according to depth. First degree (superficial) is characterized by pain and erythema from vasodilation with epidermal cell injury and subsequent desquamation but with no tissue destruction and no scar formation. Second degree (partial thickness) may be divided into superficial partial thickness and deep partial thickness. In the former, the epidermis and superficial dermis are destroyed, pain and vesicle formation are typical, and healing by

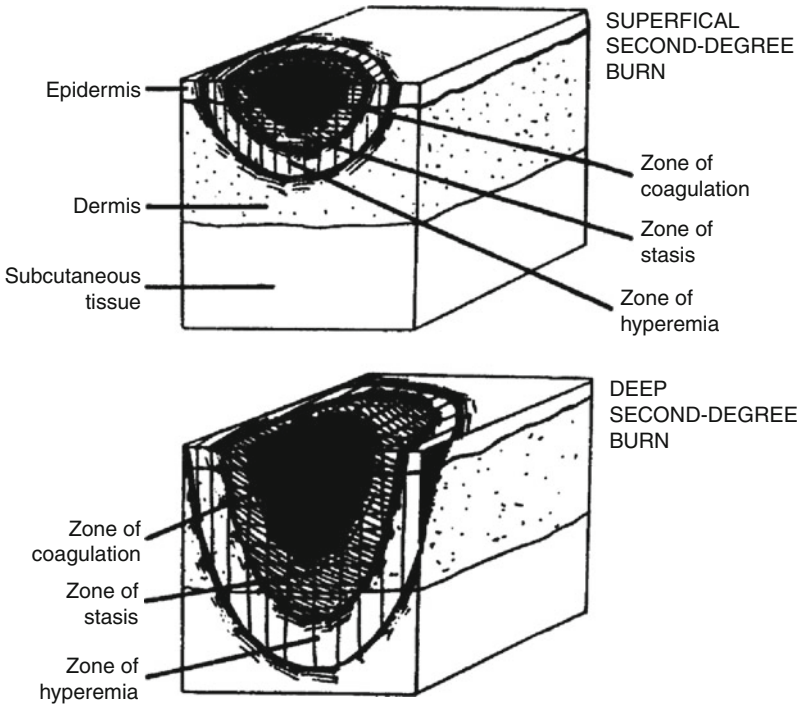

Fig. 16.2
Zones of injury for superficial and deep second-degree burns (Adapted from Roth and Hughes (2004))

reepithelialization from the dermal appendages would be expected in up to 2 weeks, assuming no adverse influences occur. Deep-partial-thickness injuries on the other hand have few epithelial lined structures remaining in the residual deep dermis, which would take many weeks to heal with severe scarring and are generally treated as full-thickness injuries. Of course, this is a continuum in terms of depth and varies from one area of the wound to another.

Third-degree (full-thickness) burns destroy both epidermis and dermis, have the potential to reepithelialize only from the periphery, and are generally treated by excision and skin grafting if other than very small in area. Fourth-degree burns involve deeper tissue including muscles, tendons, and/or bones.

Clinical assessment of burn depth is accurate for very superficial and very deep burns but not at all for differentiating wounds in between, where the decision to allow to heal or excise and skin graft will determine the outcome in terms of function and appearance as well as potentially affect systemic complications. Since early excision and skin grafting of burns of the appropriate depth has been shown to decrease mortality, morbidity, and length of hospital stay and to provide superior outcomes in terms of form and function (Janzekovic 1970; Burke et al. 1974; Hendon and Barrow 1989; Munster et al. 1994; Ong et al. 2006), exploration of optical modalities to determine burn depth has been undertaken. Several methods have been described. All have their drawbacks, and none has gained widespread implementation (Kaiser et al. 2011a).

Laser Doppler imaging relies on perfusion reflecting burn depth, as do most of the other devices, the speed and volume of blood flow being measured. Several clinical studies support its efficacy, and it is the most widely used at present (Alsbjorn et al. 1984; Riordan et al. 2003; Hoeksema et al. 2009, Kim et al. 2010; Monstrey et al. 2011; Sharma et al. 2011), but reservations exist (Jaskille et al. 2010).

Epidemiology of Burn Injury

Risk factors for burns worldwide include those related to socioeconomic status, race and ethnicity, age, gender, those pertaining to region of residence (i.e., burns are the leading cause of disability-adjusted life years lost in low- and middle-income countries), and comorbidities (Peck 2011). Burns are also one of the main forms of combat-sustained injury including trauma sustained by civilians living within war-torn areas (Atiyeh and Hayek 2010; D'Avignon et al. 2011). Children have a much higher risk of being burned than adults (Dissanaike and Rahimi 2009), and infants and young children have a higher risk of death even with burns smaller than 30 % TBSA without an inhalation injury (Morrow et al. 1996). In the United States, an estimated 158,775 children $\leq$19 years of age required emergency care for burn-related injuries per annum, and approximately 730 children die (National SafeKids Burn Campaign 2004). Older children commonly sustained flame injuries while infants and young children $<$4 years of age are more likely to sustain injuries from scald burns caused by hot liquids or steam (National SafeKids Burn Campaign 2004; Abeyasundara et al. 2011). Male children are at increased risk of burn injury compared to females.

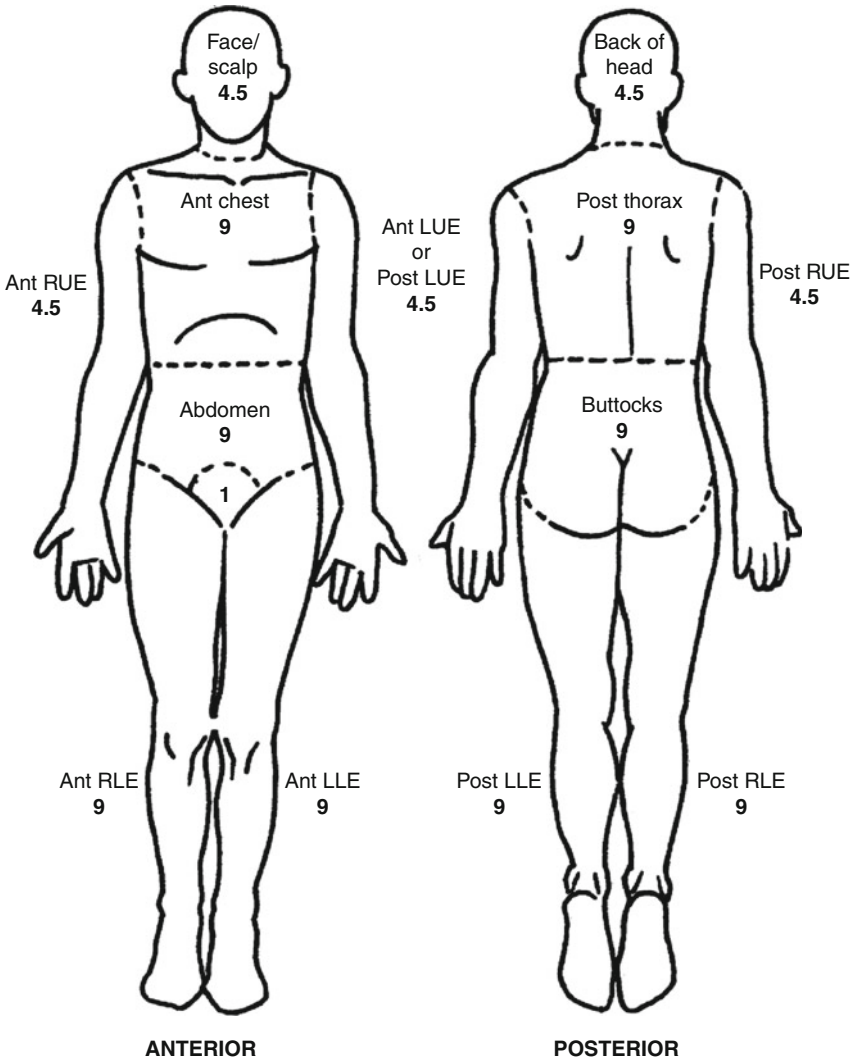

Fig. 16.3
Body diagram for estimation of total burned surface area (% TBSA) in adults using the rule of nines (numbers are for anterior only and posterior only) (Adapted from Roth and Hughes (2004))

Elderly patients also have an increased risk of being burned and worse clinical outcomes than other adults (Hunt and Purdue 1992; McGill et al. 2000; Wibbenmeyer et al. 2001; Dissanaike and Rahimi 2009; Albornoz et al. 2011). A review of adult patients admitted to a burn center over a 7-year period showed that 221/1557 (11 %) were >59 years of age and a higher proportion were women than men (McGill et al. 2000). Most elderly burn patients have one or more preexisting medical conditions and impaired judgment and/or mobility (McGill et al. 2000; Alden et al. 2005; Dissanaike and Rahimi 2009). Toxicology screening in one study also showed that substance abuse played a role: 10 % had used alcohol and almost one-third tested positive for other drugs (McGill et al. 2000). In a recent case–control study of elderly burn patients, those over age 65 years had less TBSA burned, but the proportion of deep TBSA burned was higher (Albornoz et al. 2011). Elderly patients had significantly higher mortality than patients under 65 years of age (48 % vs. 24 %), and their probability of dying was 1.9 times as high, but this increased to 12 times when adjusted for TBSA and the amount of the TBSA that was deeply burned (Albornoz et al. 2011).

Persons with disabilities and those with self-inflicted burns also have more severe burns and have longer hospital stays than those with accidental injury (Backstein et al. 1993; Horner et al. 2005; Chen et al. 2007). Diabetic patients not only have a higher attributable morbidity and mortality with burn injury, but the time to index wound closure is significantly prolonged despite increased grafting (McCampbell et al. 2002; Schwartz et al. 2011) (169,276,290). Delayed wound healing, more infection complications, and increased mortality have also been reported for patients with severe immunosuppression due to human immunodeficiency virus infections (HIV)/AIDS or preexisting medical conditions such as metastatic cancer or liver or renal diseases

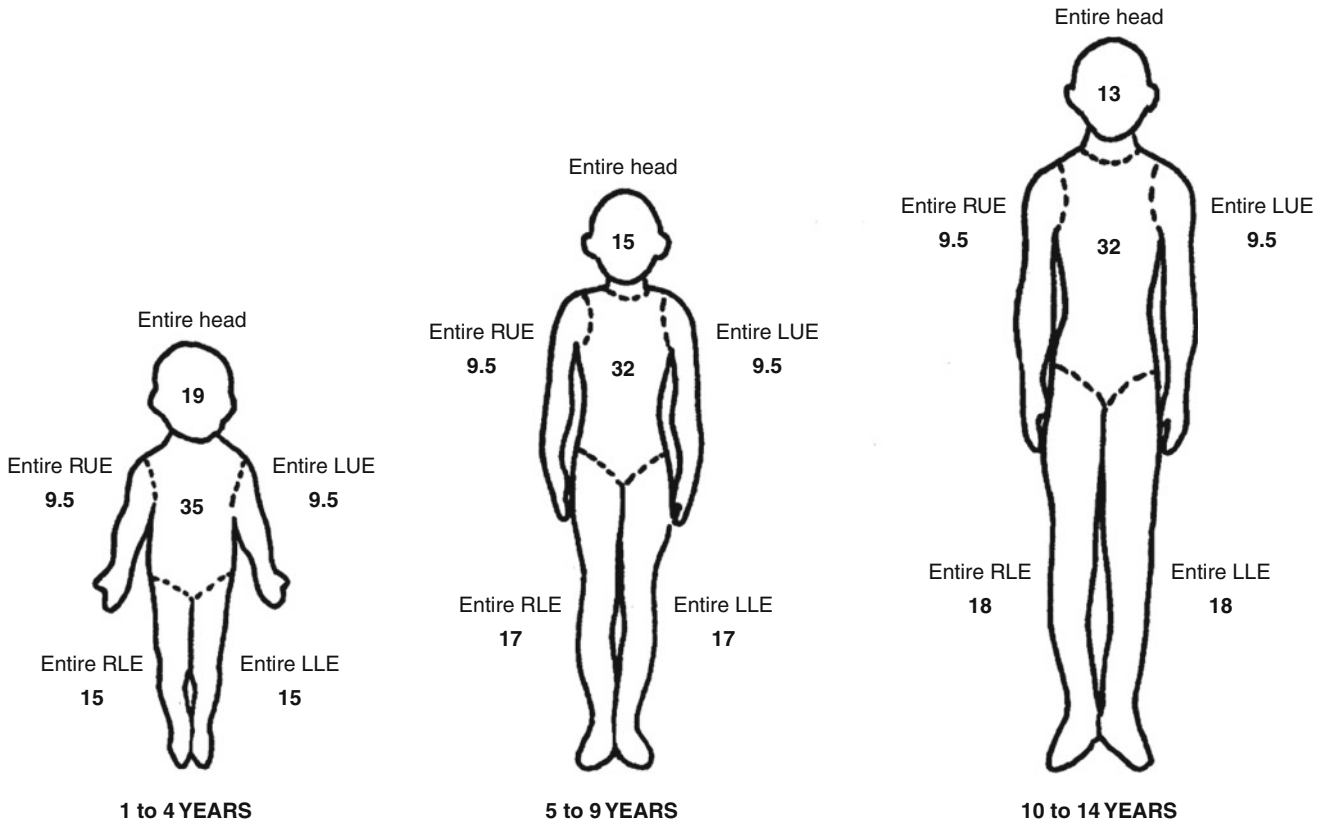

Fig. 16.4
Body diagram for estimation of total burned surface area (% TBSA) in children using the rule of nines (numbers include anterior and posterior) (Adapted from Roth and Hughes (2004))

(Thombs et al. 2007; Edge et al. 2001; Mzezewa et al. 2003; Sjoberg et al. 2004; Chalya et al. 2011).

Burn Wound Excision

Advances in burn wound care, particularly the early excision of necrotic tissue and closure of the wound, has improved patient survival in cases without inhalational injury, in part, by reducing induction of an overwhelming systemic immune response (SIRS) and by preventing burn wound infection and sepsis by temporary or permanent closure of the burn wound (Heimbach 1987; Sheridan et al. 1994; Herndon and Spies 2001; Roth and Hughes 2004; Ong et al. 2006; Chang et al. 2010; Chen et al. 2010b; Kennedy et al. 2010; Purdue et al. 2011). Modern surgical burn care excises and closes full-thickness and deep-partial-thickness wounds as soon after injury as possible once the patient has been hemodynamically stabilized (Burke et al. 1974; Sheridan et al. 1994; Roth and Hughes 2004; Atiyeh et al. 2005; Mosier and Gibran 2009). Surgical debridement of burn eschar is achieved by tangential excision, removing layers of necrotic tissue until viable tissue is exposed or by excision to fascia, depending on the depth and location of the burn and the state of the patient. The area removed at any one time is limited by blood loss and body temperature decline. Measures to reduce blood loss for the wound to be tangentially excised and the skin graft donor sites include the application of topical epinephrine, topical thrombin, and subcutaneous "tumescent" infiltration of epinephrine and the use of tourniquets (Cartotto et al. 2000; O'Mara et al. 2002; Djurickovic et al. 2001).

Sequential procedures over the first several days are required to excise the whole eschar in extensive burns. Closure is with autogenous skin grafts, allografts, or synthetic temporary skin substitutes (Sheridan 2009; Meier et al. 2011), the last two of which will have to be replaced by the first, as donor sites heal and can be reharvested. The formation of hypertrophic scarring remains a major adverse outcome in the burn patient (Deitch et al. 1983). The prolonged immune response present in the burn wound is a factor in the development of such. Early wound closure may reduce this (Singer and McClain 2002; Atiyeh et al. 2003; Ladak and Tredget 2009).

Pathogenesis of Burn Wound Infections

Besides the nature and extent of destruction of the skin barrier, the host's immune response elicited by burn injury in addition

to microbial factors influences the onset of infection in the burn wound. Major risk factors for the development of an invasive burn wound infection are described in this section and include concomitant depression of local and systemic host cellular and humoral immune response, and the colonization and subsequent biofilm formation on the burn wound surface by various types and amounts of virulent microbes.

Host Immunologic Response to Burn Injury

Severe burn injury predisposes patients to infectious complications by inducing a state of immunosuppression. Wertheim, Avdakoff, Rydigier, and Kijannitzini published on "burn toxins" that were linked early on to the immunodeficiency that follows thermal injury (Wertheim 1868; Avdakoff 1876; Sevitt 1957), but the more recent findings of prolonged allograft survival, anergy, and increased susceptibility to infection in burn patients support the development of immunodeficiency as a result of the injury (Kay 1957; Casson et al. 1966; Solowey and Rapaport 1966; Ninnemann et al. 1978; Wolfe et al. 1982). Many in vitro and in vivo studies have been conducted to characterize the immune responses and the relationships between various cell types and inflammatory mediators, and several reviews discuss the findings of original works in more detail (Heideman and Bengtsson 1992; Lederer et al. 1999; Steinstraesser et al. 2004; Shankar et al. 2007). Measured effects occur in both the innate and adaptive immune systems and contribute to the immunosuppression associated with burn injuries. The innate immune response acts immediately after the integumentory system is breached and relies on a phylogenetically ancient system for microbial recognition in which germ-line-encoded receptors (pattern recognition receptors, PRRs) recognize structural components of microorganisms and viruses (pathogen-associated molecular patterns, PAMPs) (Steinstraesser 2004). The adaptive immune response often takes longer especially if it involves new antigenic exposure, but it is a more efficient system for dealing with recurrent infections relying on immune cell memory, antigen recognition, and clonal proliferation.

The Systemic Response to Burn Injury

Local inflammation following injury is essential for wound healing and host defense against infection, but trauma or burns of a sufficient magnitude incite a systemic inflammatory response, along a continuum from SIRS through a septic shock-like state, which has the ability to cause significant cellular and end-organ damage (Bone 1996; Shankar et al. 2007). Initially, the immunologic response to severe burn injury is pro-inflammatory but later becomes predominately anti-inflammatory in an effort to maintain homeostasis and restore normal physiology. Cytokines and cellular responses mediate both of these phases.

Increased serum levels of pro-inflammatory cytokines characterize the systemic response to burns. IL-1β (interleukin-1β) and TNF-α (tumor necrosis factor-α) are produced by a wide variety of cells in response to injury, of which leukocytes are key players. Both of these cytokines contribute to the production of fever, acute phase proteins, and an overall state of catabolism. They also upregulate the production of prostaglandin E_2 (PGE_2), interleukin 6 (IL-6), and platelet-activating factor (PAF) by endothelial cells and macrophages (Weissman 1990; Chaudry and Ayala 1993). Levels of IL-6 are increased after injury through its production by a number of different cells (Biffl et al. 1996). Like IL-1β and TNF-α, IL-6 induces fever and the production of acute phase reactants that contribute to T cell activation (Xing et al. 1998). Levels of IL-6 peak at approximately 1 week after injury, and high levels have been associated with increased rates of morbidity and mortality (Guo et al. 1990), for which it is likely a marker of disease severity, rather than an etiologic factor. IL-6 may also be an important mediator of burn-injury pain (Summer et al. 2008). IFN-γ (interferon γ) is another pro-inflammatory cytokine, produced by NK (natural killer) cells and T_h1 (T helper) cells, in response to injury. It has an important role in macrophage activation and the differentiation of CD4+ T cells into T_h1 cells while inhibiting their differentiation into T_h2 cells (Gosain and Gamelli 2005).

Other mediators involved in the pro-inflammatory process in burns include the following: reactive oxygen species (ROS) and reactive nitrogen species (RNS). Mediators arising from oxidative stress have beneficial antimicrobial and healing effects, but their enormous production in burns significantly contributes to systemic inflammatory response, tissue damage, and organ failure (Parihar et al. 2008). Persistent elevation of estrogens post injury correlates with the pro-inflammatory state and increased mortality. Complete deficiency of aromatase is associated with decreased production of IL-6 and partial restoration of delayed type of hypersensitivity response but not with a decrease of TNF-α (Plackett et al. 2006). The gaseous mediator and potent vasodilator hydrogen sulfide (H_2S) as well as pain and inflammatory mediator substance P were also found to play an important role in the pro-inflammatory response in burns (Sio et al. 2008; Bhatia 2010).

Cell types that are important in facilitating a pro-inflammatory response to injury are pro-inflammatory macrophages and CD4+ T helper (T_h) cells. It is thought that the proactive release of inflammatory mediators drives the development of organ dysfunction syndrome. TNF-α, IL-1β, IL-6, HMGB-1 (high-mobility group box-1), MMIF (macrophage migration inhibitory factor), heat shock proteins, apoptosis, and inflammatory caspases are all actively involved in mediating cardiac dysfunction in burns (Carlson and Horton 2006). Recently, complement factor 5a (C5a) has been directly linked to burn-induced cardiac dysfunction. LPS may also contribute to the cardiac dysfunction in burn injury (Hoesel et al. 2007). Copious amounts of reactive oxygen species, reactive nitrogen species, proteases, cytokines, chemokines, and complement proteins released by inflammatory cells also lead to neuroinflammation characterized by blood–brain barrier breakdown, neuronal damage, and even life-threatening cerebral cytotoxic edema (Flierl et al. 2009).

Locally, burn induces a robust Th17 response, characterized by secretion of a Th17 group of cytokines (IL-6, IL-17, IL-22, IL-23, IL-27, and TGF-β). Th17 response is considered to play an important role in wound immunology and healing (Sasaki et al. 2011). Dermal inflammatory response acts as an ongoing trigger of systemic inflammation and organ dysfunction. In mice, attenuation of burn wound inflammation signaling by topical p38 MAPK inhibitor significantly decreased the inflammatory response in the lungs: decreased neutrophil sequestration, pulmonary cytokine expression, microvascular injury, and edema formation (Ipaktchi et al. 2006). Also, serum levels of CRP (C-reactive protein) were significantly decreased in patients when xenogenic acellular dermal matrix was placed on second-degree burns when compared to patients with betadine ointment coverage (Feng et al. 2007).

The anti-inflammatory response and the subsequent immunosuppression following burn injury are characterized by a set of opposing cell types and cytokines. The production and recruitment of monocytes/macrophages are decreased following burn injury and sepsis (Gamelli et al. 1994). Under these circumstances, macrophages produce increased amounts of PGE_2 and decreased amounts of IL-12, which have a cooperative effect on T cell differentiation (Goebel et al. 2000). T helper cells begin to preferentially differentiate into T_h 2 cells that produce the anti-inflammatory cytokines, IL-4 and IL-10 (DiPiro et al. 1995; Gosain and Gamelli 2005). T cell dysfunction has a complex etiology in burns, including specific lipid complexes derived from burned skin, alterations of lymphocyte subpopulation (e.g., decreased CD4+/CD8+ ratio), shift to T_h 2-type immune responses, macrophage-derived mediators (e.g., PGE_2 and NO), and also defects in CD3 signaling pathway (Duan et al. 2008). The exact sequence of events that results in immunosuppression after burn injury remains unknown; however, biochemical changes that may affect the immune system include those to the endocrine system, the arachidonic acid cascade, and the cytokine network. Following severe burn injury, there is an increase in the levels of vasopressin, aldosterone, growth hormone, cortisol, glucagon, and catecholamines (Gosain and Gamelli 2005). Elevated levels of glucocorticoids inhibit the production of IFN-γ and IL-2 but not IL-4 and IL-10 (Faist et al. 1986; Ramirez et al. 1996; Gosain and Gamelli 2005). Norepinephrine released early after injury similarly inhibits T_h 1 cell function but not that of T_h 2 cells (Sanders et al. 1997) and also plays a role in the generation of polymorphonuclear neutrophils with immunosuppressive ability (PMN II) in association with SIRS or injury, which may be responsible for increased susceptibility to infections in burn patients (Tsuda et al. 2008). Increased production of PGE_2 by inhibitory macrophages has been observed after severe injury (Weissman 1990). PGE_2 may have an important role in secondary immunosuppression as it has been shown to decrease lymphocyte proliferation, to decrease the levels of the pro-inflammatory cytokines IL-1β and IL-2, to diminish the lymphocyte response to IL-2, to inhibit the activity of NK cells, and to activate suppressor T cells (Alexander 1990; Gosain and Gamelli 2005). Many of the changes in cytokine levels following burn injury represent alterations of the adaptive immune system particularly within the T lymphocyte population. Some of the molecules with anti-inflammatory properties were identified as potentially protective. Carbon monoxide (CO) liberated from CORM (carbon monoxide-releasing molecules) has been shown to decrease leukocyte sequestration, to decrease expression of ICAM-1-(intercellular adhesion molecule-1), to decrease activation of NF-kappaB (nuclear factor kappaB) in the lungs, to inhibit the expression of iNOS (inducible nitric oxide synthase), to decrease NO (nitric oxide) production, and to downregulate the expression of pro-inflammatory mediators (TNF-α, IL-1β) in the liver (Sun et al. 2008). Ubiquitin is known to have pleiotropic effect on host defense mechanisms, especially to reduce fluid shifts into tissues during inflammation. Ubiquitin reflects the extent of tissue damage, and its systemic release may be protective, as burn patients who develop sepsis and MOF (multiorgan failure) appear to have a relative ubiquitin deficiency (Majetschak et al. 2008). Diminished level of defensins in burns may also facilitate infection and sepsis and alter function of B and T lymphocytes, neutrophils, macrophages, and complement (Bhat and Milner 2007).

The Innate Immune System in Response to Burn Injury

Natural resistance to infection in traumatic wounds is predominately a function of the innate immune system. Following thermal injury, the innate immune system responds immediately by stimulating localized and systemic inflammatory reactions. The innate immune response participates in activating the adaptive immune response; however, in so doing, it may have an adverse affect on the burn victim's ability to mount a vigorous immune response to invading microorganisms and, therefore, predisposes the burn victim to infectious complications. The innate immune system itself is composed of natural barriers to microbial invasion as well as cellular (leukocyte) and humoral (complement) elements.

Before a pathogen can establish invasive infection within the host, it must break through the natural barriers of the skin or mucosa. For example, there is a loss of barrier function of the gastrointestinal epithelium in burn patients, which may be induced by upregulation of the nitric oxide synthetase gene and the overproduction of nitric oxide (Nadler et al. 1999); postoperative changes, such as decreased intestinal motility and mucus secretion; as well as increased exposure to endotoxin (LPS) (Alexander 1990). The development of MODS (multiorgan dysfunction syndrome) in critically ill patients has also been associated with a derangement in intestinal permeability (Doig et al. 1998). As a result, higher rates of bacterial translocation and endotoxin absorption through the gastrointestinal mucosa may contribute to the inflammatory response seen in burn patients. Induction of TLR4 (toll-like receptor 4) expression by commensal microflora decreases bacterial translocation in burn patients, which increases bacterial killing (Chen et al. 2010a). Increased susceptibility to bacterial translocation

in burns is caused by CCL2 (chemokine C-C motif ligand 2) which converts resident macrophages to alternatively activated macrophages M2a and M2c with decreased antibacterial killing properties (Shigematsu et al. 2009). Concomitant intoxication with ethanol is associated with increased bacterial translocation and increased production of IL-6 by enterocytes (Choudhry et al. 2006; Scalfani et al. 2007).

The cellular elements of the innate immune system play an important role in antimicrobial killing and in coordinating the immune response. Decreased macrophage and natural killer (NK) cell activation result in reduced levels of IFN-γ following burn injury (Collart et al. 1986; Hayes et al. 1995). The function of NK cells is diminished following significant injury (Miyazaki et al. 2011). Neutrophil dysfunction after significant thermal injuries has also been reported (Grogan and Miller 1973; Fikrig et al. 1977; Bjornson et al. 1981; Lavaud et al. 1988). Endothelial adherence of neutrophils is initially decreased after injury and then increases (Salo 1992); however, the site of endothelial adhesion may not be at the point of injury, and this misguided neutrophil adhesion and activation contributes to neutrophil-mediated endothelial injury, which may play a significant role in the pathogenesis of SIRS (systemic inflammatory response syndrome) and MODS (multiorgan dysfunction syndrome). Neutrophil chemotaxis and intracellular killing are impaired following major burns (Grogan 1976; Griswold 1993). Diminished cytotoxic activity follows from a surge of degranulation early after injury and a subsequent inability to replenish intralysosomal enzymes and defensins (Griswold 1993). Furthermore, PMN II (immunosuppressive polymorphonuclear cells) appearing in response to burn injury impair host antibacterial resistance against translocation of bacteria through the conversion of resident macrophages to alternatively activated macrophages (Tsuda et al. 2008). Macrophages also demonstrate diminished phagocytic capacity following severe injury (Schildt 1970; Altman et al. 1977). Lower levels of MHC class II (major histocompatibility complex class II) expression and antigen presentation disrupt their role in coordination of the immune response (Stephan et al. 1989). They also produce larger quantities of PGE_2, resulting in the suppression of B and T cell reactivity (McLoughlin et al. 1979). Increased levels of IL-4 and IL-10 inhibit macrophage antigen presentation, decrease the production of pro-inflammatory cytokines, like IL-1β, and suppress bactericidal and fungicidal activity (Essner et al. 1989; Donnelly et al. 1991; Fiorentino et al. 1991; Oswald et al. 1992; Vannier et al. 1992). Altered cytokine secretion profiles are associated with altered TLR expression on innate immune cells (TLR 2, TLR 4) and unique changes in the macrophage population (Cairns et al. 2008).

The complement cascade represents an important humoral component of the innate immune system. Following significant burn injuries, the alternate pathway of the complement cascade is primarily depressed while there is little effect on the classical pathway (Gallinaro et al. 1992). Complement levels fall in proportion to injury severity and then rise to supranormal levels (Gallinaro et al. 1992). Activation of the complement cascade by thermal injury increases levels of C3a and C5a (complement factors 3a and 5a) which may result in changes in blood pressure, vascular permeability, and leukocyte function (Hugli 1984; Yurt and Pruitt 1986; Bengtson and Heideman 1987). Small amounts of C5a have been shown to stimulate leukocyte function; however, large amounts lead to suppression of activity (Webster et al. 1980). Membrane attack complexes may target normal cells near the site of injury, contributing to reactive cell lysis, which may induce end-organ damage (Heideman and Bengtsson 1992). Lastly, increased levels of C3b may be directly immunosuppressive as they have been shown to decrease phagocytosis and contribute to lymphocyte dysfunction (Alexander 1990).

Dendritic cells (DCs) are potent antigen presenting cells that serve as a critical link between the innate and acquired immunity. DCs are essential for coordinating the host response to pathogens. In burns, dendritic cell function is impaired. In septic burn patients, the number of circulating DCs, both myeloid and plasmacytoid, is significantly decreased (D'Arpa et al. 2009). The ability of DCs to prime naive T cells when triggered by the TLR4 signaling cascade (especially TLR reactivity on CD8α + DCs) is altered (Patenaude et al. 2010). Dendritic cells also modify neutrophil responses to infection, and impaired DC function in burns leads to decreased neutrophil-mediated control of bacterial spread (Bohannon et al. 2009).

These alterations to the innate immune system have the combined effect of increasing the burn patient's exposure to pathogens and decreasing the natural defenses that are responsible for counteracting them. Exposure to pathogens occurs via the burn wound, invasive monitoring devices, as well as the gastrointestinal tract that loses some of its capacity to act as an effective barrier to bacterial translocation. The effects of an anti-inflammatory cytokine milieu on NK cells, neutrophils, and macrophages impair the eradication of these pathogens by the innate immune system. Furthermore, the activation of complement following burn injury may be directly immunosuppressive. As a result of these phenomena and subsequent alterations to the adaptive immune system, burn patients are more susceptible to wound infections, severe sepsis, and multiple organ failure.

The Adaptive Immune System in Response to Burn Injury

Following significant injury, several changes in the T lymphocyte population have been observed. Total numbers of T lymphocytes fall in proportion to injury severity during the first week after injury (Heideman and Bengtsson 1992), and there is a decrease in T cell-dependent immune functions (Kay 1957; Casson et al. 1966; Wolfe et al. 1982). Diminished T cell proliferation in response to mitogens (Wolfe et al. 1982; Horgan et al. 1994) is associated with, and may be the result of, decreased production of IL-2 and IFN-γ by monocytes (Wood et al. 1984; Faist et al. 1986). Thermal injury together with septic complications is associated with decreased survivability of CD4+ T cells via apoptosis in mesenteric lymph nodes (Fazal and Al-Ghoul

2007). The production of IgG in response to T cell-dependent antigens is also impaired after serious burn injury; however, no impairment of antibody formation to T cell-independent antigens has been observed (Nohr et al. 1984). There is a decreased ratio of CD4+ T_h helper cells to CD8+ T suppressor cells (O'Mahony et al. 1985; Burleson et al. 1988). After an initial pro-inflammatory phase, injury results in a loss of T_h1 cells associated with depressed levels of IL-1β and IFN-γ. Concomitantly, T_h2 lymphocytes are present in increased numbers along with higher levels of the anti-inflammatory cytokines, IL-4 and IL-10, which may inhibit T_h1 cell activation by suppressing antigen presentation. A correlation between increased levels of IL-10 and septic events has been reported (Sherry et al. 1996; Lyons et al. 1997). It remains uncertain as to whether the relative predominance of T_h2 cells over T_h1 cells represents a phenotypic change or an increase in the rate of apoptosis of T_h1 cells (Lederer et al. 1999). Alterations in the balance between T suppressor lymphocytes and T helper lymphocytes as well as the ratio of T_h1 to T_h2 cells appear to be important etiologic factors in the suppression of the adaptive immune response. Recent findings show the dendritic cell growth factor, fms-like tyrosine kinase-3 ligand (FL), has an ability to promote Th1-associated antigen-specific responses (Bohannon et al. 2009). In addition, toll-like receptors (TLRs) were identified on activated and memory T cells. More specifically, TLR4 expression on memory T cells generated late after injury could represent a mechanism for enhanced T cells response late post injury (Cairns et al. 2008). Other mediators, like high-mobility group box-1 protein (HMGB-1) release, are associated with T cell suppression after thermal injury. Interestingly, TLR4 was identified as a receptor essential for the negative effect of HMGB1 on CD4(+)CD25(+) T regulatory cells (T regs) (Zhang et al. 2008; Zhu et al. 2011). Further, small subsets of T cells, gamma-delta T cells, were found to play a central role in regulation of the immunopathological response following burn injury and may become a potential target to affect inflammation and healing (Schwacha 2009).

Inflammatory Markers in Burn Injury

Over the last 5 years, a significant effort has been undertaken to establish reliable soluble receptor or genetic markers monitoring systemic inflammatory responses in burn injury which could be used for early detection of complications and evaluation of severity of disease and prognostication. Some of these markers will be reviewed below.

Serum procalcitonin has been shown to be a reliable marker of early septic complications in burn patients (Lavrentieva et al. 2007). Simultaneous monitoring of soluble cytokine inhibitors (sTNFR I, sTNFR II, and IL-IRa) and anti-inflammatory cytokines (IL 10, IL 13) plus inflammatory markers such as C-reactive protein (CRP) has shown to be useful tools in the evaluation of inflammatory response in burned children (Sikora et al. 2009). Combined measurement of macrophage migration inhibitory factor-A (MIF-A) and procalcitonin (PCT) was shown to discriminate between post-burn inflammation and SIRS versus sepsis with lethal outcome (Grieb et al. 2010).

The decrease of mHLA-DR expression on monocytes has also been shown to be a predictor of septic complications in critically ill patients, and monitoring of mHLA-DR expression could be a biomarker for detection of nosocomial infections in critically ill patients, including burn patients (Cheron et al. 2010).

Finally, on a genome level, the carrying of variant alleles of IL-1β, IL-6, TNF-α, CD14, and TLR4 is associated with significant risk for severe sepsis after burn injury (Barber et al. 2006). Development of microfluidic devices (biomicroelectromechanical systems) represents a new platform in near-patient molecular diagnostic and personalized medicine and may be used widely in the near future to identify burn patients at risk of increased morbidity and mortality (Rosenbach et al. 2011).

Altering the Immunologic Response to Burn Injury

Despite our increasingly detailed understanding of the immunologic suppression that follows thermal injuries, no attempts at directly modulating the immune response at a specific site have been shown to be clinically effective. It is becoming increasingly clear that any therapies directed at addressing the immunodeficiency in burn patients will likely have to target multiple points in the inflammatory response and the neuroendocrine axis.

Immune function in burn patients can only be restored through intensive resuscitation and support. Early excision of burn eschar and prompt wound coverage removes a significant inflammatory stimulus and restores the barrier function of the skin. Providing adequate analgesia and maintaining adequate tissue perfusion, ambient temperature, and blood volume help optimize the oxidative killing capacity of neutrophils (Kuroda et al. 1997). Adequate fluid resuscitation in burns is a matter of current debate. It has been recently shown that adequate fluid resuscitation guided with ITBV (intrathoracic blood volume monitoring) suppresses the shift toward anti-inflammatory immune response measured by expression of circulating cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α) and leukocyte surface markers (CD11a, CD11b, CD14, CD18, CD49d, and CD97) more than hourly urine output-guided fluid resuscitation (Foldi et al. 2010). Patients treated with hydroxyethyl starch (HES)-supplemented fluid resuscitation required less fluid, showed less interstitial edema, and dampened inflammatory response (CRP levels) compared with patients receiving isotonic crystalloid alone (Vlachou et al. 2010).

Early and adequate nutritional support is also important in restoring protein synthesis and normal immune function. Despite the fact that tight glucose control has been generally abandoned in the critically ill, in a subgroup of burn patients, it may be an important factor in treatment: insulin increased resistance to burn wound infection-associated sepsis in an

animal model (Gauglitz et al. 2010), markedly improved immune function, enhanced phagocytosis by monocytes (Zhao et al. 2007), and decreased morbidity in post-burn pediatric patients (Jeschke et al. 2010). The mechanism of insulin effect on the immune system in sepsis, severe trauma, and burns has been summarized in a recent review (Deng and Chai 2009).

Research efforts have focused on the topic of immune-modifying diets, such as glutamine-enriched diets and their clinical benefits (Garrel et al. 2003). Glutamine levels are significantly reduced in burn patients. Glutamine supplementation in enteral nutrition improved cellular immunity functions (lymphocyte transformation ratio, neutrophil phagocytic index (NPI), CD4+/CD8+ ratio, and IL-2) and wound healing but did not affect humoral immunity (concentration of IgG, IgM, C3, C4) (Peng et al. 2006). Current evidence supports glutamine supplementation for patients with severe burn injuries (Kurmis et al. 2010).

Many molecules have been shown to have immunomodulatory and therapeutic benefit in systemic and local treatment of burns in experimental models. This area of investigation will continue to yield possible therapeutic options in the future.

Microbial Factors Contributing to Burn Wound Infection

Although burn wound surfaces are sterile immediately following thermal injury, these wounds eventually become colonized with microorganisms that originate from the patient's endogenous skin, gastrointestinal and respiratory flora (Manson et al. 1992a, b, c; Ramzy et al. 1998; Barret and Herndon 2003; Altoparlak et al. 2004; Erol et al. 2004; van Saene et al. 2008; de Smet et al. 2009). Microorganisms may also be transferred to a patient's skin surface via contact with contaminated external environmental surfaces, water, fomites, air, and the contaminated hands of healthcare workers (Tredget et al. 2004; Ribeiro et al. 2010; Rafla and Tredget 2011). Burn wound infections may be caused by bacteria, fungi, or viruses (Schofield et al. 2007; D'Avignon et al. 2010). The most common microorganisms causing burn wound colonization and infection is outlined in *Table 16.1*. Although *Streptococcus pyogenes* was the most common cause of burn wound infection in the pre-penicillin era (Durtschi et al. 1982), *S. aureus* has become the most common gram-positive bacteria to immediately colonize the burn wound after injury (Barret and Herndon 2003; Altoparlak et al. 2004; Erol et al. 2004). *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have recently been documented to be the major cause of morbidity and mortality in burn patients worldwide (Branski et al. 2009; Guggenheim et al. 2009; Kaiser et al. 2011b; Rezaei et al. 2011). Endogenous gram-negative bacteria including most commonly *P. aeruginosa*, *Acinetobacter* spp., or Enterobacteriaceae may also colonize the burn wound surface a few days (i.e., 3–7) after injury (Manson et al. 1992c; Tredget et al. 2004; Hodle et al. 2006; Branski et al. 2009; Rezaei et al. 2011). A recent survey of 104 US burn units showed that 44 % identified *P. aeruginosa* as the most prevalent gram-negative pathogen, followed by *Acinetobacter* spp. (Hodle et al. 2006). Enterobacteriaceae are more frequently isolated in other parts of the world (Guggenheim et al. 2009; Rezaei et al. 2011). Another recent study of 62 burn patients showed that 24 (39 %) developed a surgical burn wound infections (SWIs), and the identified pathogen had been nosocomially transmitted 56 % of the time (Posluszny et al. 2011). In these 24 patients, 70 distinct infections were identified, of which 46 % required regrafting; *Candida* spp. (24 %), *P. aeruginosa* (22 %), *Serratia marcescens* (11 %), and *S. aureus* (11 %) comprised most of the pathogens. Development of an SWI not only increased the overall length of stay but also increased the need for regrafting, the area of autograft, and the number of operative procedures in patient with higher %TBSA and depth of burn injury.

Table 16.1
Microorganisms causing invasive burn wound infection

Gram-positive	*Staphylococcus aureus*
	Methicillin-resistant *S. aureus* (MRSA)
	Coagulase-negative staphylococci
	Enterococcus spp.
	Vancomycin-resistant enterococci (VRE)
Gram-negative	*Pseudomonas aeruginosa*
	Acinetobacter spp.
	Escherichia coli
	Klebsiella pneumonia
	Serratia marcescens
	Enterobacter spp.
	Proteus spp.
	Bacteroides spp.
Fungi	*Candida* spp.
	Aspergillus spp.
	Fusarium spp.
	Alternaria spp.
	Rhizopus spp.
	Mucor spp.
Viruses	Herpes simplex virus
	Cytomegalovirus
	Varicella-zoster virus

Microorganisms transmitted from the hospital environment also tend to be more resistant to antimicrobial agents than those originating from the patient's normal flora (Fuchs et al. 2002; Clark et al. 2003; Branski et al. 2009; Guggenheim et al. 2009; Rezaei et al. 2011). Emerging antimicrobial resistance trends in burn wound bacterial pathogens represent a serious therapeutic challenge for clinicians caring for burn patients. MRSA, methicillin-resistant coagulase-negative staphylococci (MRSE), vancomycin-resistant enterococci (VRE), and multiply resistant gram-negative bacteria (*P. aeruginosa*, *Acinetobacter* spp., and various members of the family *Enterobacteriaceae*) that possess several types of beta-lactamases including

extended-spectrum beta-lactamases (ESBLs), *Amp*C beta-lactamases, and metallo-beta-lactamases (MBLs) have increasingly caused burn wound infection, sepsis, or other infection complications in burn patients, and transmission within the nosocomial environment has resulted in outbreaks (Laupland et al. 2005; Walsh et al. 2005; Teare et al. 2010; Zavascki et al. 2010; Altoparlak et al. 2011; Kaiser et al. 2011b; Livermore et al. 2011; Steinmann et al. 2011). Resistance to methicillin in *S. aureus* and more recently emergence of resistance to glycopeptides and oxazolidinones complicate the treatment of burn wound infections and sepsis caused by this highly virulent organism (Haraga et al. 2002; Meka et al. 2004; Kaiser et al. 2011b). *P. aeruginosa* and *Acinetobacter* spp. also carry many intrinsic and acquired antimicrobial resistance traits that make microbial burn wound difficult to treat (Laupland et al. 2005; Keen et al. 2010a; Zavascki et al. 2010). Risk factors for acquisition of an ARO include receipt of antibiotics prior to the development of infection, extended duration of hospitalization, previous hospitalization, invasive procedures, comatose state, and advancing age.

Important emerging causes of late-onset morbidity and mortality in patients with major burn injury, invasive burn wound infection, and immunodeficiency include a number of yeast and molds (◉ *Table 16.1*). Most commonly, the burn wound surface becomes colonized a few weeks after injury with either *Candida* or *Aspergillus* spp. or less often with other opportunistic fungi (i.e., *Alternaria* spp., *Fusarium* spp., *Rhizopus* spp., and *Mucor* spp.) (Stone et al. 1979; Becker et al. 1991; Pruitt and McManus 1992; Mousa and al-Bader 2001; Moore et al. 2010; Capoor et al. 2012; Pedrosa and Rodrigues 2011). Fungal infection, particularly with Aspergillus or Zygomycetes, presents as a darkening of the burn wound surface due to ischemic necrosis, similar in appearance to ecthyma gangrenosum (Stone et al. 1979; Becker et al. 1991). Although rare, burn wound infection due to Zygomycetes such as *Rhizopus* spp. and *Mucor* spp. has a very high mortality due to their unique ability to rapidly spread across fascial tissue planes and to invade vasculature (Cooter et al. 1990; Tang and Wang 1998; Ribeiro et al. 2010; Struck et al. 2010). Survival requires aggressive wide surgical excision of the infected areas of the burn wound before the development of deep fungal invasion, along with administration of systemic antifungal therapy.

Herpes viruses including herpes simplex virus (HSV) and herpes zoster virus (HZV) but less commonly cytomegalovirus (CMV) have also increasingly been reported to cause burn wound infections either due to a primary viral infection, reactivation of latent virus, or exogenous reinfection in previously infected patients (Gallagher 1970; Bourdarias et al. 1996; Sheridan et al. 2000; D'Avignon et al. 2010; Peppercorn et al. 2010; Bordes et al. 2011a). HSV most commonly infects healing or recently healed partial-thickness burns (Gallagher 1970; Brandt et al. 1985; Kagan et al. 1985; Bourdarias et al. 1996; Sheridan et al. 1999, 2000), particularly those in the nasolabial area or involving skin graft donor sites, while those due to CMV typically involve full-thickness burns (Kagan et al. 1985; Bordes et al. 2011a). HSV burn wound infection usually occurs 2–6 weeks after injury, and typical clusters of small vesicles or vesiculopustules are found within or around the wound margins. Although most herpetic burn wound infections are self-limited, systemic spread may occur (Kagan et al. 1985; Sheridan et al. 1999, 2000).

A number of virulence factors produced by common burn wound pathogens such as *P. aeruginosa* and *S. aureus* are important in the pathogenesis of invasive infection. *P. aeruginosa* produces a number of cell-associated [adhesins, alginate, pili, flagella, lipopolysaccharide (LPS)] and extracellular virulence factors (elastase, exoenzyme S, exotoxin A, hemolysins, iron-binding proteins, leukocidins, proteases) that mediate a number of pathogenic processes including adhesion, nutrient acquisition, immune system evasion, leukocyte killing, tissue destruction, and bloodstream invasion (Van Delden and Iglewski 1998; Tredget et al. 2004; Bielecki et al. 2008). Bielecki and colleagues recently reviewed research efforts that have revealed specific traits in *P. aeruginosa* that are pivotal for the development of burn wound infections (Bielecki et al. 2008). A series of gene expression studies show that the three *P. aeruginosa* quorum-sensing systems (*las*R, lasI, *rhl*) allow this bacterium to "sense" the density of bacterial population on the burn wound surface and respond in an organized manner by regulation of a large battery of genes, including those encoding virulence factors (Rumbaugh et al. 2000). In addition, studies by Ha and Jin using in vivo expression technology in a burn mouse model have identified the importance of genes for iron acquisition (*fpt*A) and oxidative stress responses (*sox*R) in the pathogenesis of *P. aeruginosa* burn wound infection (Ha and Jin 1999; Ha et al. 2004). *S. aureus* also has a diverse array of virulence factors that facilitate adherence to host tissues, immune system evasion, and destruction of host cells and tissues, including coagulase, protein A, leukocidins, hemolysins, and superantigens (Foster 2004).

Biofilms are complex communities of surface-attached aggregates of microorganisms embedded in a self-secreted extracellular polysaccharide matrix (EPS) or "slime," and the ability to form biofilms is essential for the development of infection (Hall-Stoodley et al. 2004). Mature biofilms act as efficient barriers against host immune defenses, protect against the environment (i.e., exposure to UV, metal toxicity, acids, dehydration, and salinity), and allow bacteria to resist the effects of antimicrobial agents (Hall-Stoodley et al. 2004; Hoiby et al. 2010). Intercellular signaling molecules produced by bacteria are able to traverse channels in the biofilm and influence their overall growth pattern and behavior in response to various host and environmental factors (Hall-Stoodley et al. 2004; Archer et al. 2011).

Bacteria-causing burn wound infection aggregate to produce a biofilm on the burn wound surface (Kennedy et al. 2010). In animals with experimentally inflicted partial-thickness cutaneous burns, mature biofilms develop in a 48-72-h time period, while in vitro experiments with *Pseudomonas aeruginosa* strains recovered from human burn wounds demonstrate that mature biofilms can form in about 10 h (Harrison-Balestra et al. 2003). Schaber and colleagues recently studied the role of biofilm due to *P. aeruginosa* in a burn-injured mouse model of acute

Table 16.2
Classification of burn wound infections

Type of infection	Description	Diagnosis	Comments
Impetigo	Loss of epidermis in a previously reepithelialized site	Superficial culture or tissue biopsy culture	Usually involves grafts, partial-thickness burns healing by secondary intention or healed donor sites
Surgical wound infection	Purulent exudates, loss of synthetic or biological covering or the wound, erythema and hyperemia of the wound and surrounding tissues	Gram stain shows purulence, and superficial or tissue biopsy cultures are positive for pathogen(s)	Includes both excised burn and donor sites that have not yet epithelialized
Cellulitis	Extension of infection into healthy, uninjured skin and soft tissues. Erythema, pain and tenderness, swelling and warm in the skin surrounding the wound area, and associated lymphangitis	Gram stain shows purulence and superficial or tissue biopsy cultures are positive for pathogen(s)	Includes both excised burn and donor sites
Invasive infection in unexcised wounds	Rapid change in the burn wound appearance with separation and/or dark brown, black, or violaceous discoloration of the eschar	Gram stain shows purulence, and superficial or tissue biopsy cultures are positive for pathogen(s). Histology shows microbial invasion of adjacent tissues	Includes both unexcised partial-thickness and full-thickness burn wounds

Adapted from Peck et al. (1998)

infection and showed that a biofilm was formed within 8 h of burn wound colonization (Schaber et al. 2007). The *P. aeruginosa* quorum-sensing system is also required for efficient blood vessel invasion during burn wound infection, after the biofilm is formed and following the increase in local cell density (Schaber et al. 2007). Bacteria within a biofilm are also able to resist treatment with antimicrobial agents, thereby becoming more resistant (Hoiby et al. 2010). Cells that persist after treatment with antimicrobial agents and antiseptics disable their inherent mechanisms of programmed cell death in the presence of harsh environmental conditions and help in repopulating the biofilm, often leading to failure in biofilm eradication (Hoiby et al. 2010; Archer et al. 2011).

Diagnosis of Burn Wound Infections

Burn wound infection is clinically diagnosed by regular monitoring of the patient's vital signs, blood work, and inspection of the entire burn wound surface, preferably during each dressing change. Important signs of infection include conversion of a partial-thickness injury to a full-thickness wound, rapidly extending cellulitis into healthy tissue surrounding the injury, rapid eschar separation, and tissue necrosis. The classification of burn wound infections was developed by a subcommittee of the Committee on the Organization and Delivery of Burn Care of the American Burn Association (*Table 16.2*) (Peck et al. 1998). However, with early excision and grafting, most burn wound infections now involve open burn-related surgical wound infections (SWIs) (Posluszny et al. 2011).

Laboratory diagnosis of burn wound infection requires culture of regularly collected surface swab or tissue biopsy samples and correlation of the culture results to clinical data in order to distinguish colonization from infection of the burn wound surface (*Table 16.3*). Church and colleagues have previously described the methods that should be used for collecting and transporting burn wound samples to the laboratory (Church et al. 2006). Prior studies that compared the results of surface swab and tissue biopsy cultures provide conflicting results for the following reasons: (a) burn patients do not have homogenous injury (e.g., the severity and extent of burn injury greatly varies from patient to patient), (b) various sampling techniques and laboratory methods have been used, and (c) most studies were done before the advent of early excision therapy (Church et al. 2006). Superficial swabs are the most convenient and least invasive sampling technique for areas of the burn wound that have been excised and provide an adequate sampling of the bacterial microbial flora present on the wound surface. Surface swabs are also the only type of sample that may be taken from areas where the skin is too thin to do a biopsy such as over the ears, eyes, and digits. Anaerobic swab systems and pre-reduced anaerobic media are commercially available that provide an optimal environment for the transport of inoculated surface swabs for culture (Citron 1984), but collection of tissue biopsies placed in nonbacteriostatic saline-moistened gauze in a sterile container may be more reliable for recovery of all anaerobic species from burn wounds. However, the preferred sampling approach for nonexcised areas of the burn wound (i.e., eschar) is to perform quantitative cultures of burn wound tissue biopsies along with concomitant histological analysis (Church

Table 16.3
Criteria used to determine colonization versus infection of the burn wound

Criteria	Colonization[a]	Infection	Comments
Fever, tachycardia, hypotension	Absent	One or more signs may be present	Fever is an early sign of burn wound infection
Redness, pain around burn wound(s)	Absent	Present	Redness spreading into surrounding tissue characteristic of secondary cellulitis
Gram stain (↑PMNs on wound surface)	Little or no ↑PMNs	Moderate to heavy ↑PMNs	PMNs may be present secondary to inflammatory response to the injury
Gram stain (presence of typical bacterial pathogens)	Mixture of normal skin flora and potential pathogens with none predominant	Predominance of one or more pathogens	Correlate the Gram stain appearance (cells and bacteria) with semiquantitative or quantitative culture results
Superficial swab or tissue culture	Mixture of normal skin flora and potential pathogens with none predominant	Predominant growth of one or more pathogens	Correlate semiquantitative or quantitative culture results to the Gram stain result
Tissue histology	Superficial colonization by bacteria but no invasion to deep tissues	Invasion of bacteria into the dermis beneath the eschar and into surrounding tissues	Histological analysis may be required to definitively diagnose invasive infection
Blood cultures	Multiple sets remain negative	One or more sets positive for a pathogen(s) cultured from the burn wound surface or a tissue biopsy	Septic work-up should be done when invasive burn wound infection is suspected

[a]Normal skin flora (i.e., *Staphylococcus*, especially coagulase-negative staphylococci, *Micrococcus*, *Corynebacterium* spp., *Propionibacterium acnes*, *Streptococcus viridans* group spp., *Neisseria* spp., *Brevibacterium*, and *Peptococcus*)

et al. 2006), which provides an accurate assessment of the depth and extent of burn infection in areas of indeterminate injury. Multiple superficial swabs or tissue biopsies should be taken from several areas of the burn wound in order to obtain the most accurate assessment of the burn wound microbiology. Frequent sampling should also be done in the first few days to weeks following burn injury (e.g., daily or every 48 h during dressing changes) when the microbial flora is evolving. Sampling frequency may be decreased to weekly once the burn wound has been excised provided there are no clinical signs of infection.

Gram stain of surface swabs should assess the presence of purulence (i.e., increased amount of PMNs) as well as the numbers and type of bacteria present, although its diagnostic sensitivity is low for detection of burn wound infection (Taddonio et al. 1988; Elsayed et al. 2003). Detailed methods for performing both semiquantitative and quantitative cultures of burn wound surface swabs have been previously reported (Levine et al. 1976; Steer et al. 1996a, b; Church et al. 2006). Semiquantitative culture includes the isolation and identification of each bacterial pathogen and their relative amounts according to the extent of growth on agar media (e.g., 1+, 2+, 3+, 4+). Quantitative counts may be reported from surface swab cultures provided a standard area was swabbed (e.g., 4 cm^2 of the burn wound surface). Colony counts are done to obtain the counts per cm^2 of the surface of the burn for all potential pathogens isolated, and the culture report provides the identification and exact amount per cm^2 of each pathogen. Quantitative tissue biopsy cultures are performed using the original method described by Loebl and others (Loebl et al. 1974a, b; Church et al. 2006). A pathogen tissue density of $>10^5$ cfu/g of tissue was shown to be diagnostic of burn wound infection, correlated well with histological evidence of bacterial tissue invasion beneath the eschar, predicted the development of sepsis, and has been associated with a high mortality rate (McManus et al. 1987). This level of bacterial density in tissue is therefore used by clinical microbiology laboratories to identify all potential pathogens present in a significant amount and to perform antibiotic susceptibility testing. A semiquantitative modification of this method has also been reported by Buchanan and colleagues that provide a predictive index of burn wound sepsis similar to that of quantitative biopsy cultures (Buchanan et al. 1986). Molecular methods may also be used to quantify pathogens in infected burn tissues, but the organism still needs to be cultured from tissue biopsies in order to perform antibiotic susceptibility testing. *P. aeruginosa* has been quantified in burn wound biopsy specimens using real-time polymerase chain reaction (PCR) (Pirnay et al. 2000).

Burn wound infection is confirmed on histological tissue analysis by finding microorganisms invading viable tissue beneath the eschar surface. Histological examination should also be performed on tissue biopsies because high tissue bacterial density may be found during colonization that does not necessarily correlate with microscopic tissue invasion (McManus et al. 1987; Mitchell et al. 1989). A grading system for histological diagnosis of burn wound infection was

Table 16.4
Tissue biopsy histological grading for burn wound infection

Grade	Histological description
0	No microorganisms were observed throughout the entire section
I Ia Ib	Microorganisms were limited to burn wound surface: • Contamination by a few bacteria • Colonization by numerous organisms
II	Microorganisms penetrated superficial dermis
III	Bacterial colonization could be observed throughout entire dermis
IV	An important microbial invasion occurred in the burn wound eschar of subjacent viable tissue and the hypodermis

Adapted from Mitchell et al. (1989)

developed by comparing histological analysis with the results from quantitative cultures of tissue biopsies and is based on the degree and depth of microbial penetration (*Table 16.4*) (Mitchell et al. 1989). Lower histological grades indicate colonization of the burn wound, middle grades (1b or II) indicate increased colonization and early invasion of microorganisms into the superficial dermis, and higher grades document burn wound infection. Histological measurement can also accurately assess the depth of the burn injury using dermal microvascular occlusion and correlate well with both clinical and LDI measurements (Watts et al. 2001). Histological examination of tissue biopsies along with specialized cultures is also necessary to diagnose burn wound infections due to either yeasts, fungi, or viruses (Church et al. 2006). Molecular methods can also be used to monitor infection due to CMV (Bordes et al. 2011b).

Burn Wound Sepsis

Sepsis in the burn patient has recently been defined as a change that triggers the concern for infection (Greenhalgh et al. 2007; Chipp et al. 2010). The clinical triggers include temperature changes (>39 °C or <36.5 °C), progressive tachycardia and/or tachypnea, thrombocytopenia, hyperglycemia, or an inability to continue enteral feedings >24 h (Greenhalgh et al. 2007). However, sepsis is clinically difficult to separate from SIRS in the burn patient because of the altered metabolic, physiological, and immunologic changes that accompany burn injury. An initial sepsis diagnosis is therefore presumptive, and although empiric antimicrobial therapy is started, sepsis cannot be confirmed pending documentation of an infection by either positive cultures or pathological tissue diagnosis or a clinical response to empiric antimicrobial therapy (Greenhalgh et al. 2007).

Although bloodstream infections (BSIs) are a major cause of morbidity and mortality in burn patients, these infections have not been well defined in this patient population (Fitzwater et al. 2003; Williams et al. 2009; Chipp et al. 2010; Shupp et al. 2010). Prior hospital studies have documented the microbiology and septic consequences of burn wound infections, particularly in the preexcision era (Ekenna et al. 1993; Bang et al. 1998; Church et al. 2006). Although the incidence of sepsis as a result of invasive burn wound infection has substantially decreased since the advent of early excision therapy, secondary sources of invasive infection have increased as important contributors including catheter-related infections and ventilator-associated pneumonia (Church et al. 2006; Chipp et al. 2010).

A retrospective review of all patients in the North American National Burn Repository database between 1981 and 2007 with infection listed as a complication was recently performed to determine its contribution to mortality (Shupp et al. 2010). Case matching of patients with blood stream infections (BSI) was done to patients without BSI using TBSA deciles and other potential confounders, and a total of 11, 793 patients (3,931 cases and 7,862 controls) were included. *S. aureus* (32 %) was the most common cause of BSI overall, and among cases due to gram-negative bacteria, *P. aeruginosa* was the most prominent etiology. Infected patients were older (40.9 vs. 32.8 years, $p < 0.05$) and had higher % TBSA burn injury (22.2 vs. 7.9, $p < 0.05$). BSI was associated with significantly higher overall mortality (21.9 % vs. 3.09 %), hospital length of stay (47.4 vs. 8.8 days) including ICU stay (30.8 vs. 2.6 days), ventilator days (29.2 vs. 1.4 days), and hospital charges in US funds ($339, 909.91 vs. $33,272.43) ($p < 0.001$ for all values) (Shupp et al. 2010). Mortality also remained higher for patients with <% TBSA compared to case-matched controls.

Burn patients with sepsis should be immediately examined to determine the site and source of infection including inspection of the entire burn wound surface, skin graft donor sites, and current and previous vascular access sites. Intravascular cannulae should be changed and the removed devices cultured. Diagnostic tests should be done to identify the site/source of infection including blood, urine, and lower respiratory (i.e., sputa, bronchoalveolar lavage) cultures. Empiric broad-spectrum antibiotic therapy directed at the most recent bacteria isolated from burn wound cultures and other sources should be promptly instituted (Murphy et al. 2003; Cohen et al. 2004; Dellinger et al. 2004; Chipp et al. 2010; Shupp et al. 2010). Patients with bloodstream infections in the ICU may develop severe sepsis if initial empiric antimicrobial treatment provides inadequate antimicrobial activity against the organism causing infection (Zaragoza et al. 2003). Additional drugs that block a part of the septic cascade may be also be administered to burn patients with severe sepsis. Activated protein C appears to hold the most promise for improving outcomes in ICU patients with severe sepsis, but sepsis drug intervention trials have shown divergent results for other agents including cytokine inhibitors, anti-endotoxin, and other naturally occurring anti-coagulants (Polderman and Girbes 2004).

Table 16.5
Profile of commonly used topical antimicrobial agents

Topical Agent	Preparation	Depth of eschar penetration	Antibacterial activity	Major toxicity
Silver nitrate ($AgN0_3$)	0.5 % Solution	None	Bacteriostatic against:	Electrolyte imbalance
			Aerobic gram-negative bacilli	
			P. aeruginosa	
			Limited antifungal	
Silver sulfadiazine (Silvadene®, Flamazine®, Thermazene®, Burnazine®)	1 % Water-soluble cream (oil-in-water emulsion)	None	Bactericidal against:	Leucopenia
			Aerobic gram-negative bacilli	
			P. aeruginosa	
			Some *C. albicans*	
Mafenide acetate (Sulfamylon®)	10 % Water-soluble cream (oil-in-water emulsion)	Limited	Broad-spectrum against:	Metabolic acidosis
			Aerobic gram-negative bacilli	
			P. aeruginosa	
	5 % Solution		Anaerobes	
Nanocrystalline silver dressings (Acticoat® A.B. dressing, Silverlon®)	Dressing consisting of two sheets of high-density polyethylene mesh coated with nanocrystalline silver	Moderate	Potent activity against:	Limited toxicity
			Aerobic gram-negative bacilli	
			P. aeruginosa	
			Aerobic gram-positive bacilli	
			MRSA, VRE	
			Multidrug-resistant *Enterobacteriaceae*	

Adapted from Murphy et al. (2003), Lansdown et al. (2005), Church et al. (2006), Dai et al. (2010)

Prevention of Infection in the Burn Patient

Aside from intensive supportive care (i.e., early fluid resuscitation, burn wound excision, enteral feeding, and the use of insulin) that helps restore metabolic, physiological, and immunologic imbalance in the critically ill burn patient, prevention of infection requires adoption of rigorous infection control practices within the burn unit as well as the use of effective topical antimicrobial agents, tetanus immunization, and the prescription of preoperative prophylactic systemic antibiotic(s).

Table 16.5 outlines the profile of the most widely used topical antimicrobial agents and newer silver nanocrystalline dressings that are based on the bactericidal properties of the silver ion (Lansdown et al. 2005; Dai et al. 2010), which strongly interacts with thiol groups in the respiratory enzymes in the bacterial cell (Lansdown 2002a, b). Silver has also been shown to interact with structural proteins and preferentially bind with DNA nucleic acid bases to inhibit replication (Lansdown 2002a, b). For this reason, silver may also be highly toxic to keratinocytes and fibroblasts and delay burn wound healing if applied indiscriminately to debrided healing tissue areas (Cooper et al. 1990; Lansdown 2002). Widespread application of effective topical antimicrobial agents substantially reduces the microbial load on the open burn wound surface, thereby reducing the risk of infection and conversion of partial-thickness to full-thickness wounds (Church et al. 2006; Kowalske 2011). The use of topical antibiotic agents however is adjunctive to early excision therapy in the overall burn care plan (Herndon and Spies 2001; Mosier and Gibran 2009). Selection of topical antimicrobial therapy should be based on the agent's ability to inhibit the microorganisms recovered from burn wound surveillance cultures and to monitor the nosocomial infections acquired in the burn unit (i.e., burn unit antibiogram). Prescription is also based on the individual preparation of the topical agent (e.g., ointment/cream vs. solutions or dressings) and their pharmacokinetic properties (Murphy et al. 2003; Dai et al. 2010). Topical antimicrobial preparations in use in a given burn unit are rotated in order to decrease the potential development of antibiotic resistance (Altoparlak et al. 2004; Church et al. 2006; Rezaei et al. 2011).

Prophylactic systemic antibiotic therapy may also be given to prevent secondary episodes of bacteremia immediately before, during, and for 1 or 2 doses after surgical procedure, particularly in burn patients with extensive injury (e.g., 40% TBSA) (Mozingo et al. 1997). Culture-specific laboratory information obtained from bacterial culture and susceptibility results of the burn wound and other sources (i.e., blood, urine, and respiratory cultures) should be used to guide the selection of effective antimicrobial agents for use as preoperative prophylaxis as well as treatment of overt clinical infections (Murphy et al. 2003).

However, there is no benefit of prescription of a prophylactic course of systemic antibiotics in adult burn patients in either decreasing the occurrence of burn wound infections or improving clinical outcomes compared to the use of topical therapy along with surgical excision (Chipp et al. 2010). Antibiotic prophylaxis may be given in pediatric burn units to prevent the risk of toxic shock syndrome (Rashid et al. 2005), although evidence for and against this practice remains limited (Chipp et al. 2010). Exposure to systemic antibiotic therapy may also cause other complications including antibiotic-associated diarrhea due to the overgrowth of toxigenic strains of *Clostridium difficile* (Crabtree et al. 2011) and colonization with endogenous and pathogenic bacteria with increased resistance to a wide variety of antibiotics (Keen et al. 2010b; Rezaei et al. 2011).

Burn patients are also prone to develop tetanus since thermal injury creates an open dirty wound (Sherman 1970; Culbertson et al. 2004). Human tetanus immune globulin (HTIG 250–500 IU) is given to provide immediate passive immunization, followed by active immunization with tetanus toxoid (0.5 ml I.M.) if the patient has not received a complete primary immunizing series or has not received a tetanus toxoid booster within the past 10 years (Culbertson et al. 2004). Adults are given tetanus-diphtheria vaccine (Td) while a trivalent diphtheria-pertussis-tetanus (DPT) vaccine is administered in young children (i.e., <7 years of age). Subsequent doses of tetanus vaccine should be given to patients who have not had prior or only partial tetanus immunization to ensure protective antibody levels are achieved.

Modern infection control practice in the burn unit has been previously reviewed; so only a brief description of the key measures is outlined herein (Weber and McManus 2004; Rafla and Tredget 2011). Burn patients are uniquely susceptible to being colonized by one or more nosocomial pathogens, and the larger the burn injury, the higher the number and density of microorganisms that may be transmitted in the burn unit environment. Nosocomial pathogens are therefore commonly transmitted in the burn unit by direct or indirect contact, either by the hands of the personnel caring for the patient or from contact with inappropriately decontaminated equipment (Weber and McManus 2004; Church et al. 2006; Rafla and Tredget 2011). Infection control in the burn unit requires strict compliance with enforced hand washing and the universal use of standard precautions including personal protective equipment (i.e., gowns, gloves, and masks) (Weber and McManus 2004; Rafla and Tredget 2011). Healthcare personnel must be gowned (including use of disposable or reusable gowns and disposable plastic aprons to prevent soilage of healthcare workers clothing during wound care procedures) and gloved at each entry to the burn patient's isolation room. Monitoring and diagnostic equipment is housed in each burn patient's room to prevent cross-contamination between patients. All common equipment should be regularly cleaned with appropriate disinfectants between patient use, but sometimes, this is difficult to achieve (i.e., tanks, stretchers, shower tables, straps, mattresses) (Rafla and Tredget 2011). Procedures that have a high risk of contamination such as hydrotherapy or other common treatment areas are minimized (Mayhall 2003).

Hospitalized burn patients are placed on contact isolation in their own room. Nurses and other healthcare personnel are assigned to care for a specific patient or cohort of patients as a team, and the movements of assigned personnel between patients are strictly limited (Thompson et al. 2002). Admission surveillance cultures are done to screen burn patients for colonization by antibiotic-resistant organisms (e.g., MRSA and VRE) (Wibbenmeyer et al. 2009; Altoparlak et al. 2011; Kaiser et al. 2011b). Patients who are colonized on admission or who acquire an antibiotic-resistant organism during their burn unit stay must be physically isolated from other burn unit patient.

Infection control practitioners also play an integral part in any burn center's prevention program. Surgical burn wound infections should be rigorously monitored, and other types of nosocomial infection should also be surveyed including catheter-related infections, pneumonia, and urinary tract infections. In all cases, published standard definitions should be used in identifying these types of infection complications (Emori et al. 1991; Gaynes 1997; Gaynes et al. 2001). Laboratory surveillance cultures (e.g., MRSA culture of nasal, rectal, or groin swabs and VRE culture of rectal swab) as well as routine microbial surveillance cultures of the burn wound and other sources (i.e., blood, respiratory, and urine samples) should be monitored to rapidly identify epidemic pathogens and/or antibiotic-resistant strains so that control measures can be immediately implemented (Weber and McManus 2004; Rafla and Tredget 2011). A burn unit-specific antibiogram should be developed to determine and track the specific pattern of burn wound microbial colonization, time-related changes in the predominant microbial flora of the burn wound in individual patients, the antimicrobial susceptibility profiles of microorganisms implicated in burn wound infections in a given time period, and trends in the nosocomial spread of these pathogens (Altoparlak et al. 2011; Kaiser et al. 2011b; van Duijn et al. 2011). Antibiotic utilization should be rotated or changed based on monitoring antibiotic resistance trends (e.g., antibiograms) within individual burn centers (Agnihotri et al. 2004). Finally, adverse outcomes including morbidity and mortality due to burn wound infection, sepsis, or another nosocomial infection complication should be monitored in burn patients according to the extent of burn injury in order to assess the effectiveness of existing infection control practices within the institute's modern burn therapy program.

Future Perspectives

Due to the dramatic advances that have occurred in the surgical and intensive care of burn patients over the past 100 years (Pruitt and Wolf 2009), most patients with burn injury survive. Because the vast majority of deaths in burn patients occur as a result of bloodstream infections due to invasive surgical burn wound infections, catheter-related infections, or pneumonia, the

continued availability of effective antimicrobial therapy is essential for their survival. Rapidly emerging multidrug resistance in common burn wound pathogens (*S. aureus*, *P. aeruginosa*, *Acinetobacter*, Enterobacteriaceae, and *Enterococcus* spp.) has the potential to erode the progress in survival made by modern burn care over the past century, because limited or no effective therapy is available for some highly resistant organisms (Clark et al. 2003; Walsh et al. 2005; Branski et al. 2009; Livermore et al. 2011). New antibiotic agents with effective activity against common burn pathogens that have acquired multidrug-resistant profiles are therefore urgently needed if mortality due to infection complications in burn patients is to remain low.

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17 Typhoid

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Abstract
Typhoid fever is a severe systemic infection caused by the human-adapted *Salmonella enterica* serovar Typhi (*S.* Typhi). Paratyphoid fever is indistinguishable from typhoid fever in its clinical presentation but is associated with other typhoidal *Salmonella* serovars, including the human-adapted *S. enterica* serovars Paratyphi A, Paratyphi B, Paratyphi C, or Sendai. Although eradicated in most developed countries, these illnesses continue to be a major contributor to morbidity and mortality worldwide, and the emergence of antibiotic resistance is beginning to limit treatment options. Sanitation of drinking water is effective in reducing transmission of typhoid and paratyphoid fever, but eradication of these diseases also requires management of human carriers. Typhoidal *Salmonella* serovars are closely related genetically to nontyphoidal *Salmonella* serovars, which are associated with a localized gastroenteritis in humans. Recent studies suggest that differences in the clinical presentation of typhoid fever and gastroenteritis can be attributed to virulence mechanisms that enable typhoidal *Salmonella* serovars to evade innate immunity but that are absent from nontyphoidal *Salmonella* serovars. One such factor is the virulence capsular polysaccharide (Vi antigen) of *S.* Typhi and *S.* Paratyphi C. Studies on immunity to typhoid fever have resulted in licensing of a killed whole-cell parenteral typhoid vaccine, a live-attenuated oral typhoid vaccine, and a parenteral vaccine consisting of purified Vi antigen. Our entry will review basic and applied research on this enigmatic human disease.

History

Typhoid fever has been an important disease throughout history. A prominent typhoid fever victim in antiquity might have been Alexander the Great, who died from a febrile illness in Babylon on June 10, 323 BC (Oldach et al. 1998). Although the disease has likely contributed to morbidity and mortality for millennia, the nonspecific symptoms make it difficult to distinguish from other illnesses based on historic accounts. The term typhous (τυφωδης) fever, meaning smoky or hazy fever, was applied broadly since the time of Hippocrates (460 BC–*ca.* 370 BC) to describe continued febrile illnesses associated with delirium or stupor which likely included, but were not limited to, typhoid fever.

The first accurate account of the symptoms associated with typhoid fever is that of "putrid fever" described by the British physician Thomas Willis in 1659 (Willis 1682). However, an

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influential treatise on classifying diseases published in 1763 by François Boissier de Sauvages de la Croix continued to apply the term typhus broadly to continued fevers (Sauvages de la Croix 1763). Pierre Charles Alexandre Louis was the first to define typhoid fever as a distinct disease entity in 1829. He used analytical methods to differentiate François Boissier de Sauvages de la Croix's term typhus into two distinct diseases, typhus and typhoid fever, the latter being characterized by lesions in the Peyer's patches and mesenteric lymph nodes (Louis 1836). Subsequent work in the 1830 s by William Wood Gerhard, a disciple of Pierre Charles Alexandre Louis, helped establish the concept that typhoid fever is an independent disease entity in the United States (US). It was not until 1849, through case reports published by Sir William Jenner, that the concept of typhoid fever being distinct from typhus became accepted in the United Kingdom (Jenner 1849).

In 1856, the British physician William Budd published a pioneering epidemiological study in which he demonstrated the communicable nature of typhoid fever and its spread through contagious dejecta (Budd 1856). William Budd's contribution was remarkable as it preceded identification of the causative agent by decades and helped erect the modern discipline of epidemiology. Furthermore, the finding that typhoid fever spreads by fecal-oral transmission led William Budd to propose water chlorination as a preventive strategy. However, this idea was initially met with skepticism by those in the medical community, who remained convinced that typhoid fever spreads through the gases rising from putrid matter (miasma) rather than by contagion (germ theory). Eventually, William Budd's recommendations were heeded with the passing of the British Public Health Act in 1875, and this resulted in improved sanitary practices.

In 1880, the German pathologist Carl Joseph Eberth provided direct support for the theory that contagious germs cause typhoid fever by visualizing the causative agent, termed typhoid bacillus, in histological sections (Eberth 1880). In 1884, the typhoid bacillus was isolated and grown in pure culture by the German physician Georg Theodor August Gaffky, who worked under Robert Koch at the Imperial Health Office (Kaiserliches Gesundheitsamt) in Berlin (Gaffky 1884). One year after Georg Theodor August Gaffky's characterization of the typhoid bacillus, the bacteriologist Theobald Smith isolated *Bacterium cholerae-suis* from a pig while working under Daniel Elmer Salmon at the Veterinary Division of the United States Department of Agriculture (USDA) (Salmon and Smith 1885). In honor of Daniel Elmer Salmon, the genus *Salmonella* was erected in 1900 with *Salmonella cholerae-suis* as the type species (Salmonella-Subcommittee 1934). In 1904, the British physician Albert Sidney Grünbaum fulfilled the last Koch's postulates by demonstrating that experimental oral infection of chimpanzees with the typhoid bacillus produces a typhoid-like disease with the characteristic intestinal lesions (Grunbaum 1904). In 1918, the typhoid bacillus was renamed *Eberthella typhi* to recognize Carl Joseph Eberth's contribution to its discovery (Buchanan 1918). In 1933, the designation *Eberthella typhi* was changed to *Salmonella typhi* (Salmonella-Subcommittee 1934). At this time, each species within the genus *Salmonella* was defined based on its fermentative and serological characteristics and was referred to by a Latin binomial (Kelterborn 1967). This nomenclature was eventually abandoned when it became clear that members of the genus *Salmonella* are closely related genetically (Crosa et al. 1973). Current nomenclature distinguishes only two species within the genus, *Salmonella enterica* (Le Minor and Popoff 1987) and *Salmonella bongori* (Reeves et al. 1989). All other former *Salmonella* species are now considered to be serovars (also known as serotypes). For example, the causative agent of typhoid fever is now regarded as a serovar belonging to the species *S. enterica*, and it is correctly referred to as *S. enterica* serovar Typhi (*S.* Typhi) (Brenner et al. 2000).

The identification of the causative agent of typhoid fever by Carl Joseph Eberth and Georg Theodor August Gaffky in the 1880s (Eberth 1880; Gaffky 1884) not only helped to fortify the concept that typhoid fever spreads by contagious germs, but it also spawned important discoveries related to diagnosis and prevention. For example, the ability to grow the organism in pure culture enabled Georges-Fernand-Isidor Widal to demonstrate in 1896 that serum from typhoid fever patients could agglutinate *S.* Typhi (Widal 1896). This serological test for typhoid fever was termed the Widal test and is still in use today. In the same year, bacteria isolated from two individuals with symptoms of typhoid fever that tested negative in the Widal reaction were termed *Bacille paratyphique* (Achard and Bensaude 1896), a pathogen now known as *S. enterica* serovar Paratyphi B (*S.* Paratyphi B).

Cultivation of *S.* Typhi also paved the way in 1896 for the development of the first heat-killed parenteral vaccine (Groschel and Hornick 1981), a strategy pursued in parallel by the British pathologist Sir Almroth Edward Wright at the Army Medical College in Netley (Wright 1896) and the German bacteriologist Richard Pfeiffer, who worked under Robert Koch at the Imperial Health Office in Berlin (Pfeiffer and Kolle 1896). Identification of the virulence (Vi) antigen, a capsular polysaccharide of *S.* Typhi, in 1934 (Felix and Pitt 1934) set the stage for the development of a purified Vi polysaccharide parenteral vaccine (Robbins and Robbins 1984). Finally, René Germanier and Emil Fürer from the Swiss Serum and Vaccine Institute introduced a third immunization strategy in 1975 by developing the first live attenuated oral typhoid vaccine strain (Ty21a) (Germanier and Fuer 1975).

In 1903, Robert Koch gave a lecture at a conference in Kassel in which he proposed the new concept that typhoid fever spreads solely through human-to-human transmission and that healthy humans that chronically shed *S.* Typhi could serve as a reservoir. This presentation initiated measures to improve sanitary practices in Germany and launched public health measures to identify and isolate human carriers of the disease (Drigalski 1904). These public health measures for managing carriers proved essential for eradicating typhoid fever in North America and Europe. While it was established at the time that *S.* Typhi is regularly present in the gallbladder (Anton and Fütterer 1888), Robert Koch's lecture sparked renewed interest, and soon

persistence in the gallbladder was proposed as a mechanism for chronic human carriage (Kelly 1906; Porster 1906). In 1906, Heinrich Kayser, who worked under Robert Koch at the Imperial Health Office in Berlin, estimated that 13.5% of typhoid fever cases could be traced to apparently healthy human carriers (Kayser 1906). Perhaps the best-known case of chronic carriage was the one identified by the American sanitary engineer George Soper. In his 1907 report, he traced an outbreak of typhoid fever in the state of New York back to a cook named Mary Mallon, better known as typhoid Mary (Soper 1907).

Before improved sanitary practices were implemented, the annual incidence of typhoid fever remained as high as 500 cases per 100,000 people in the nineteenth century in Europe and North America. The discovery by William Budd that typhoid fever is transmissible led to improved sanitation of drinking water, while identification of the causative agent by Carl Joseph Eberth and Georg Theodor August Gaffky set the stage for vaccination and the identification and isolation of human carriers. Together, these public health measures reduced the incidence of typhoid fever in Europe and North America throughout the first half of the twentieth century to their current level of less than 1 case per 100,000 people.

Taxonomy

Salmonella Species

The genus *Salmonella* belongs to the phylum Proteobacteria, the class Gammaproteobacteria, the order Enterobacteriales, and the family Enterobacteriaceae. The genus consists of two species, *Salmonella enterica* (Le Minor and Popoff 1987) and *Salmonella bongori* (Reeves et al. 1989). The species *S. enterica* is further subdivided into six subspecies, which can be referred to either by roman numerals (subspecies I, II, IIIa, IIIb, IV, and VI) or by names (subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*, respectively) (Brenner et al. 2000; Tindall et al. 2005). All *Salmonella* serovars that are associated with typhoid fever or paratyphoid fever in humans belong to *S. enterica* subspecies *enterica* (*S. enterica* subspecies I). However, the subspecies designation is commonly omitted when referring to a serovar (e.g., *S. enterica* serovar Typhi instead of *S. enterica* subspecies *enterica* serovar Typhi), and, in order to shorten reports, this designation is often further abbreviated (e.g., *S.* Typhi).

Salmonella Serovars

Each *Salmonella* serovar is defined by an antigen formula, which lists O factors, H1 factors, and H2 factors separated by colons (*Table 17.1*). O factors (also known as O antigens or somatic antigens) correspond to antigenic determinants present in the oligosaccharide repeat units of lipopolysaccharide (LPS). Capsule expression is uncommon in the genus *Salmonella* but occurs in *S.* Typhi and *S. enterica* serovar Paratyphi C, which express the Vi antigen. Expression of the Vi antigen is indicated along with O factors before the first colon in the antigen formula. H factors (H antigens) represent antigenic determinants present in flagellin, the major protein subunit of flagella. The majority of *S. enterica* serovars express two flagellins, termed FliC (H1 factor) and FljB (H2 factor), respectively. Flagellin expression oscillates between states characterized by exclusive production of FliC (H1-phase flagellin) and exclusive production of FljB (H2-phase flagellin), a phenomenon known as flagellar phase variation (Andrews 1922). Sequence variants of FliC and FljB proteins can be distinguished serologically, and their reactivity with typing sera is indicated in the antigen formula of a serovar. Flagellar phase variation is mediated by a heritable molecular switching mechanism composed of an invertible promoter element controlling expression of the *fljBA* genes (Silverman and Simon 1980). If the invertible promoter element is oriented such that it drives expression of *fljBA* (phase H2 orientation), the cell produces FljB (H2-phase flagellin) and FljA. In turn, FljA prevents the production of H1-phase flagellin by binding to the 5′-untranslated region of the *fliC*

Table 17.1
Typhoidal *Salmonella* serovars and related nontyphoidal serovars

S. enterica serotype	O antigen : H1-phase : H2-phase	Other	Serogroup (immunodominant O factor)	H_2S production	D-tartrate utilization	Citrate utilization
Typhi	9, 12, [Vi] : d : -	[Z_{66}]	D1 (O : 9)	+/–	+	+/–
Paratyphi A	*1*, 2, 12 : a : –		A (O : 2)	+/–	–	–
Paratyphi B	*1*, 4, [5], 12 : b : 1, 2		B (O : 4)	+	–	+/–
Paratyphi B var. Java (nontyphoidal)	*1*, 4, [5], 12 : b : 1, 2		B (O : 4)	+	+	+
Paratyphi C	6, 7, Vi : c :1, 5		C1 (O : 7)	+	+	+
Sendai	*1*, 9, 12 : a : 1, 5		D1 (O : 9)	+/–	–	–
Miami (nontyphoidal)	*1*, 9, 12 : a : 1, 5		D1 (O : 9)	+	x	+

[ ] can be absent in isolates from this serotype, *1* the O factor 1 can be present due to phage conversion, : the H2 factor is absent, x weakly positive, +/– reaction can be positive or negative

transcripts (Bonifield and Hughes 2003), thereby blocking its translation (Yamamoto and Kutsukake 2006). Phase variation requires inversion of the controlling DNA element (phase H1 orientation), thereby silencing *fljBA*, which relieves the translational block of *fliC* mRNA and allows the cell to produce H1-phase flagellin (Silverman and Simon 1980).

S. Typhi has the O antigen formula 9, 12, [Vi] : d : -, which is a numeric representation of four antigenic epitopes: O factor 9, O factor 12, the Vi antigen, and H1 factor d (Table 17.1). O factor 9 corresponds to a tyvelose branch that is α-(1,3)-linked to D-mannose in the trisaccharide backbone of the O antigen and represents the immunodominant epitope in the LPS of *S.* Typhi. O factor 12 corresponds to the trisaccharide backbone of the O antigen repeat unit consisting of α-D-mannose-(1,4)-α-L-rhamnose-(1,3)-α-D-galactose (Fig. 17.1a). The Vi antigen is a homopolymer composed of (1,4)-2-acetamido-3-*O*-acetyl-2-deoxy-α-D-galacturonic acid that forms a capsular polysaccharide on the bacterial surface (Heyns and Kiessling 1967) (Fig. 17.1b). Expression of the Vi antigen prevents agglutination of *S.* Typhi with serum against O factors 9 and 12 (Felix et al. 1934; Felix and Pitt 1934). The parenthesis in the antigen formula indicates that the Vi antigen is encoded on a genetically unstable DNA region that can be lost upon laboratory passage of *S.* Typhi (Bueno et al. 2004; Nair et al. 2004). H1 factor d indicates that the FliC protein of *S.* Typhi reacts with H1 typing serum d. Finally, the absence of an H2 factor (-) indicates that *S.* Typhi is a monophasic serovar in which the *fljBA* genes have been lost by deletion. An exception to the monophasic phenotype of *S.* Typhi has been described for some Indonesian isolates which carry a *fljBA*-like operon on a linear plasmid that encodes the H : z_{66} antigen (Huang et al. 2004; Baker et al. 2007).

Typhoid fever caused by *S.* Typhi is indistinguishable in its symptoms from paratyphoid fever, which can be caused by *S. enterica* serovar Paratyphi A (*S.* Paratyphi A), *S.* Paratyphi B, *S.* Paratyphi C, or *S. enterica* serovar Sendai (*S.* Sendai) (Kelterborn 1967). Pathogens causing typhoid fever or paratyphoid fever are collectively referred to as typhoidal *Salmonella* serovars (Table 17.1). *S.* Sendai expresses O factors 9 and 12 but lacks the Vi antigen. *S.* Paratyphi A and *S.* Paratyphi B possess the same trisaccharide repeating unit, O factor 12, but differ from *S.* Typhi and *S.* Sendai in their immunodominant branching carbohydrate moieties. *S.* Paratyphi A has paratose α-(1,3)-linked to D-mannose (O factor 2), while the branching carbohydrate moiety of

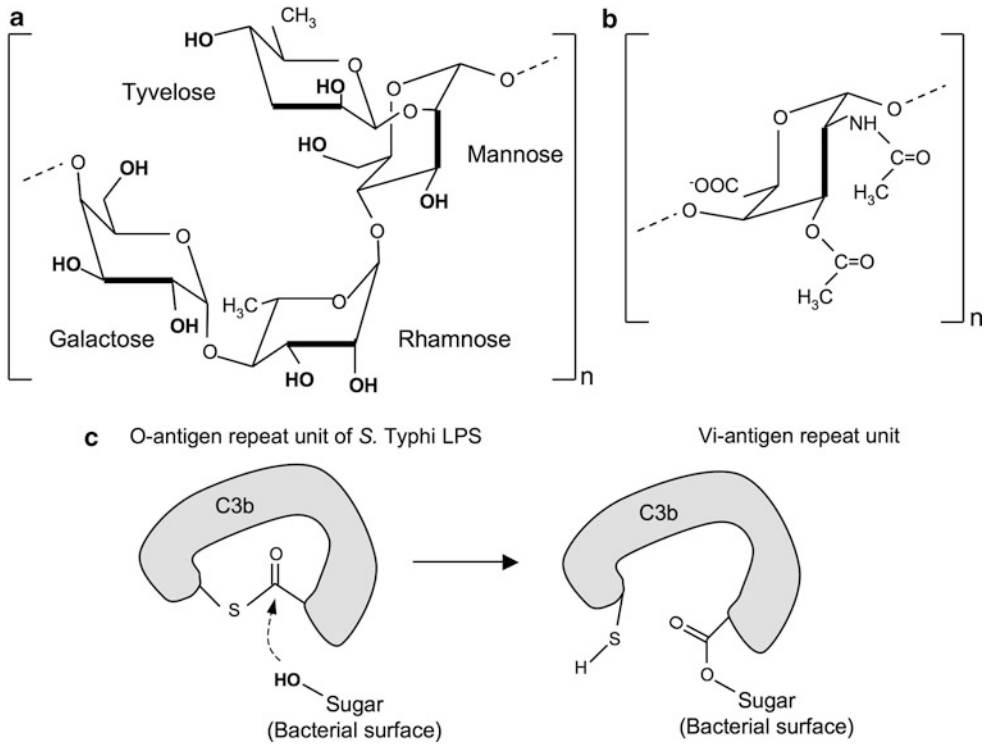

Fig. 17.1

***O antigen, Vi capsule, and complement deposition*. (a) Chemical composition of the *S.* Typhi O antigen repeat unit (reproduced from (Wilson et al. 2011) with permission). Free hydroxyl groups available for complement deposition are indicated in bold font. (b) Chemical composition of the Vi antigen repeat unit (reproduced from (Wilson et al. 2011) with permission). Note the absence of free hydroxyl groups in the Vi antigen. (c) Mechanism of complement deposition (opsonization). An intramolecular thioester bond within C3b reacts with free hydroxyl groups in sugar moieties of the O antigen to form an ester bond that covalently attaches complement to the bacterial surface**

S. Paratyphi B is abequose (O factor 4). In some isolates of *S.* Paratyphi B, the abequose branch can be acetylated, giving rise to O factor 5. O factor 12 serves as a receptor for P22 and related bacteriophages. Lysogenic conversion by these phages can result in expression of an additional epitope, O factor 1 (Zinder 1958), which represents a glucosyl branch linked to the galactose residue in the trisaccharide repeat unit. Finally, *S.* Paratyphi C (O antigen formula 6, 7, Vi) expresses the Vi antigen and an O antigen with pentasaccharide repeat units that are composed of β-D-mannose-(1,2)-α-D-mannose-(1,2)-α-D-mannose-(1,2)-β-D-mannose-(1,3)-β-*N*-acetyl-D-glucosamine (Lindberg et al. 1988). While *S.* Paratyphi B, *S.* Paratyphi C, and *S.* Sendai are biphasic (i.e., expressing H1 and H2 factors), *S.* Paratyphi A is a monophasic serovar due to mutations in the *hin* and *fin* genes (Kutsukake et al. 2006), which encode proteins that control inversion of the *fljBA* promoter element (Silverman and Simon 1980). As a result, the invertible *fljBA* promoter element is locked in the H1 orientation in *S.* Paratyphi A, resulting in exclusive expression of H1-phase flagellin (McClelland et al. 2004).

Salmonella Serogroups

Salmonella serovars that express identical immunodominant O factors can be further organized into serogroups (*Table 17.1*). For example, *S.* Typhi and *S.* Sendai both express the immunodominant O factor 9 and thus belong to the same serogroup, designated D1. The majority (95.7%) of *Salmonella* serovars isolated from humans or food animals belong to only five serogroups, including B (O factor 4), C1 (O factor 7), C2 (O factor 8), D1 (O factor 9), and E1 (O factor 10) (Kelterborn 1967).

S. Typhi Phage Types

Phage typing is a method to distinguish different isolates of the same serovar for epidemiological purposes. The method is based on differences in the susceptibility of isolates to a set of bacteriophages (typing phages). Phage typing schemes are available for two typhoidal *Salmonella* serovars, *S.* Typhi and *S.* Paratyphi B (Anderson and Williams 1956; Craigie and Felix 1947). *S.* Typhi typing phages are derived from a bacteriophage isolated by Craige and Yen in 1938, which is specific for the Vi antigen and is termed the type II Vi phage (CRAIGIE and YEN 1938). Derivatives of the type II Vi phage can be generated by sequential adaptation to growth on specific *S.* Typhi host strains. An adapted phage can yield progeny that forms more plaques on the parent strain than on genetically distinct *S.* Typhi isolates, and the respective phage stock can thus be used as a typing phage. Differences in the infectivity of these typing phages for different *S.* Typhi isolates are largely based on the restriction/modification systems encoded by the *S.* Typhi host strain used for sequential adaptation of the respective type II Vi phage stock (Pickard et al. 2008).

Isolation and Identification

Isolation

Salmonella serovars can be isolated from stool by streaking samples on low selective agar plates containing bile salts to inhibit growth of Gram-positive bacteria (e.g., MacConkey agar or deoxycholate agar) and on intermediate-selective agar plates containing bile salts, citrate, and a low concentration of Brilliant green (0.0003 g/l) to inhibit growth of Gram-positive bacteria and most coliform bacteria (e.g., *Salmonella-Shigella* agar). This method is commonly used for stool samples from febrile individuals with diarrhea because it screens for two possible causes, *Shigella* species and *Salmonella* serovars.

Isolation of *S.* Typhi from individuals with typhoid fever is best achieved using their bone marrow cultures, followed by duodenal content cultures, while blood cultures, stool cultures, or urine cultures are less reliable (Khourieh et al. 1989; Avendano et al. 1986; Gilman et al. 1975; Gilman and Hornick 1976; Benavente et al. 1984; Hoffman et al. 1984; Farooqui et al. 1991). A combination of these methods succeeds in isolating typhoidal *Salmonella* serovars from more than 90% of typhoid fever patients. However, due to the discomfort associated with bone marrow aspirate cultures and duodenal string-capsule cultures, isolation frequently relies on blood cultures, which reduces sensitivity to 40–70% (Hoffman et al. 1984; Benavente et al. 1984; Gilman et al. 1975; Farooqui et al. 1991).

A medium that is highly selective for *Salmonella* serovars is Brilliant green agar, which contains a high concentration of Brilliant green (12.5 g/l) to inhibit growth of Gram-positive bacteria and most Gram-negative bacilli (Kauffmann 1935). However, typhoidal *Salmonella* serovars do not adequately grow on Brilliant green agar. The medium of choice for isolating typhoidal *Salmonella* serovars is bismuth sulfite agar, which contains an intermediate concentration of Brilliant green (0.025 g/l) to inhibit growth of coliforms and Gram-positive bacteria (Wilson and Blair 1927, 1931). Bismuth sulfite agar contains ferrous sulfate ($FeSO_4$), which can be reduced to hydrogen sulfide (H_2S) by proteins encoded in the *phsABC* operon (Heinzinger et al. 1995) and the *asrABC* operon (Huang and Barrett 1991) of *Salmonella* serovars. Hydrogen sulfide (H_2S) reacts with the bismuth sulfite indicator ($Bi_2[SO_3]_3$) in the medium to produce bismuth sulfide (Bi_2S_3), a black insoluble precipitate that produces a characteristic black to brown coloration of colonies. H_2S production in typhoidal *Salmonella* serovars is variable and can be absent (*Table 17.1*).

Enrichment can be performed for samples containing a low number of bacteria prior to streaking samples on selective agar plates. A common enrichment method for *Salmonella* serovars is static growth in tetrathionate broth (Muller 1923) or in derivatives of this medium. The *ttrRS ttrBAC* gene cluster (Hensel et al. 1995) enables *Salmonella* serovars to utilize tetrathionate ($S_4O_6^{2-}$) as a terminal respiratory electron acceptor (Barrett and Clark 1987), thereby promoting their outgrowth over competing microbes in tetrathionate broth under microaerophilic conditions.

Identification

Salmonella serovars are Gram-negative, rod-shaped, and commonly peritrichously flagellated bacteria. They can be distinguished based on their biochemical properties from closely related members of the Enterobacteriaceae that can grow on selective media used for isolation of *Salmonella* serovars. For instance, *Escherichia coli* and *Citrobacter* species utilize lactose, a property absent in *Shigella* species and most *Salmonella* serovars. However, some serovars of *S. enterica* subspecies *arizonae* and most serovars of *S. enterica* subspecies *diarizonae* can ferment lactose and therefore form pink colonies on MacConkey agar plates, which contain lactose and a pH indicator to visualize fermentation of this carbon source. *Citrobacter* species and most *E.* coli isolates can ferment sucrose, while *Salmonella* serovars are negative for this reaction. Citrobacter species and *Salmonella* serovars can reduce ferrous sulfate ($FeSO_4$) to hydrogen sulfide (H_2S), which distinguishes them from *E. coli* and *Shigella* species.

The above biochemical properties can be assessed, for example, by using triple sugar iron (TSI) agar slants, a medium containing lactose (1%), sucrose (1%), a small amount of glucose (0.1%), ferrous sulfate ($FeSO_4$), and the pH indicator phenol red (Russell 1911). A TSI agar slant is inoculated by streaking a pure culture over the surface, followed by stabbing the inoculum deep into the agar. An organism that can ferment lactose and/or sucrose, such as *E. coli* or *Citrobacter* species, will turn the medium yellow after incubation for 24 h. *Citrobacter* species produce H_2S, which forms a black ferrous sulfide (FeS) precipitate in TSI agar, with yellow-colored agar remaining visible at the top of the agar slant. No black precipitate is visible with slants inoculated with *Escherichia coli* and *Shigella* species. Since *Salmonella* serovars only ferment the small amount of glucose present in the medium, the top of the TSI agar slant remains red while H_2S production turns the agar black. It should be mentioned that H_2S production can be absent in typhoidal *Salmonella* serovars (*Table 17.1*).

A quick test of whether a colony isolated on selective media represents a *Salmonella* serovar is slide agglutination with polyvalent O antiserum, which allows a rapid detection of the most common *Salmonella* serogroups. *Salmonella* serovars can then be assigned to *S. bongori* or to one of the subspecies of *S. enterica* using biochemical criteria. Most isolates belonging to *S. enterica* subspecies *enterica* can utilize tartrate as a carbon source, a property absent from *S. bongori* and other *S. enterica* subspecies. However, tartrate utilization is commonly absent in typhoidal *Salmonella* serovars, which are all members of *S. enterica* subspecies *enterica* (*Table 17.1*).

Traditionally, the most common method for subdividing members of the genus *Salmonella* is serotyping. This is still the gold standard, but the procedure is time consuming and requires the availability of a collection of typing sera. A colony isolated on selective media can be immediately typed by slide-agglutination for its O factor and its H1 factor, but not for its H2 factor, because *Salmonella* serovars consist predominantly of bacteria in the H1-phase after in vitro passage (Stocker 1949). Isolation of H2-phase bacteria is performed by stabbing a culture into motility agar supplemented with antiserum against its H1 factor. Since H1-phase bacteria become entrapped in the agar by antibodies, halos formed after overnight incubation consist of H2-phase bacteria, which can then be isolated to type their H2 factor.

In some cases, biochemical characterization is performed to distinguish biotypes within a serovar. For example, *S.* Paratyphi B isolates from patients with paratyphoid fever are generally tartrate utilization-negative, while isolates from individuals with gastroenteritis are commonly tartrate utilization-positive, and the latter biotype is referred to as *S.* Paratyphi B variety Java (*Table 17.1*) (Kauffmann 1955; Ezquerra et al. 1993; Malorny et al. 2003). *S.* Sendai isolates associated with paratyphoid fever do not produce hydrogen sulfide (H_2S) and/or cannot grow on citrate, which distinguishes them from gastroenteritis isolates that share the same antigen formula but represent a different biotype classified as *S.* Miami (*Table 17.1*) (Edwards and Moran 1945; Chau and Huang 1979).

Epidemiology

Habitat

Humans are the only known reservoir for *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi C, and for biotypes of *S.* Sendai and *S.* Paratyphi B that are associated with paratyphoid fever. Individuals with active typhoid fever commonly shed the organism with their feces or urine. During convalescence, at the time of hospital discharge, approximately 20% of individuals still excrete *S.* Typhi (Gould and Qualls 1912). This number declines to approximately 4% of individuals who develop chronic carriage in the gallbladder or, less frequently, in the urinary bladder (Stone 1912). Chronic carriage in the gallbladder leads to intermittent release of *S.* Typhi into the intestine, resulting in fecal shedding. In the absence of adequate treatment, this state can persist for the remainder of an individual's life (Gregg 1908). Contact carriers represent another important reservoir for excretion of *S.* Typhi. Contact carriers have no history of typhoid fever but shed *S.* Typhi, presumably because they developed a subclinical infection, commonly through contact with a typhoid fever patient, a condition that can arise in hospital workers (Stone 1912).

Routes of Transmission

Typhoid fever spreads by means of water, milk, and food products that become contaminated by individuals excreting *S.* Typhi (Stone 1912). Carriers handling water, milk, or food can introduce the contamination directly into these products. Infections can also occur remotely, as *S.* Typhi can survive outside its host and contaminate water supplies or spread through products distributed by trade.

Observations made during the eradication of typhoid fever in the United States illustrate the relative importance of different

routes of transmission. Typhoid fever was an important cause of death in major US cities when monitoring began. Typhoid fever death rates per 100,000 people were 65 for Boston in 1865, 73 for Chicago in 1867, 62 for Baltimore in 1875, 66 for Buffalo in 1882, 42 for Providence in 1884, and 43 for New Orleans in 1895 (Putnam 1927). A crucial initial step in the eradication of typhoid fever was improved sanitation of drinking water either by filtration or by chlorination (Howard 1920; Putnam 1927; Ferrie and Troesken 2008). The incidence of typhoid fever in the USA declined following the 1870s, but records during the initial period are sparse (Ausubel et al. 2001; Putnam 1927). Concurrently with the implementation of improved drinking water sanitation, the annual typhoid fever death rates in the USA dropped from an average of 31.3 per 100,000 people in 1900 to 3.8 per 100,000 people in 1925 (Putnam 1927). These data suggest that contaminated drinking water was the most important vehicle for typhoid fever transmission in the USA at the beginning of the twentieth century.

Given that 14.5% of typhoid fever cases had a fatal outcome in the USA during the first decades of the twentieth century (Graham 1916), a death rate of 3.8 per 100,000 people reported in 1925 (Putnam 1927) suggests an incidence of approximately 26 illnesses per 100,000 people, which is more than 100-fold higher than the current annual incidence of 0.2 illnesses per 100,000 people (Scallan et al. 2011). Thus, while sanitation of drinking water markedly reduced the incidence of typhoid fever, it did not result in an eradication of the disease (Leavitt 1992). This does not suggest that drinking water was not successfully treated because the measures taken were sufficient to eradicate the water-borne disease cholera in the USA by 1911 (Weber et al. 1994). The fact that typhoid fever remained endemic in the USA, even after the broad implementation of drinking water sanitation by 1925, has been attributed to healthy human carriers (Putnam 1927; Leavitt 1992). Chronic carriage of *Vibrio cholerae* does not develop in individuals that recover from an episode of cholera, which made it possible to eradicate the disease solely by providing access to safe drinking water. In contrast, the eradication of typhoid fever ultimately requires the identification and management of healthy human carriers in addition to improving water sanitation.

After drinking water in the USA became safe, the discovery and control of healthy typhoid carriers became an increasingly important goal in the effort to eradicate typhoid fever. For example, starting in the second and third decades of the twentieth century, confirmed chronic typhoid carriers in New York were placed under supervision by the local or state health department, which involved quarterly visits. Chronic typhoid carriers were not permitted to engage in any occupation involving the handling of food for other than members of their own household and were not permitted to reside on farms on which cows were kept. Furthermore, contacts of chronic carriers were advised to be vaccinated against typhoid fever. Chronic carriers remained under supervision by the local or state health department for the remainder of their lives, unless the carrier state was resolved by cholecystectomy, a procedure associated with 15% mortality in the 1920s and 1930s (Senftner and Coughlin 1933). Although chloramphenicol therapy did not succeed in clearing *S.* Typhi carriage (Woodward et al. 1950), the advent of antibiotic therapy in the 1940s made cholecystectomy a safe procedure to cure chronic carriers (Freitag 1964). Eventually, these public health measures led to an eradication of typhoid fever in the USA by the second half of the twentieth century (Armstrong et al. 1999).

The Global Burden of Typhoid Fever

The most recent estimate suggests that 21,650,974 illnesses of typhoid fever occurred worldwide during the year 2000 (Crump et al. 2004). The incidence of typhoid fever is highest in south-central and Southeast Asia with more than 100 illnesses per 100,000 people each year. For comparison, the annual incidence in the USA at the beginning of the twentieth century was approximately 216 illnesses per 100,000 people (Putnam 1927). Typhoid fever is also endemic in the rest of Asia, Africa, Latin America, the Caribbean, and Oceania (with the exception of Australia and New Zealand) where it is responsible for between 10 and 100 illnesses per 100,000 people each year (Crump et al. 2004). For comparison, the incidence of typhoid fever in the USA was within this range in 1925, with 26 illnesses per 100,000 people, after improved sanitation of drinking water had been implemented, but before the problem of chronic carriage had been resolved (Putnam 1927). Currently, the disease is essentially eradicated in North America, Europe, and other developed countries. The Centers for Disease Control and Prevention (CDC) estimates 433 cases of typhoid fever occur each year in the USA (Scallan et al. 2011), and most of these cases can be traced back to foreign travel into endemic areas (Lynch et al. 2009).

Paratyphoid fever was responsible for an estimated 5,412,744 illnesses worldwide during the year 2000 (Crump et al. 2004). Globally, *S.* Paratyphi A is responsible for most episodes of paratyphoid fever. In southeast China, *S.* Paratyphi A has recently surpassed *S.* Typhi as the most common typhoidal serovar isolated from blood cultures (Ochiai et al. 2005). This might be due in part to successes in typhoid fever vaccination, which does not protect against *S.* Paratyphi A (Simanjuntak et al. 1991).

Using the conservative estimate of 1% for the case fatality derived from hospital-based studies and the estimate that 21,650,974 illnesses occurred worldwide in the year 2000, it has been approximated that typhoid fever was responsible for 216,510 deaths that year (Crump et al. 2004). A limitation of this estimate is that case fatality rate data from population-based studies in areas with a high incidence of the disease are lacking. A more recent meta-analysis of hospital-based studies conducted between 1984 and 2005 in Africa, Asia, Latin America, and Oceania suggests that 2% is a more realistic estimate for the current median case fatality rate of typhoid fever (Crump et al. 2008). When this more recent approximation for the case fatality rate is applied to predict the global death toll, it can be estimated that *S.* Typhi was responsible for 433,020 deaths in the year 2000.

Antibiotic Resistance

In 1948, chloramphenicol was identified as the first antibiotic to be effective in the treatment of typhoid fever (Woodward et al. 1948), revolutionizing management of the disease and quickly becoming the drug of choice in its treatment (Rosove et al. 1950). However, *S.* Typhi strains carrying plasmid-encoded resistance against chloramphenicol emerged in the early 1970s in Latin America and Asia (Anderson and Smith 1972), which led to a search for alternative drugs.

Amoxicillin/ampicillin and oral cotrimoxazole (trimethoprim/sulfamethoxazole) therapy surfaced as an effective alternative for treatment of typhoid fever in the 1960s and 1970s (Uwaydah and Shammaa 1964; Calderon 1974; Snyder et al. 1976). However, multidrug-resistant (MDR) *S.* Typhi strains carrying plasmid-encoded resistance to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole appeared in the late 1980s in Asia (Anand et al. 1990). MDR *S.* Typhi currently comprises 7% of isolates from India, 22% of isolates from Vietnam, and 65% of isolates from Pakistan (Parry and Threlfall 2008).

In the 1980s and 1990s, new antibiotics for the treatment of MDR *S.* Typhi were introduced, which included extended spectrum cephalosporins (Soe and Overturf 1987; Islam et al. 1988) and fluoroquinolones (Wang et al. 1989; Dutta et al. 1993). Partial or full resistance to fluoroquinolones emerged in Vietnam in 1993 (decreased ciprofloxacin susceptible [DCS] *S.* Typhi), which has begun to limit treatment options (Wain et al. 1997; Chinh et al. 2000; Parry 2004). A recent survey suggests that 44% of typhoid fever isolates from Vietnam, 57% of isolates from India, and 59% of isolates from Pakistan represent DCS *S.* Typhi (Parry and Threlfall 2008). Current treatment recommendations for typhoid fever include the extended spectrum cephalosporin antibiotic ceftriaxone, the macrolide antibiotic azithromycin, the fluoroquinolone antibiotic gatifloxacin, and, in areas with low prevalence of plasmid-mediated resistance, chloramphenicol (Butler 2011).

Disease

Distribution of Bacteria

Typhoid fever is an infection characterized by systemic bacterial dissemination in immunocompetent individuals. Upon ingestion of contaminated food, milk, or water, *S.* Typhi enters the ileal mucosa at areas of Peyer's patches. From here, the pathogen passes through the mesenteric lymph nodes and the efferent lymphatics into the blood stream, from which it disseminates to internal organs. Postmortem examination reveals *S.* Typhi to be present more commonly in the ileum and less frequently in the colon (Tonney et al. 1916). In the absence of antibiotic treatment, *S.* Typhi is isolated consistently from the Peyer's patches, the mesenteric lymph nodes, the liver and the spleen, with great frequency from the bone marrow and the gallbladder, in about half of the cases from blood, and not commonly from the kidneys and the lungs (Horton-Smith 1900; Levy and Gaehtgens 1908). Bacteriuria occurs in approximately 22% of individuals toward the end of the disease or during convalescence, which is commonly associated with bacterial numbers high enough to turn the urine turbid (Richardson 1903; Horton-Smith 1900).

With the advent of antibiotic therapy, bacteriuria caused by *S.* Typhi has become rare (Mathai et al. 1995). However, *S.* Typhi can be isolated from rectal swab culture or stool culture in 26–37% of typhoid fever patients treated with antibiotics (Gilman et al. 1975; Hoffman et al. 1984; Benavente et al. 1984). Chronic gallbladder carriage develops in approximately 3% of patients despite antibiotic therapy (Huang and DuPont 2005; Gilman 1989).

Clinical Presentation

One striking feature of typhoid fever is that after ingestion of contaminated food, milk or water, the initial spread of *S.* Typhi through the Peyer's patches and mesenteric lymph nodes into the circulation does not evoke overt host responses, as indicated by the fact that the average incubation period of the disease is 2 weeks (Olsen et al. 2003). After this incubation period, the disease presents with nonspecific symptoms, most commonly including fever and a relative slowing of the heart rate (bradycardia) (Nasrallah and Nassar 1978). Splenomegaly, hepatomegaly, or rose spots on the skin are encountered less frequently (Nasrallah and Nassar 1978). Less than half of typhoid patients present with neurological symptoms, cough, abdominal pain, or headache (Secmeer et al. 1995; Chow et al. 1989). Typhoid fever is not considered a diarrheal disease as this symptom develops in only a fraction (approximately one third) of typhoid fever patients, while the remaining individuals remain either diarrhea-free or become constipated (Chow et al. 1989; Yap and Puthucheary 1998). Paratyphoid fever is indistinguishable in its symptoms from typhoid fever but milder in its course.

Histopathology

The pathology of typhoid fever is characterized by the predominance of mononuclear phagocytes (macrophages and/or dendritic cells) and a paucity of neutrophils (Mallory 1898; Hornick et al. 1970). An exception is the accumulation of neutrophils that is occasionally seen in typhoidal meningitis, typhoidal osteomyelitis, or typhoidal pneumonia. However, neutrophils are typically scarce in typhoid fever lesions and the pathology consists of clusters of mononuclear phagocytes and lymphocytes, termed typhoid nodules, which are observed with variable frequency and size depending upon the severity of the condition (Nasrallah and Nassar 1978; Shin et al. 1994). These histopathological changes can be seen as early as 3 days after oral infection of volunteers in biopsies collected from the small intestinal mucosa, which exhibit granulomatous inflammatory lesions formed largely by mononuclear phagocytes (Sprinz et al. 1966). The characteristic swelling

of the Peyer's patches, the mesenteric lymph nodes, and the spleen during typhoid fever is due largely to the formation of mononuclear infiltrates (Mallory 1898). Mononuclear phagocytes can also be present in the urine (Pepper 1920) or in stool samples (Harris et al. 1972; Alvarado 1983).

Mononuclear phagocytes containing erythrocytes are a common finding in typhoid lesions (Shin et al. 1994; Ponfick 1872). These so-called typhoid cells represent hemophagocytic macrophages that engulf neutrophils, red blood cells, and platelets (Mallouh and Sa'di 1987). A general increase in the phagocytic activity of macrophages in the reticuloendothelial system is suggested by an increased clearance of tagged albumen particles from the blood of typhoid fever patients (Greisman et al. 1964). Hemophagocytosis is thought to be a mechanism contributing to complete blood count changes that can accompany typhoid fever, including neutropenia, anemia, and thrombocytopenia (Serefhanoglu et al. 2003; Khosla et al. 1995; Abdool Gaffar et al. 1992; Butler et al. 1978; Houston 1901).

Complications

A relapse occurs in approximately 8–10% of patients receiving no antibiotic therapy and in 15–20% of patients treated with chloramphenicol (Hornick et al. 1970). Relapses in typhoid fever represent a complete renewal of the primary disease, producing the same symptoms but commonly being of shorter duration (Horton-Smith 1900).

Intestinal perforation develops in approximately 5% of typhoid fever patients (Butler et al. 1985). Typhoid intestinal perforation is a significant cause of mortality from typhoid fever affecting both children and adults (Uba et al. 2007; Bitar and Tarpley 1985). The mononuclear cell infiltrate in ileal lymphoid tissue can be associated with fibrin deposition in capillaries, resulting in capillary thrombosis and ulceration at areas of Peyer's patches, usually observed in the second week of fever. Continuous ulceration can produce hemorrhage and intestinal perforation associated with peritonitis, which usually occurs in the third week of fever (Bitar and Tarpley 1985). Infiltrates observed in histological studies from perforated small intestines remain mononuclear while neutrophils are scarce (Mukawi 1978; Kraus et al. 1999).

Several complications of the hepatobiliary system have been described. Typhoid nodules may coalesce to form a liver abscess or hepatic necrosis or produce typhoid hepatitis with jaundice (Durrani 1995; Nasrallah and Nassar 1978). Jaundice can also be associated with cholecystitis, which develops in some individuals (Morison 1913). Spontaneous splenic rupture has also been reported (Huang and DuPont 2005). Finally, chronic gallbladder carriage is a risk factor for developing gallbladder cancer (Caygill et al. 1995; Caygill et al. 1994; Welton et al. 1979).

Approximately 1–6% of typhoid fever patients develop pneumonia, which is commonly associated with underlying abnormalities, such as immunosuppression (Dutta et al. 2001). Other atypical manifestations include osteomyelitis, arthritis, meningitis, and cardiac abnormalities (Huang and DuPont 2005).

Differences Between Typhoid Fever and Gastroenteritis

Typhoidal *Salmonella* serovars are closely related genetically to nontyphoidal *Salmonella* serovars (Selander et al. 1990) but cause strikingly different diseases in humans (Tsolis et al. 2008). Nontyphoidal *Salmonella* serovars are commonly associated with gastroenteritis in immunocompetent individuals (NTS gastroenteritis), with *S. enterica* serovars Typhimurium and Enteritidis being isolated most frequently (Rabsch et al. 2001). NTS gastroenteritis is a typical diarrheal disease that manifests as a localized infection of the terminal ileum, mesenteric lymph nodes, and less frequently the colon. Common symptoms include, in order of frequency, diarrhea, abdominal pain, fever, headache, muscle pains, chills, and vomiting. NTS gastroenteritis has a short incubation period, which is less than 1 day on an average (Glynn and Palmer 1992). The histopathological hallmark of NTS gastroenteritis is acute intestinal inflammation with infiltrates that are dominated by neutrophils (McGovern and Slavutin 1979; Murphy and Gorbach 1982). Similarly, fecal leukocytes from NTS gastroenteritis patients are dominated by neutrophils (Harris et al. 1972; Alvarado 1983). The disease is self-limited and symptoms subside within less than 10 days after onset (Glynn and Palmer 1992). Thus, the characteristics of NTS gastroenteritis differ markedly from the clinical features of typhoid fever (*Table 17.2*).

Infections with nontyphoidal *Salmonella* serovars can be complicated by bacteremia in immunocompromised individuals (NTS bacteremia) (Gordon 2008; Reddy et al. 2010), a condition accompanied by neutrophilia (Saballs et al. 1993; Fleisher 1991), an antibacterial response triggered by an interleukin (IL)-1β-mediated release of neutrophils from bone marrow stores (Tewari et al. 1990). In contrast, neutrophilia does not develop during *S.* Typhi bacteremia. Instead, 25% of individuals with typhoid fever develop neutropenia (Khosla et al. 1995; Abdool Gaffar et al. 1992). Leukocytes in the blood respond to NTS bacteremia by mounting a respiratory burst, and the resulting production of reactive oxygen intermediates can be visualized by an increased ability of blood samples to reduce nitroblue tetrazolium (NBT) (Miller et al. 1976). In contrast, typhoid fever is associated with a negative NBT blood test, indicating a markedly reduced oxidative response in the blood of patients infected with *S.* Typhi (Miller et al. 1976). Collectively, these observations suggest that nontyphoidal *Salmonella* serovars trigger some innate antibacterial responses in the human host, which are not observed in individuals with typhoid fever.

Phylogeny and Evolution

Evolutionary History of the Genus *Salmonella*

The genus *Salmonella* is a clonal lineage, which is closely related to *E. coli*, *Shigella* species, and *Citrobacter* species (*Fig. 17.2*) (Petty et al. 2010). It has been estimated that the point of divergence between the genus *Salmonella* and the genus

Table 17.2
Differences between typhoid fever and NTS gastroenteritis

	Typhoid fever	NTS gastroenteritis
Etiology	*S.* Typhi	Nontyphoidal *Salmonella* serovars (e.g., *S.* Typhimurium, *S.* Enteritidis)
Distribution of bacteria in immunocompetent host	Systemic infection	Infection remains localized to intestine and mesenteric lymph nodes
Incubation period	14 days	<1 day
Common symptoms	Fever, relative bradycardia	Diarrhea, abdominal pain, fever, headache, muscle pains
Duration of symptoms	3 weeks	<10 days
Predominant cell type in intestinal infiltrates	Mononuclear cells and lymphocytes	Neutrophils
Fecal leukocytes	Mononuclear cells	Neutrophils

Escherichia was approximately 100 Ma ago (Ochman and Wilson 1987). *S. bongori* and the different *S. enterica* subspecies form a hierarchical population structure (*Fig. 17.3*) (McQuiston et al. 2008). A low rate of recombination between these lineages is inferred from sequence data of housekeeping genes, which differ between 2.8% and 4.4% of nucleotides between species and subspecies (Nelson et al. 1991; Nelson and Selander 1992; Boyd et al. 1994). It has been estimated that *S. enterica* diverged from *S. bongori* during the Eocene period between 40 and 63 Ma ago, while *S. enterica* subspecies *enterica* emerged as a separate lineage 4 to 6 Ma ago (McQuiston et al. 2008). While other lineages within the genus *Salmonella* are largely reptile-associated, *S. enterica* subspecies *enterica* is commonly isolated from mammals and birds, contains the largest number of serovars (*Fig. 17.3*), and is responsible for more than 99% of human clinical isolates (Aleksic et al. 1996; Bäumler 1997). Recombination within *S. enterica* subspecies *enterica* is not uncommon (Lan et al. 2009; Didelot et al. 2007; Li et al. 1994; Smith et al. 1990).

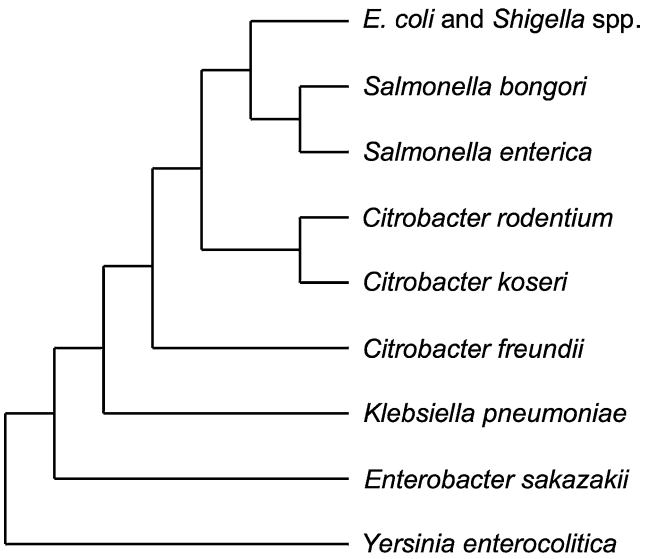

Fig. 17.2
***Phylogenetic connection of S. enterica and S. bongori to related species within the Enterobacteriaceae*. The tree is not drawn to scale. Phylogenetic relationships are based on genome analysis (Petty et al. 2010)**

Evolutionary History of Typhoidal *Salmonella* Serovars

The vast majority of *S. enterica* subspecies *enterica* serovars are associated with gastroenteritis in humans (Aleksic et al. 1996). Within this group, typhoidal *Salmonella* serovars developed in four phylogenetically unrelated clonal lineages, presumably from ancestral organisms that were originally associated with gastroenteritis (Selander et al. 1990). *S.* Typhi isolates, *S.* Paratyphi C isolates, and *S.* Paratyphi B isolates each form one of these distinct lineages. The fourth lineage is formed by isolates of *S.* Paratyphi A and *S.* Sendai. These observations suggest that *S.* Sendai evolved recently from *S.* Paratyphi A by acquiring an *rfb* biosynthesis gene cluster through horizontal gene transfer that encodes an O antigen of serogroup D1 (*Table 17.1*) (Selander et al. 1990).

S. Typhi represents a clonal lineage within *S. enterica* subspecies *enterica* that likely emerged after human migrations out of Africa but before the agricultural revolution of the Neolithic age (Roumagnac et al. 2006; Kidgell et al. 2002). This scenario is supported by sequence analysis of seven housekeeping genes from different *S.* Typhi isolates, which indicates that the lineage is between 15,000 and 150,000 years old (Kidgell et al. 2002). A more recent single-nucleotide polymorphism (SNP) analysis of 200 *S.* Typhi genes suggests a similar time of origin for the most recent common ancestor of the lineage, which lived an estimated 10,000–71,000 years ago (Roumagnac et al. 2006).

Although typhoidal *Salmonella* serovars form four phylogenetically distant lineages, evolution of the typhoidal lifestyle may have not occurred four times independently. For instance, the

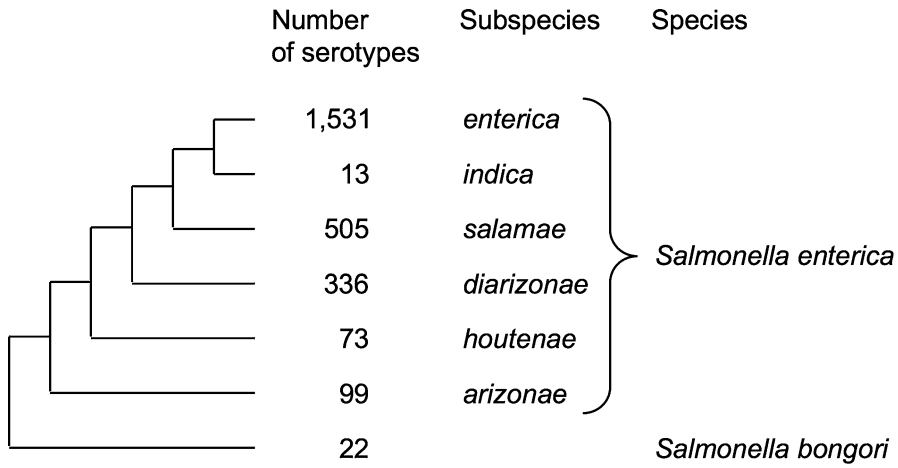

Fig. 17.3

***The genus Salmonella*. The phylogenetic tree shown on the left is not drawn to scale and is based on sequence analysis of housekeeping genes (McQuiston et al. 2008). The number of serovars present in each species and subspecies has been reported recently (Grimont and Weill 2007). The classification into species and subspecies has been reviewed previously (Brenner et al. 2000)**

viaB region encoding production of the Vi antigen is present in *S.* Typhi and *S.* Paratyphi C, suggesting that convergence by horizontal transfer of this DNA region occurred in their evolutionary history (Selander et al. 1990). Furthermore, horizontal gene transfer between *S.* Typhi and *S.* Paratyphi A/*S.* Sendai lineage has occurred on a scale so massive (Didelot et al. 2007) that it blurs the evolutionary relationship of their lineages (Chan et al. 2003). This large-scale recombination between the lineages of *S.* Typhi and *S.* Paratyphi A/*S.* Sendai occurred before the common ancestors of their contemporary clinical isolates emerged. During this phase, both lineages exchanged an astonishing 23% of their genome by horizontal gene transfer, presumably during coexistence in a shared human reservoir (Didelot et al. 2007).

It has been speculated that extensive horizontal gene transfer between *S.* Typhi and *S.* Paratyphi A might have conferred new genetic traits required for causing disseminated infections (Holt et al. 2008), and this event could thus mark the origin of typhoid and paratyphoid fever. Large-scale recombination with *S.* Paratyphi A occurred after the *S.* Typhi lineage had already passed through much (approximately 75%) of its evolutionary history (Holt et al. 2008). The clock rate and age of the *S.* Typhi lineage estimated by Achtman and coworkers (Roumagnac et al. 2006) would suggest that this large-scale exchange took place between 1,500 and 10,700 years ago. Typhoid fever might thus have its origin somewhere between antiquity and the agricultural revolution of the Neolithic age.

Subsequent to the large-scale recombination between *S.* Typhi and *S.* Paratyphi A, both lineages became isolated again, which was accompanied by the formation of pseudogenes at an accelerated rate, a process that is still ongoing (Holt et al. 2008). Many pseudogenes of typhoidal *Salmonella* serovars encode functions required for the gastrointestinal lifestyle of nontyphoidal *Salmonella* serovars, suggesting that the phase of isolation and accelerated pseudogene formation might represent the transition to chronic gallbladder carriage, a route of transmission that became accessible to *S.* Typhi after it acquired the ability to cause disseminated infections. The transition to gallbladder carriage would have made functions obsolete that are required for transmission during gastroenteritis. Inactivation of such functions through pseudogene formation might have been accelerated because the lineage passed through bottlenecks as it became host-adapted (Holt et al. 2008). Finally, the lineage diversified to give rise to all contemporary *S.* Typhi isolates (Roumagnac et al. 2006).

Analysis of the contemporary population structure using SNP analysis suggests that extant *S.* Typhi lineages arose by clonal diversification from a monophasic, antibiotic-sensitive ancestral organism (Lan et al. 2009). A relatively recently emerged clonal lineage (Lan et al. 2009) acquired a linear plasmid (Baker et al. 2007) carrying a *fljBA*-like operon that encodes the H:z_{66} antigen (Roumagnac et al. 2006; Octavia and Lan 2007). Isolates from this clonal lineage are biphasic and currently circulate in Indonesia (Huang et al. 2004). Analysis of the variation within the contemporary *S.* Typhi population does not reveal evidence for adaptive selection (Holt et al. 2008), with the exception of mutations in *gyrA*, which confer resistance to ciprofloxacin (Wain et al. 1997). Separate clonal lineages emerged in response to antibiotic selection either by acquisition of plasmid-encoded antibiotic resistance determinants or by gaining ciprofloxacin resistance through mutations in the chromosomal *gyrA* gene (Holt et al. 2010; Kariuki et al. 2010; Octavia and Lan 2009; Lan et al. 2009; Baker et al. 2008; Octavia and Lan 2007; Wain et al. 1997; Roumagnac et al. 2006). The absence of adaptive selection, other than that imposed by antibiotic resistance, might be explained by small population size and genetic drift, a scenario consistent with the proposed key role of chronic *S.* Typhi carriers as the main reservoir of this pathogen during evolution (Holt et al. 2008). Furthermore, an absence of adaptive selection suggests that *S.* Typhi is not under strong selective pressure from the host immune system in the niche the pathogen occupies during chronic carriage (Holt et al. 2008).

Pathogenesis and Genetics

Animal Models for Typhoid Fever

Oral inoculation of chimpanzees with *S.* Typhi produces an illness that closely mimics typhoid fever in its clinical course and its pathological alterations (Engelhardt 1972; Gaines et al. 1968; Edsall et al. 1960; Grunbaum 1904). However, lower primates, such as rhesus macaques, are resistant to experimental *S.* Typhi infection (Gaines et al. 1968; Grunbaum 1904). Similarly, nonprimate vertebrate animals generally do not show signs of infection upon ingesting *S* Typhi. Historically, virulence of *S.* Typhi has been assessed by intraperitoneal or intravenous inoculation of mice, sometimes after administration of iron (Findlay 1951; Carter and Collins 1974; Powell et al. 1980; O'Brien 1982; Furman et al. 1994), but these models have fallen out of favor because they only poorly reproduce the course of infection and pathology characteristic of typhoid fever. More recently, *S.* Typhi has been shown to grow in the liver and spleen of humanized mice, which represents a promising new animal model for studying aspects of typhoid fever pathogenesis (Libby et al. 2010; Song et al. 2010; Firoz Mian et al. 2011).

Analysis of its phylogenetic history suggests that *S.* Typhi evolved from an ancestral nontyphoidal organism associated with gastroenteritis (Selander et al. 1990). Some virulence mechanisms inherited from this ancestral organism are likely shared with nontyphoidal *Salmonella* serovars, such as *S.* Typhimurium. This assumption provides the principle rationale for using *S.* Typhimurium infection in the mouse as an animal model for studying *S.* Typhi infection in humans. Mice ingesting *S.* Typhimurium develop a systemic illness characterized by bacterial multiplication in the liver, spleen, mesenteric lymph nodes, and Peyer's patches (Loeffler 1892; Müller 1912; Ørskov and Moltke 1929). This animal model has been used extensively to study the pathogenesis of typhoid fever (mouse typhoid model) (reviewed in (Tsolis et al. 1999b; Santos et al. 2001)). Work using the mouse typhoid model provides important insights into the basic pathogenic strategies deployed by nontyphoidal *Salmonella* serovars and into the functions of major virulence factors shared by typhoidal and nontyphoidal *Salmonella* serovars.

Ancestral Virulence Mechanisms Shared by Typhoidal and Nontyphoidal *Salmonella* Serovars

One virulence strategy conserved among typhoidal and nontyphoidal *Salmonella* serovars is the ability to invade the intestinal epithelium. Flagella contribute to epithelial invasion by increasing bacterial contact with host cells. Flagella-mediated motility contributes markedly to the efficiency of epithelial invasion observed in vitro for *S.* Typhimurium (Jones et al. 1992; Khoramian-Falsafi et al. 1990) as well as *S.* Typhi (Winter et al. 2009b; Liu et al. 1988). A second virulence factor required for invasion is the invasion-associated type III secretion system (T3SS-1) of *S.* Typhi (Elsinghorst et al. 1989) and *S.* Typhimurium (Galán & Curtiss 1989). The T3SS-1 apparatus is encoded by *Salmonella* pathogenicity island 1 (SPI1) (Mills et al. 1995), a 40-kb DNA region conserved among isolates of *S. bongori* and *S. enterica* but absent from closely related bacterial species such as *E. coli* (Li et al. 1995). The T3SS-1 functions as a molecular syringe that injects proteins, termed effectors, into the host cell cytosol (Fu and Galan 1998). The T3SS-1 effector proteins SipA, SipC (SspC), SopA, SopB (SigD), SopD, SopE, and SopE2 cooperate in inducing actin rearrangements in the host cell cytosol, thereby promoting bacterial entry into the epithelium (Hong and Miller 1998; Hardt et al. 1998; Hayward and Koronakis 1999; Zhou et al. 1999; Friebel et al. 2001; Jepson et al. 2001; McGhie et al. 2001; Raffatellu et al. 2005b).

A second virulence strategy shared by typhoidal and nontyphoidal *Salmonella* serovars is the ability to survive in macrophages. Macrophage survival of *S.* Typhimurium (Ochman et al. 1996) and *S.* Typhi (Forest et al. 2010) requires the action of a second type III secretion system (T3SS-2) encoded by SPI2. SPI2 is a DNA region that is absent from *S. bongori* but highly conserved within *S. enterica* (Ochman and Groisman 1996; Hensel et al. 1997). Acting as a molecular syringe, the T3SS-2 injects the effector proteins SpiC, SseF, SseG, SlrP, SspH1, SspH2, SifA, SifB, SseI, SseJ, PipB, PipB2, SseK1, SseK2, GogB, and SopD2 into the macrophage cytosol (reviewed in (Abrahams and Hensel 2006)). One function of the T3SS-2 is to manipulate vesicular trafficking events in macrophages (Vazquez-Torres et al. 2000; Uchiya et al. 1999; Beuzon et al. 2000), but it remains unclear in most cases how individual effector proteins contribute to T3SS-2-mediated macrophage survival. Inactivation of T3SS-2 in *S.* Typhimurium attenuates virulence in the mouse typhoid model 10,000-fold (Hensel et al. 1995), suggesting that this virulence factor is important in the setting of a disseminated infection.

Induction of Innate Immunity During Gastroenteritis

To cause gastroenteritis in a calf model, *S.* Typhimurium uses flagella and T3SS-1 to invade the intestinal mucosa and T3SS-2 to survive in tissue (Tsolis et al. 1999a, c; Schmitt et al. 2001; Zhang et al. 2002; Winter et al. 2009a). The presence of *S.* Typhimurium in tissue is sensed by the innate immune system through a multitude of pathways, including the detection of cytosolic access by T3SS-1 through NLRC4 (NOD-like receptor [NLR] family caspase-associated recruitment domain [CARD]-containing protein 4) (Miao et al. 2006, 2010b; Franchi et al. 2006), the activation of NLRP3 (NLR family pyrin domain-containing protein 3) by an unknown mechanism (Broz et al. 2010), the detection of cell wall fragments by nucleotide-binding oligomerization domain (NOD)1 and NOD2 (Geddes et al. 2010, 2011; Le Bourhis et al. 2009), the detection of flagellin by TLR5 (Gewirtz et al. 2001), the detection of curli amyloid fibrils through TLR1/TLR2 (Tukel et al. 2005, 2009, 2010), and the detection of the lipid A moiety of LPS by TLR4 (Vazquez-Torres et al. 2004). Finally, the O antigen moiety of LPS is detected through complement, which results in the deposition of C3b,

a cleavage product of complement component 3 (C3), on the bacterial surface (Joiner et al. 1989) through covalent attachment of C3b by an ester bond formed with free hydroxyl groups in LPS sugar moieties (Sahu et al. 1994) (Fig. 17.1c).

Detection of *S.* Typhimurium through these pathways triggers a cytokine storm in the intestinal mucosa that helps to orchestrate antibacterial responses, including macrophage activation, neutrophil recruitment, and the epithelial release of antimicrobials (reviewed in (Winter et al. 2010a; Santos et al. 2009)). Orchestration of these innate immune responses occurs rapidly, which explains the short incubation period of gastroenteritis (Table 17.2). Infected macrophages sense T3SS-mediated cytosolic access and respond by releasing *S.* Typhimurium through pyroptosis, which renders the pathogen vulnerable to neutrophil attack (Miao et al. 2010a). Neutrophils are important for preventing spread of the pathogen beyond the mesenteric lymph node in humans (Noriega et al. 1994; Tumbarello et al. 1995) because C3-deposition on *S.* Typhimurium facilitates complement receptor 3 (CR3)-mediated phagocytosis (Joiner et al. 1989), an uptake mechanism that is linked to a respiratory burst in neutrophils (Joiner et al. 1989). Eventually, the pathogen is cleared from intestinal tissues through these mechanisms without transmitting to a susceptible host, which suggests that localization in tissue represents a dead end for *S.* Typhimurium.

However, growth of *S.* Typhimurium in the intestinal lumen is enhanced during intestinal inflammation (Stecher et al. 2007; Barman et al. 2008). Neutrophils migrate into the intestinal lumen, which gives rise to fecal leukocyte populations that are dominated by neutrophils, one of the characteristic clinical features of gastroenteritis (Table 17.2) (Harris et al. 1972; Alvarado 1983). Neutrophil transepithelial migration promotes luminal growth of *S.* Typhimurium (Sekirov et al. 2010) because a by-product of the phagocyte respiratory burst is the generation in the intestine of a new respiratory electron acceptor, tetrathionate ($S_4O_6^{2-}$) (Winter et al. 2010b). Tetrathionate is a chemical long used for the enrichment of *Salmonella* serovars in samples containing competing microbes (Muller 1923). Growth by tetrathionate respiration in the inflamed intestine enables *S.* Typhimurium to outgrow competing microbes (Winter et al. 2010b) by using nutrients, such as ethanolamine, that do not support growth by fermentation (Thiennimitr et al. 2011). The resulting outgrowth of *S.* Typhimurium in the intestinal lumen promotes transmission of the pathogen to the next susceptible host through the fecal oral route (Lawley et al. 2008).

In conclusion, the principal virulence strategy of nontyphoidal *Salmonella* serovars is to use their virulence factors, flagella, T3SS-1, and T3SS-2, to attract neutrophils into the intestinal lumen, thereby gaining a luminal growth advantage that ensures their transmission (reviewed in (Santos et al. 2009)).

Evasion of Innate Immunity During Typhoid Fever

While nontyphoidal and typhoidal *Salmonella* serovars share flagella, T3SS-1, and T3SS-2, the former group is associated with gastroenteritis in humans while the latter group causes typhoid and paratyphoid fever (Table 17.2). This clinical correlation suggests that some of the genes that enable *S.* Typhi to cause typhoid fever in humans are not present in *S.* Typhimurium. It follows that the virulence mechanisms responsible for the differences between gastroenteritis and typhoid fever (Table 17.2) cannot be interrogated by studying *S.* Typhimurium infection in mice (Tsolis et al. 2011). Since the vast majority of investigators studying typhoid fever pathogenesis rely on *S.* Typhimurium infection in the mouse as a model, the differences between gastroenteritis and typhoid fever remain less studied than the virulence mechanisms that are shared by nontyphoidal and typhoidal *Salmonella* serovars.

One striking difference between gastroenteritis and typhoid fever is that the latter has a very long incubation period (Table 17.2), which illustrates that flagella/T3SS-1-mediated epithelial invasion and T3SS-2-mediated survival in macrophages are not accompanied by host responses severe enough to elicit symptoms within the first 2 weeks of an *S.* Typhi infection. Since the acute onset of intestinal inflammation that produces symptoms of gastroenteritis within less than 1 day is triggered by innate immune recognition of *S.* Typhimurium, the long incubation period of typhoid fever suggests that *S.* Typhi evades or prevents detection by the innate immune surveillance system. A DNA region involved in this process is the *viaB* locus of *S.* Typhi (Hirose et al. 1997; Sharma and Qadri 2004; Raffatellu et al. 2005a, 2007).

In addition to genes for the biosynthesis (*tviBCDE*) and the export (*vexABCDE*) of the Vi antigen, the *viaB* locus contains *tviA*, which encodes a positive regulator of capsule expression (Virlogeux et al. 1995). Expression of *tviA* is induced at tissue osmolarity by the two-component system EnvZ/OmpR, while high osmolarity encountered in the intestinal lumen prevents *tviA* expression (Fig. 17.4) (Winter et al. 2009b, 2010c). TviA changes gene expression by forming heterodimers with the response regulator RcsB (Virlogeux et al. 1996), a repressor of the *flhDC* genes (Cano et al. 2002). In turn, FlhDC acts as a positive regulator for expression of flagella and T3SS-1 invasion genes (Frye et al. 2006). As a result, *S.* Typhi remains invasive, motile, and noncapsulated while it resides in the high-osmolarity environment of the intestinal lumen. However, expression of TviA is induced during the transit though the intestinal epithelium, resulting in a rapid repression of flagellin and T3SS-1 expression (Winter et al. 2010c), while expression of the Vi antigen, a capsular polysaccharide, is induced (Tran et al. 2010).

TviA regulation has two important consequences. First, some of the signals that enable the innate immune system to detect *S.* Typhimurium in tissue, such as cytosolic access by the T3SS-1 sensed through NLRC4 (Miao et al. 2006, 2010b; Franchi et al. 2006) and the presence of flagellin sensed through TLR5 (Gewirtz et al. 2001), are rapidly turned off by TviA when *S.* Typhi enters the intestinal mucosa (Fig. 17.4). Second, the Vi antigen is expressed when *S.* Typhi transits through the intestinal epithelium (Tran et al. 2010), which ensures that the

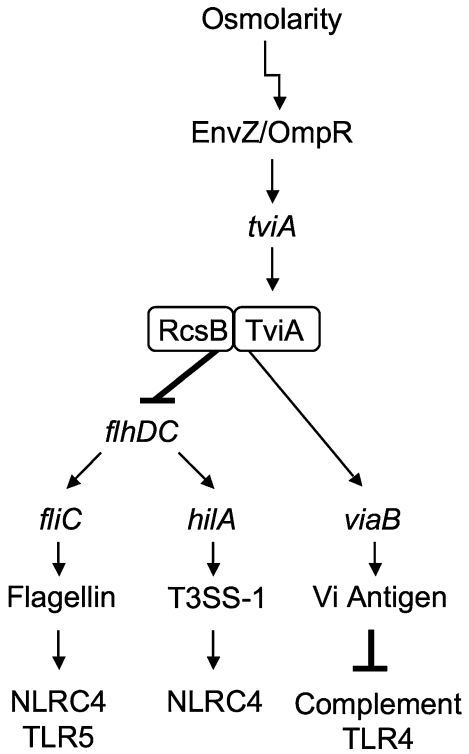

Fig. 17.4

***TviA-mediated evasion of innate immunity*. The EnvZ/OmpR two-component system activates *tviA* expression in response to changes in osmolarity that *S.* Typhi encounters when it transits from the intestinal lumen into tissue. TviA forms heterodimers with RcsB, which in turn represses *flhDC* expression. Through this mechanism, TviA prevents innate immune recognition of flagella and T3SS-1 through TLR5 and NLRC4. Furthermore, TviA/RcsB activates genes in the *viaB* locus which are involved in the biosynthesis of the Vi antigen, a surface structure preventing complement activation and recognition through TLR4**

pathogen is encapsulated by the time it encounters complement. Expression of the Vi antigen inhibits complement deposition (Wilson et al. 2011; Looney and Steigbigel 1986) because this capsular polysaccharide does not contain free hydroxyl groups available for ester formation with C3b (*Fig. 17.1b*). The complement fragments C3a and C5a generated during complement deposition are also known as the anaphylatoxins due to their potency in inducing inflammatory responses (reviewed in (Haas and van Strijp 2007)). C5a synergizes with TLR4 to generate responses against LPS (Li et al. 2005; Riedemann et al. 2003), which might explain why expression of the Vi antigen enables *S.* Typhi to evade detection through TLR4 (Hirose et al. 1997; Wilson et al. 2008; Jansen et al. 2011). Thus, TviA regulation enables *S.* Typhi to evade detection through multiple innate immune sensors by inducing Vi antigen expression and repressing motility and T3SS-1 (*Fig. 17.4*). These innate immune evasion mechanisms contribute to the scarcity of neutrophils in lesions of typhoid fever (Jansen et al. 2011; Haneda et al. 2009; Raffatellu et al. 2007) and might help explain the long incubation period of typhoid fever (*Table 17.2*).

Inhibition of complement deposition by the Vi antigen also has consequences for the interaction of *S.* Typhi with neutrophils. CR3-mediated phagocytosis by neutrophils is coupled to a respiratory burst (Joiner et al. 1989). Inhibition of complement deposition by the Vi antigen prevents CR3-mediated phagocytosis (Looney and Steigbigel 1986; Wilson et al. 2011) and the generation of a respiratory burst during the interaction of *S.* Typhi with neutrophils (Miller et al. 1972; Kossack et al. 1981). The inhibition of a respiratory burst in neutrophils helps explain why *S.* Typhi bacteremia is associated with a negative NBT blood test (Miller et al. 1976). Collectively, these properties might aid *S.* Typhi in overcoming a neutrophil barrier and spread beyond the mesenteric lymph node to cause a disseminated infection in humans (*Table 17.2*).

Importantly, the ability to disseminate throughout the host and take residence in internal organs is a prerequisite for transmission through gallbladder carriage. Similar to *Mycobacterium tuberculosis*, *S.* Typhi is able to persist in tissue in a state of dormancy, a property that is poorly studied but likely important for developing chronic carriage. Persistence in tissue may occur in hemophagocytic macrophages, or typhoid cells (Silva-Herzog and Detweiler 2010; Nix et al. 2007), that are commonly observed in typhoid lesions (Shin et al. 1994; Ponfick 1872). Finally, persistence in the gallbladder can be accompanied by the formation of *S.* Typhi biofilms on the surface of gallstones (Crawford et al. 2010). Formation of these biofilms requires an O antigen capsule that is distinct from the Vi antigen (Crawford et al. 2008).

In conclusion, the principal virulence strategy of *S.* Typhi is based on virulence mechanisms shared with nontyphoidal *Salmonella* serovars. These include flagella/T3SS-1-mediated epithelial invasion and T3SS-2-mediated macrophage survival. However, while nontyphoidal Salmonella serovars stimulate innate immunity to promote their growth in the intestinal lumen, S. Typhi evades innate immunity to disseminate systemically and ensure its transmission through chronic gallbladder carriage (reviewed in (Raffatellu et al. 2006; Tsolis et al. 2008)).

Genomic Rearrangements

The gene order on the chromosomes of *E. coli* and *S.* Typhimurium is highly conserved (Riley and Anilionis 1978) and very few inversions are observed between their sequenced genomes (Rocha 2004), although both lineages diverged approximately 100 Ma ago (Ochman and Wilson 1987). This high degree of conservation suggests that the gene order may be under selection. One effect commonly associated with genome rearrangements is a lower growth rate because inversions may alter gene dosage, gene strand bias, or chromosome symmetry (Rebollo et al. 1988; Hill and Gray 1988; Campo et al. 2004). It is therefore thought that any advantage gained by multiplying at a maximum growth rate places the gene

order on the chromosome under selection. In the case of *S.* Typhimurium, selective pressure arises from the need to transmit from an infected host to a new susceptible host (Kingsley and Bäumler 2000). Transmission requires outgrowth of competing microbes in the gut (Lawley et al. 2008), which likely necessitates multiplication at a maximum growth rate. Thus, the maximum growth rate needed for transmission of *Salmonella* serovars associated with gastroenteritis likely imposes selective constraints that prevent chromosomal rearrangements.

In contrast to nontyphoidal *Salmonella* serovars, transmission of *S.* Typhi no longer depends on being able to multiply at a maximum growth rate to successfully compete with other microbes residing in the intestine. Instead, transmission is likely aided by a state of dormancy or low growth rate in tissue of chronic carriers. This would suggest that selective constraints that prevent chromosomal rearrangements no longer apply to *S.* Typhi. Consistent with this idea, major genomic rearrangements due to homologous recombination between the *rrn* operons, leading to inversions and translocations, are commonly detected in genomes of *S.* Typhi isolates (Kothapalli et al. 2005; Liu and Sanderson 1995, 1996). Similar genome rearrangements are also common in *S.* Paratyphi C (Liu and Sanderson 1998). Recombination between *rrn* operons occurs with equal frequency in *S.* Typhi and *S.* Typhimurium in the laboratory, but *S.* Typhimurium strains carrying rearrangements have never been isolated from nature (Kothapalli et al. 2005). In contrast, analysis of *S.* Typhi isolated from human carriers over multiple years shows that chromosome rearrangements occur within the host over time (Matthews et al. 2011). The finding that gene order on the *S.* Typhi chromosome is not under strong selective pressure in the niche the pathogen occupies during chronic carriage supports the concept that multiplication at a maximum growth rate is not needed for transmission of typhoid fever.

Pseudogene Formation in Typhoidal *Salmonella* Serovars

Typhoidal serovars contain substantially more pseudogenes (genes with inactivating mutations) than their nontyphoidal relatives (*Fig. 17.5*). Indeed, this accumulation of pseudogenes in typhoidal serovars is hypothesized to be both a cause and a consequence of their human host restriction, extraintestinal tissue residence and shift in disease manifestation. This process is not unique to human typhoidal isolates, however. Genome sequencing reveals substantial pseudogene accumulation has occurred independently in a variety of systemic, host-restricted pathogens (Chiu et al. 2004; Thomson et al. 2008; McClelland et al. 2004; Liu et al. 2009; Parkhill et al. 2001; Deng et al. 2003; Jarvik et al. 2010; Holt et al. 2009). Direct gene-for-gene comparisons between *S.* Typhi and *S.* Paratyphi A have revealed the two serovars share 25 independently evolved and 44 inherited or recombined pseudogenes (Holt et al. 2009). While understanding the initial changes that lead a *Salmonella* strain down the path of typhoidal evolution can provide insight into host specificity and disease, every alteration in this process can be informative for the pathogenesis of both typhoidal and nontyphoidal serovars: While genes that have been inactivated in typhoidal serovars can be seen as dispensable for an extraintestinal lifestyle, these genes likely persist in nontyphoidal serovars because they aid in intestinal growth and transmission during gastroenteritis.

For example, *S.* Typhi strain CT18 contains pseudogenes in 7 of its 11 fimbrial operons of the chaperone usher assembly class (Townsend et al. 2001), which encode intestinal colonization factors (Weening et al. 2005). The *shdA* and *misL* genes encode nonfimbrial adhesins that enhance intestinal colonization of *S.* Typhimurium (Dorsey et al. 2005; Kingsley et al. 2000, 2002). Pseudogene formation in typhoidal serotypes led to inactivation of both *shdA* (*S.* Typhi and *S.* Paratyphi A) and *misL* (*S.* Typhi) (Parkhill et al. 2001; McClelland et al. 2004). Another pseudogene in *S.* Typhi strain CT18 is *ttrS* (Parkhill et al. 2001), which is required by *S.* Typhimurium to outgrow competing microbes in the inflamed intestine by tetrathionate respiration (Winter et al. 2010b). Tetrathionate respiration supports growth of *S.* Typhimurium on ethanolamine as a carbon source in the lumen of the inflamed gut (Thiennimitr et al. 2011). Growth on ethanolamine under anaerobic conditions necessitates biosynthesis of vitamin B12 by the *cob/cbi* gene cluster (Roof and Roth 1988), which carries pseudogenes in *S.* Typhi (*cbiM*, *cbiK*, *cbiJ*, and *cbiC*) (Parkhill et al. 2001) and *S.* Paratyphi A (*cbiA*) (McClelland et al. 2004). Additional pathways with shared pseudogenes in *S.* Typhi and *S.* Paratyphi A include iron uptake and chemotaxis (Holt et al. 2009). Interestingly, iron acquisition and chemotaxis are both required by *S.* Typhimurium to edge out competing microbes in the gut lumen during gastroenteritis (Raffatellu et al. 2009; Stecher et al. 2008). In conclusion, many of the pseudogenes found in the genomes of typhoidal *Salmonella* serovars encode functions that aid intestinal colonization and competition with the microbiota during gastroenteritis, which in turn enhances transmission of nontyphoidal *Salmonella* serovars by the fecal oral route (Lawley et al. 2008). Thus, one driving force responsible for pseudogene formation in typhoidal *Salmonella* serovars might be that functions required for gaining an advantage during growth competition with the resident microbiota are no longer under selection, presumably because the main route of transmission changed to chronic gallbladder carriage during evolution of the typhoidal lifestyle.

One group of genes affected by pseudogene formation in *S.* Typhi and *S.* Paratyphi A are those encoding pathogenicity-associated T3SS effector proteins. While the stable of effectors in nontyphoidal serovars can be seen as relatively constant, with each serovar differing in status between four and five loci, the typhoidal effector repertoire appears to be more plastic, differing between nine and fifteen loci (*Fig. 17.5*). This observation suggests that some T3SS effectors play a greater role in the development of gastroenteritis than they do in enabling a typhoidal lifestyle.

	Typhoidal			Non-Typhoidal	
Serovar	Paratyphi A	Paratyphi C	Typhi	Enteritidis	Typhimurium
Strain(s)	AKU_12601	RKS4594	CT18	P125109	14028s
	ATCC 9150		Ty2		SL1344
Chromosome statistics					
Chromosome size (bp)	4581797 / 4585229	4833080	4809037 / 4791961	4685848	4857432 **
Predicted CDS	4285 / 4263	4578	4395 / 4339	4318	4450 **
Pseudogenes	204 / 173	149	204 / 206	113	39 **
SPI-1 T3SS Effectors					
AvrA	-	-	-	■	□ / ■
SipA (SspA)	■	■	■	■	■
SipB (SspB)	■	■	■	■	■
SipC (SspC)	■	■	■	■	■
SopA	□	□	□	■	■
SopB (SigD)	■	■	■	■	■
SopD	■	■	■	■	■
SopE	■	-	■	■	- / ■
SopE2	■	■	□	■	■
SptP	■	■	■	■	■
SPI-2 T3SS Effectors					
GogB	-	■	-	-	■
PipB	■	■	■	■	■
PipB2	■	■	■	■	■
SifA	■	■	■	■	■
SifB	□	■	■	■	■
SopD2	□	■	□	■	■
SpiC (SsaB)	■	■	■	■	■
SpvB	-	■	-	-	■ / -
SseF	■	■	■	■	■
SseG	■	■	■	■	■
SseI (SrfH)	-	□	-	■	■
SseJ	-	■	□	■	■
SseK1	-	■	-	■	■
SseK2	□	□	□	□	■
SseL	■	■	■	■	■
SspH2	-	□	■	■	■
SteA	■	■	■	■	■
SteB	-	■	-	■	■
SteC	□	□	■	■	■
Effectors of Both					
SlrP	□	□	□	□	■
SspH1	-	-	-	-	■ / -

Fig. 17.5

***Chromosome statistics and type III secretion system effector gene status*. Chromosomal gene and pseudogene content for typhoidal and nontyphoidal serovars is provided (** Typhimurium strain LT2). Genes encoding effector proteins secreted by T3SS-1, T3SS-2, and by both systems are represented in the table as follows: black box, intact; white box, pseudogene; and dash, absent. Human typhoidal isolates are listed in white cells while nontyphoidal isolates are listed in gray cells for comparison. Differing results from multiple isolates of a serovar are presented in respective order separated by a forward slash. For more information on these effector proteins, please consult the review by Haraga et al. (2008)**

Vaccination

Killed Parenteral Vaccines

The first killed parenteral typhoid vaccines were developed independently by Sir Almroth Edward Wright in England and Richard Pfeiffer in Germany (Wright 1896; Pfeiffer and Kolle 1896). Killed parenteral typhoid vaccines are effective against a low-dose challenge, which might result from a waterborne exposure, but offer little protection against high-dose challenge (Ashcroft et al. 1964; Hornick and Woodward 1967). Killed parenteral vaccines have a high reactogenicity, and their use has therefore been discontinued in most countries.

Live Attenuated Oral Vaccines

René Germanier and colleagues used the mouse typhoid model to develop the first live attenuated oral typhoid vaccine strain. *S.* Typhimurium mutants defective for the biosynthesis of uridine-5′-diphosphate galactose epimerase (GalE) are deficient in the biosynthesis of the outer core of LPS when grown in the absence of galactose and are avirulent and immunogenic for mice (Germanier 1970, 1972). Oral vaccination with a *S.* Typhimurium *galE* mutant confers better protection in the mouse typhoid model than parenteral vaccination with a killed *S.* Typhimurium vaccine (Germanier 1972). Using chemical mutagenesis, a *galE* mutant of *S.* Typhi strain Ty2 was derived (Ty21a) (Germanier and Fuer 1975) and shown to be protective as live attenuated oral vaccine in field trials (Wahdan et al. 1982; Black et al. 1983, 1990).

The *galE* mutation is likely not the sole reason for the attenuation of Ty21a because introduction of a defined mutation in *galE* does not render *S.* Typhi strain Ty2 avirulent for humans (Hone et al. 1988). Therefore, it remains unknown which chemically induced mutations contribute to the attenuation of the vaccine strain Ty21a. Subsequent studies have demonstrated that *S.* Typhi strains attenuated by defined mutations in *cya crp* (χ3927) (Tacket et al. 1992a), *cya crp cdt* (χ4073) (Tacket et al. 1992a), *aroC aroD* (CVD908) (Tacket et al. 1992b), *aroC aroD htrA* (CVD908-*htrA*) (Tacket et al. 1997, 2000), *phoPQ* (Ty800) (Hohmann et al. 1996), or *aroC ssaV* (ZH9) (Hindle et al. 2002) are immunogenic in humans. Nonetheless, Ty21a currently remains the only available live attenuated oral typhoid vaccine.

Vi Parenteral Vaccines

Antibodies against the Vi antigen are not elicited by immunization with the live attenuated oral typhoid vaccine because the vaccine strain Ty21a carries a mutation in the *viaB* locus (Germanier and Furer 1983). However, antibodies against the Vi antigen are present in the serum of typhoid fever patients (Felix et al. 1935). Early studies show that parenteral administration of purified Vi antigen confers protection in mouse models (Landy and Webster 1952; Landy 1954, 1957). Subsequent reports indicated variability in the efficacy by which purified Vi antigen conferred protection against *S.* Typhi (Gaines et al. 1961; Hornick et al. 1966). This variability in protection has been attributed to removal of O- and N-acetyl moieties from the Vi antigen during purification (*Fig. 17.1b*), which alters antigenic properties of the polysaccharide (Robbins and Robbins 1984). Purification of the Vi antigen by methods that do not remove its O- and N-acetyl moieties yields capsular polysaccharide that confers protection in animals and humans (Wong et al. 1974; Levin et al. 1975; Robbins and Robbins 1984). Vi antigen prepared by this method proofed efficacious in clinical trials (Acharya et al. 1987; Klugman et al. 1987) and is now a licensed parenteral typhoid fever vaccine (Klugman et al. 1996; Hessel et al. 1999).

Antibodies elicited by immunization with purified Vi antigen are strictly T cell independent, and the vaccine therefore does not induce increased antibody titers to a recall injection (Landy 1954). Conjugation of the Vi antigen with a recombinant, nontoxic, exotoxin A from *Pseudomonas aeruginosa* (rEPA) yields a vaccine that elicits T cell–dependent responses and confers superior protection in children (Thiem et al. 2011; Canh et al. 2004; Lin et al. 2001; Szu et al. 1994). These data suggest that licensing of the Vi-rEPA vaccine could improve typhoid fever vaccination in children in the future.

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18 Meningitis

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Abstract
Bacterial meningitis is an inflammation of the meninges, including the pia, arachnoid, and subarachnoid space, that occurs in response to infection with bacteria and/or bacterial products. Bacterial meningitis is a significant cause of mortality and morbidity worldwide, with considerable variation in incidence depending on age and geographic location of the patient and the causative agent. Young children are at highest risk for mortality and morbidity, especially those from lower socioeconomic strata in countries with poor medical infrastructure and those infected with *Neisseria meningitidis* (the meningococcus) or *Streptococcus pneumoniae* (the pneumococcus). Additional risk factors for poor prognosis after infection include the severity/stage of illness on presentation, exposure to an antibiotic-resistant organism, and the fact that medical professionals lack understanding of mechanisms underlying the pathological features of meningitis. When bacterial meningitis is suspected, immediate action is imperative to establish a definitive diagnosis, and antimicrobial treatment must be initiated immediately as a precautionary measure, because the mortality rate for untreated bacterial meningitis approaches 100 %; even with optimal treatment, mortality and morbidity remain high. Neurological sequelae are relatively common in meningitis survivors, especially if the agent of disease is a pneumococcal microorganism.

Most pathogenic microbes could potentially cause meningitis in the human brain; however, only two pathogens, *N. meningitidis* and *S. pneumoniae*, account for most cases of acute bacterial meningitis, when patients in all age groups are considered. In contrast, in very young children and neonates, most cases are caused by group B streptococcus, *Escherichia coli*, and *Listeria monocytogenes*. In developing countries, *Haemophilus influenza* type b and *Salmonella* species are still major causes of meningitis in infants and young children. *Salmonella meningitis* has a particularly dismal prognosis. Meningitis is, in the majority of the cases, a consequence of a preceding bacteremia with encapsulated strains. Although the reasons for this association are incompletely understood, bacterial agents that cause meningitis tend to express surface structures mimicking structures and epitopes on human cells and a capsule with antiphagocytic properties that protect them from phagocytosis and normal immune surveillance. Thus, the absence of opsonic or bactericidal antibodies is considered a major risk factor for meningitis. In this regard, age-related incidence of meningococcal and pneumococcal disease is inversely related to prevalence of serum bactericidal activity. Successful identification of microbial epitopes that induce opsonic or bactericidal antibodies and successful vaccination of infants and children using antigenic agents based on these epitopes has changed the epidemiology of bacterial meningitis, particularly due to reduced incidence of *Haemophilus influenzae* type b-induced meningitis more so in industrialized countries. However, antigenic epitopes suitable

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for this preventive approach have not been identified in all organisms that cause meningitis with significant frequency today.

Major Causes of Bacterial Meningitis

Bacterial meningitis, an inflammation of the meninges affecting the pia, arachnoid, and subarachnoid space in response to bacteria and bacterial products, continues to be an important cause of mortality and morbidity worldwide (WHO 2012). However, mortality and morbidity vary by age and geographic location of the patient and the causative organism. Patients at risk for high mortality and morbidity include infants and young children, those living in low-income countries [or in low socioeconomic strata], HIV-infected patients in developing countries, those infected with *Neisseria meningitidis* (the meningococcus, Mc) or *Streptococcus pneumoniae* (the pneumococcus, Pc), and infants living in resource-poor countries infected with *Salmonella* species (Chang et al. 2004; Davidsen et al. 2007; Molyneux et al. 2006). In the pre-vaccine era, *Haemophilus influenzae* was the most common bacterial pathogen causing meningitis in young children, and *H. influenzae* type b caused approximately 70 % of bacterial meningitis in children younger than 5 years of age (Dery and Hasbun 2007). However, in the early 1990s, a conjugated vaccine against *H. influenzae* type b was developed, and after a program to vaccinate against *H. influenza* type b was implemented, the global incidence of *H. influenzae*-induced meningitis decreased dramatically (55 % reduction in the annual number of cases in the United States alone) (Thigpen et al. 2011). Subsequently, *S. pneumoniae* and *N. meningitidis* have emerged as the pathogens responsible for most cases of bacterial meningitis (Dery and Hasbun 2007; Brouwer et al. 2010).

Almost all microbes that are pathogenic to human beings have the potential to cause meningitis, but a relatively small number of pathogens (primarily *N. meningitidis*, *S. pneumoniae*, *H. influenzae* type b, group B streptococcal disease, *Escherichia coli*, *Salmonella* species, and *Listeria monocytogenes*) account for most cases of acute bacterial meningitis in children and neonates, although the reasons for this remain incompletely understood (*Table 18.1*). One common denominator among bacterial agents that cause meningitis is the presence of an antiphagocytic capsule and the related fact that opsonic or bactericidal antibody is absent in most cases of meningitis (Gotschlich et al. 1969). As a correlate, age-related incidence of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* type b disease is inversely related to prevalence of serum bactericidal activity/antibodies (Gotschlich et al. 1969; Chudwin et al. 1983), and the lack of type-specific antibody is a major risk factor for neonatal group B streptococcal disease (Baker and Kasper 1976). Successful identification of microbial epitopes that induce opsonic or bactericidal antibodies and successful vaccination of infants and children using antigenic compounds based on these epitopes have changed the epidemiology of bacterial meningitis (Peltola 2000; Whitney et al. 2003; Borrow and Miller 2006; Tsai et al. 2008). However, antigenic epitopes suitable for this preventive approach have not been identified for all organisms that cause meningitis with significant frequency. Currently, the most agents that cause severe meningitis most frequently in all age groups worldwide are *N. meningitidis* and *S. pneumoniae*. This chapter reviews the extensive knowledge base, accumulated over many years by many researchers, on these organisms and their pathological effects on their human host.

Table 18.1
Meningococcal virulence factors

Virulence factor	Function
Lipopolysaccharide (LPS/LOS)	Lipooligosaccharide (LOS) has endotoxin activity and is released as bacterial outer membrane vesicles (blebs) or through cellular lysis. LOS is responsible for toxic damage to the human tissue, development of septic shock, and disseminated intravascular coagulation (DIC) through interactions with Toll-like receptors (TLR4) and cytokine induction
Polysaccharide capsule	Polysaccharide surface component which works as a protective shell and blocks the insertion of the membrane attack complex of the complement system and protects the bacteria from phagocytosis. The capsule is the main component enabling bacterial survival in blood and resisting bactericidal antibodies. The serogroup B capsule can also mimic human antigens
Type 4 pili	Major adhesins that mediate initial attachment to nonciliated human cells. Also required for efficient transformation of DNA
Outer membrane proteins (OMP)	Dominant antigens. Porin protein promotes intracellular survival. Opacity proteins mediate firm attachment to eukaryotic cells. Rmp protein can protect other antigens from bactericidal interactions with antibodies. Frequent antigenic variation makes it difficult for the host immune system to recognize these antigens
Iron-binding proteins	Transferrin-, lactoferrin-, and hemoglobin-binding proteins. Pathogenic *Neisseria* spp. are dependent on a constant iron supply for growth
IgA1 protease	Destroys mucosal IgA which is a part of the local immune system
Beta-lactamase	An enzyme that hydrolyzes the β-lactam ring of penicillin. Important for antibiotic resistance development

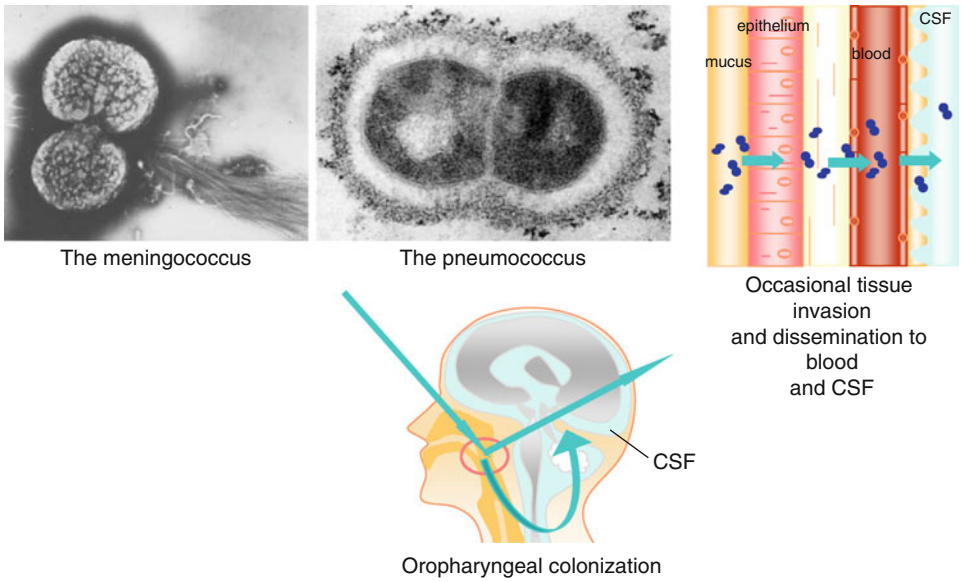

Fig. 18.1

Stages in microbial CNS pathogenesis. Neisseria meningitidis and Streptococcus pneumoniae are causative agents of meningitis. Primary adherence to mucosal epithelial cells occurs via pili and other surface components. The bacteria can then establish an intimate contact with the host cells via outer membrane proteins such as the Opa protein(s), an interaction that might allow bacterial transcytotic passage to subepithelial tissues. Bacterial interactions with the mucosal cells, submucous tissue and endothelial cells might result in its entry into the bloodstream and subsequent entry/passage of the blood–brain-barrier to cause meningitis/CNS infection

Neisseria meningitidis and Meningococcal Disease

N. meningitidis, the meningococcus, is a Gram-negative diplococcus in β-proteobacterium (Fig. 18.1) that causes endemic and epidemic meningitis and/or septicemia worldwide. Epidemic meningococcal meningitis was first described by Vieusseux in 1805 in Geneva (Vieusseux 1805). Throughout the nineteenth century, periodic epidemics occurred, involving primarily young children and adolescents, as well as military recruits. The genus *Neisseria* was named after Albert Neisser, who observed gonococci (*Neisseria gonorrhoeae*) in leukocytes in urethral exudates from patients with gonorrhea in 1879. Marchiafava and Celli (1884) described intracellular oval micrococci in a sample of cerebrospinal fluid (CSF), and Anton Weichselbaum (1887) isolated the organism from six of eight cases of primary sporadic community-acquired meningitis, identified features that distinguish pneumococci from meningococci, and gave it the name *Diplococcus intracellularis meningitidis* (Marchiafava and Celli 1884; Weichselbaum 1887). The fact that *N. meningitidis* enters human cells is an important feature of meningococcal pathogenesis. In 1896, Kiefer reported that healthy individuals can be asymptomatic "carriers" of nasopharyngeal meningococci.

Meningococci are recognized as agents that cause endemic cases, case clusters, epidemics and pandemics of meningitis, devastating septicemia, and, less commonly, pneumonia, septic arthritis, pericarditis, chronic bacteremia, and conjunctivitis in hundreds of thousands of individuals worldwide each year (WHO 2012). Mortality can be 10 % or higher in developing countries, and when patients survive, they often suffer limb loss, hearing loss, cognitive dysfunction, visual impairment, educational difficulties, developmental delays, motor nerve deficits, seizure disorders, and behavioral problems (Kim 2003; Roine et al. 2008). Curiously, certain geographic and temporal anomalies exist in the natural history of the disease; these include the fact that no outbreaks of epidemic meningococcemia or meningitis prior to 1805 have been reported, as well as no reported epidemics in the meningitis belt of sub-Saharan Africa prior to 1900 (Cartwright 1995).

Epidemiology of Meningococcal Disease. Meningococcal disease is a major global health problem (Fig. 18.1) (Stephens 2007) that causes endemic, hyperendemic, epidemic, and pandemic outbreaks at a rate that varies according to geographic region, population demographics, host susceptibility, and infectious agent/strain. In 2010, approximately 170,000 individuals died from meningococcal disease worldwide. The case fatality rate is 5–10 % in industrialized countries, and of those who survive, 10–20 % develop permanent sequelae. Transmission of meningococci occurs by respiratory droplets or kissing, requiring close contact. They colonize nonciliated epithelial cells in nasopharynx and the tonsils (Stephens 1982). Infection, which occurs within 2–10 days, leads to invasive disease in individuals who lack bactericidal antibodies that recognize the invading strain and in complement-factor-deficient individuals (Gotschlich et al. 1969; Stephens et al. 2007). Concurrent viral or mycoplasmal respiratory tract infections increase susceptibility to systemic invasion by the pathogenic bacteria.

The polysaccharide capsule is the primary determinant of the relative virulence of disease-causing meningococci. Most infections are caused by strains belonging to serogroups A, B, C, X, Y, and W-135 (Stephens et al. 2007; Khatami and Pollard 2011). In Western Europe, North America, and South America, serogroups B and C are the primary disease-causing pathogens, and these strains are endemic, causing disease at an incidence of 1–3/100,000. Periodically, local hyperendemic outbreaks occur when new lineages spread through the population. In 2001–2006, serogroup B infection spread worldwide, culminating in disease outbreaks in Australia and New Zealand (Stephens 2007; Stephens et al. 2007). In China, the Middle East, and parts of Africa, serogroups A and C predominate. Large epidemics are attributed predominantly to serogroup A strains. In the African "meningitis belt," major periodic epidemics of serogroup A disease occur every 5–12 years, with attack rates of 500/100,000 population or higher (Achtman 1995). The emergence and global importance of serogroups W-135, X, and Y were recognized only in the last 10 years. Serogroup W-135 was identified in 2002–2003 as a major threat, and it was the primary pathogen responsible for outbreaks in Africa. An unprecedented increase in incidence of serogroup X meningitis was observed in Niger in 2006 (Boisier et al. 2007). Occasionally, particularly virulent strains arise that cause pandemic outbreaks that manifest across continents (Stephens et al. 2007). In the USA, Israel, and Sweden, disease due to serogroup Y strains has increased (Rosenstein et al. 2001).

Meningococcal disease can occur when a pathogenic organism infects a susceptible host. Specific factors that increase risk of meningococcal disease include climate, age, social behavior, health status including preexisting or coinfection with other microorganisms (MacLennan et al. 2006), and hereditary factors (Schneider et al. 2007; Stephens et al. 2007). Additional risk factors for invasive meningococcal and pneumococcal disease include smoking, living in crowded conditions, exposure to pathogen by travel to epidemic areas, deficiency in terminal complement components, and asplenia (Yazdankhah and Caugant 2004). Meningococcal and pneumococcal disease can affect persons of all age groups, but higher rates of invasive disease in developed countries are seen in infants and children less than 4 years old, adolescents, military recruits, and individuals living among a transient population (e.g., college students in dormitories) (Stephens et al. 2007; Rosenstein et al. 2001). Elderly individuals are also at risk for pneumococcal disease. Meningococcal serogroup A and C disease increases during the dry season in Africa. The early stages of disease can mimic a viral infection such as influenza, but the disease course can be fulminant. Thus, it can be difficult to identify and treat meningococcal disease quickly. Rapid progression from bacteremia and/or meningitis to life-threatening septic shock can occur within the first few hours after initial symptoms appear. Because of these factors, vaccination is generally the best preventive option for controlling this disease. Although significant progress has been made in understanding meningococcal pathogenesis, and effective meningococcal vaccines along with strategies for vaccination are or are soon to be available, there remain many challenges before it will be possible to optimize and deliver effective preventive and therapeutic approaches for meningococcal disease.

Streptococcus pneumoniae and Pneumococcal Disease

The Gram-positive diplococcus *S. pneumoniae* (Fig. 18.1) is a eubacterium belonging to phylum *Firmicutes* and order *Lactobacillales*. *S. pneumoniae* is a major cause of mild respiratory tract infections (i.e., otitis media and sinusitis) and is also the worldwide leading cause of the much more severe diseases, community-acquired pneumonia, septicemia, and meningitis.

Epidemiology of S. pneumoniae. It is estimated that between 1.5 and 2 million people die from pneumococcal infection every year, a rate similar to mortality from tuberculosis. Young children, the elderly, and immunocompromised individuals (i.e., splenectomized individuals, HIV patients, and chronically ill patients suffering from renal or liver disease, alcoholism, diabetes mellitus, skull fracture, or cochlear implants) (Biernath et al. 2006; Weisfelt et al. 2006; Brouwer et al. 2010). Past history of viral infection, especially influenza A virus (IAV), also sensitizes the host for pneumococcal infection. Coinfection with pneumococcal influenza is the most important factor contributing to increased morbidity and/or mortality from *S. pneumoniae* worldwide, even accounting for the severity and increased mortality associated with the 1918 influenza pandemic. Based on 156 studies published in 2000, O'Brien et al. estimated the global burden of pneumococcal disease in children younger than 5 years of age (2009) as close to 14.5 million serious pneumococcal infections and 826,000 deaths, of which 91,000 were in individuals positive for HIV (O'Brien et al. 2009). More than 61 % of the deaths occurred in 10 African and Asian countries. On a global level, it was estimated that infection with *S. pneumoniae* accounts for approximately 11 % of mortality in this age group, when deaths due to infection with HIV were excluded. Europe had the lowest incidence rate, 6 per 100,000, while the highest rate was 38 per 100,000 in Africa. The incidence of pneumococcal meningitis in the United States in children and adults decreased from 1.09 per 100,000 in 1998–1999 to 0.81 in 2006–2007, likely reflecting implementation of a childhood vaccination program in 2000 (see below) (Thigpen et al. 2011). In children 2–23 months of age, incidence decreased from 9.68 to 3.67 in year 2006–2007 (Thigpen et al. 2011).

The case fatality rate in patients with pneumococcal meningitis is equally high in developed and undeveloped regions of the world (Molyneux et al. 2006). In the youngest children, annual global case fatality for the year 2000 was estimated to be 59 %, ranging from 29 % in the western Pacific to 73 % in Africa (O'Brien et al. 2009). In the United States, the mortality rate in all patients with pneumococcal meningitis was 17.9 % in 1998–1999 and 14.7 % in 2006–2007, hence not changing significantly after vaccine introduction (Thigpen et al. 2011). Moreover, during 2003–2007, according to the Emerging Infections Programs network in the United States, the case

fatality rate in pediatric patients was 9.4 % and 17.5 % in the adult population (Thigpen et al. 2011). The most common cause of death in patients with pneumococcal meningitis was cardiorespiratory failure, stroke, status epilepticus, or brain herniation (Brouwer et al. 2010).

Sequelae in survivors of pneumococcal meningitis are present in up to 50 % of cases (Weisfelt et al. 2006; Edmond et al. 2010; Jit 2010). In a meta-analysis of 48 studies of pneumococcal meningitis in affluent populations, the pooled prevalence of individual sequelae was 31.7 % (Jit 2010). The pooled prevalence of hearing loss, seizures, hydrocephalus, spasticity/paresis, cranial palsies, and visual impairment was 20.9 %, 6.5 %, 6.8 %, 8.7 %, 12.2 %, and 2.4 %, respectively. However, cerebral infarction was found in one study in 36 % of adult patients with pneumococcal meningitis (Schut et al. 2012).

Pathogenesis of Meningococcal and Pneumococcal Meningitis

Experimental animal models *indicate that E. coli* and group B streptococcus initially penetrate the brain via the cerebral vasculature (Ferrieri et al. 1980). The blood–brain barrier is a structural and functional barrier formed by brain microvascular endothelial cells (Rubin and Staddon 1999; Amiry-Moghaddam et al. 2004; Davidsen et al. 2007) that protects the brain from microbes and toxins in the blood. However, meningitis-causing pathogens, including *N. meningitidis* and *S. pneumoniae*, cross the blood–brain barrier as live bacteria (Ring et al. 1998; Hsu et al. 2009). Meningitis-causing pathogens cross the blood–brain barrier transcellularly, paracellularly, or by means of infected phagocytes (the "Trojan horse" mechanism) (Kim 2009; Coureuil et al. 2012). For most meningitis-causing pathogens, including *E. coli*, group B streptococcus, and *S. pneumoniae*, transcellular traversal of the blood–brain barrier in infants and children (Ring et al. 1998; Hsu et al. 2009) is mediated by physical interaction with a host cell surface receptor (Unkmeir et al. 2002; Kim 2009). For example, meningococcal and pneumococcal organisms bind to and invade human brain microvascular endothelial cells (HBMEC) (Chudwin et al. 1983; Doulet et al. 2006; Banerjee et al. 2010). The bacterial Opa and neuraminidase NanA proteins interact with CD48 and endoplasmin on HBMEC (Cundell et al. 1995). Invasion of HBMEC by meningococcal organisms also occurs through other host–pathogen receptors such as the laminin receptor (Huang and Jong 2009; Orihuela et al. 2009), a cell surface membrane receptor for the adhesive basement membrane protein laminin. Ribosomal protein SA is also a cell surface ligand for various CNS-infecting microorganisms, including *N. meningitidis*, *S. pneumoniae*, *H. influenza* type b, dengue virus, adeno-associated virus, Venezuelan equine encephalitis virus, and prion protein (Orihuela et al. 2009). It is not currently understood how the same receptor promotes penetration of different microorganisms into the CNS. Meningitis-causing pathogens binding to HBMEC via Lmb (laminin-binding protein), FbsA (fibrinogen-binding protein), pili, and IagA (via lipoteichoic acid anchoring) (Stephens et al. 2007; Orihuela et al. 2009), but whether these structures are unique to meningitis pathogens is unclear. In *N. meningitidis*, the outer membrane protein Opc binds to fibronectin, thereby anchoring the bacteria to the integrin $\alpha5\beta1$ receptor on the cell surface (Orihuela et al. 2009). In addition, *N. meningitidis* pili bind to CD46 on HBMEC (Johansson et al. 2003), and lipooligosaccharides contribute to a high degree of bacteremia and subsequent penetration into the CNS (Plant et al. 2006). CD46 is also a receptor for measles, adenovirus, and human herpesvirus 6 (Manchester et al. 2000; Gaggar et al. 2003; Santoro et al. 2003). *S. pneumoniae* crosses the blood–brain barrier partly through interaction between cell-wall phosphorylcholine and the platelet-activating factor receptor (PAFR), as shown by partial inhibition of pneumococcal invasion of HBMEC by a PAFR antagonist (Cundell et al. 1995) and delayed translocation of pneumococci from the lung to the blood and from the blood to the CSF in PAFR-knockout mice (Radin et al. 2005).

Of note, the mechanisms involved in microbial invasion of the blood–brain barrier differ from those involved in the release of cytokines and chemokines in response to meningitis-causing pathogens. For example, interleukin-8 is secreted from HBMEC infected with *E. coli* K1, but infected non-brain endothelial cells (i.e., human umbilical vein endothelial cells) do not secrete IL-8, and IL-8 secretion does not involve the same *E. coli* proteins that mediate invasion of HBMEC (Galanakis et al. 2006). In addition, c-Jun kinases 1 and 2 promote HBMEC invasion by *N. meningitidis*, and in this context, the p38 mitogen-activated protein kinase (MAPK) pathway promotes release of interleukins 6 and 8. These findings suggest that different proteins and distinct mechanisms mediate penetration of host cells into the CNS and the inflammatory process, leading to meningitis.

Meningococcal and Pneumococcal Colonization and Carriage

Meningococci and pneumococci are commensal pathogens (Yazdankhah and Caugant 2004), and even though they cause invasive diseases such as septicemia and meningitis, they also colonize the upper respiratory tract in 60–70 % of healthy children who attend day-care centers and in approximately 10 % of the general population. In children under 4 years of age, the carriage rate of meningococci is <5 %, progressively increasing to a maximum of 20–25 % in the second and third decades of life. The pathogens colonize the nasopharyngeal mucosa, subsequently spreading to the lower respiratory tract, where an acute inflammatory response is evoked, and clinical symptoms ensue. Meningococci have also been detected in tonsillar tissues in up to 45 % of patients hospitalized for tonsillectomy (Sim et al. 2000).

Asymptomatic individuals harboring these pathogens in the upper respiratory are considered to be carriers for invasive meningococcal and pneumococcal disease with subsequent

transmission occurring largely through respiratory droplets and secretions. The size of the inoculum required for transmission from one host to another is not known. Individuals harboring *N. meningitidis* and *S. pneumoniae* in the upper respiratory tract display pathology of variable severity, ranging from local inflammation to invasion of mucosal surfaces, fulminant sepsis, or focal infection (Apicella 2005). Meningococcal and pneumococcal disease usually occurs 1–14 days after acquisition of the pathogen (van Deuren et al. 2000). However, in some cases, the carrier state can persist for months or even years.

The relationship between meningococcal and pneumococcal carriage and meningococcal and pneumococcal disease has been studied to some extent, and carriage prevalence has even been used as a proxy for predicting outbreaks of meningococcal and pneumococcal disease. For example, carriage of pneumococci appears to be a risk factor for meningococcal carriage (Ridda et al. 2010). The important parameter is the rate of acquisition of hypervirulent meningococci or pneumococci, not the overall meningococcal and pneumococcal carriage. The probability of progression to meningococcal or pneumococcal disease declines very sharply 10–14 days after acquisition of the pathogen. The extent to which *N. meningitidis* and *S. pneumoniae* interact with other commensals/pathogens that reside in the upper respiratory tract is an important area for future study. Meningococcal carriage and its consequences are understood in the context of a dynamic model. Cross-sectional studies of the microbiome in meningococcal carriers can provide an incomplete "snapshot" of the coexisting flora that colonize the nasopharyngeal mucosa, especially if some flora localize primarily to intracellular or submucosal tissue in the nasopharynx. With regard to the polysaccharide capsule, its role during carriage/transmission is not well understood. Capsule-deficient strains are carried and transmitted efficiently, and the ideas that the capsule increases resistance to desiccation during transit or that it reduces adhesiveness are not well supported. It seems likely that the ability to switch between capsulate and non-capsulate forms confers adaptive and/or fitness advantage, possibly by increasing capacity for cell invasion. In this regard, it has been proposed that propensity for carriage differs in strains expressing different capsular polysaccharides (i.e., according to serogroups).

Meningococcal carriage induces bactericidal antibodies within 1–2 weeks after colonization that persist for several months. Bactericidal antibodies to *N. lactamica* cross-react with antigens from various meningococcal serogroups and serotypes. As carriage of *N. lactamica* is approximately 4 % by 3 months of age and peaks at 21 % by 18–24 months of age, this is much higher than carriage of *N. meningitidis* at this age (Yazdankhah and Caugant 2004). *N. lactamica* can protect against meningococcal disease. Development of invasive meningococcal disease correlates with the absence of bactericidal antibodies (Goldschneider et al. 1969).

Meningococci express multiple adhesins (i.e., pilus, Opa, NadA) characterized by an impressive and high degree of allelic variation, a property that likely reflects their capacity to import and incorporate genetic information laterally via genetic transformation in an addition to extensive intragenomic recombination. Nevertheless, carriage studies suggest that most individuals are colonized with a single meningococcal strain, a fact that constrains the opportunity for genetic exchange between heterologous strains and emphasizes genetic variation arising by spontaneous mutations and recombination/gene conversion within a single strain. Meningococcal adhesins in other commensal *Neisseria* sp. have not been well studied. Thus, it is not clear how allelic meningococcal diversity occurs with such frequency. One possibility is that it reflects intragenomic recombination in combination with strong selection of events providing improved fitness. Alternatively, we may be vastly underestimating the number of carriers who are colonized by multiple distinct strains of meningococci and pneumococci or similarly underestimate the size and diversity of the genetic pool available to meningococci and pneumococci in the nasopharynx.

Meningococcal and pneumococcal carriage and transmission, not disease, determine the global variation and composition of the natural population of these bacterial entities. Conjugate vaccines against a variety of encapsulated bacteria including serogroup C meningococci and multiple serotypes of pneumococci have been a powerful tool towards preventing or reducing the number of outbreaks of meningococcal and pneumococcal disease, providing a compelling rationale for continued study of the biology of the commensal behavior of meningococci. From an evolutionary perspective, the interactions between meningococci and pneumococci and the nonpathogenic flora in the human nasopharynx are key denominators.

Meningococcal Adhesion and Cell Invasion. Adhesion to human mucosal surfaces is essential for meningococcal survival, and adhesins are the bacterial proteins that mediate binding to cell surface receptors on target host cells. Furthermore, adhesin redundancy is a hallmark of the meningococcus. Recognized adhesins include pili, PilC, PilQ, Opa, Opc, LOS, factor H-binding protein, PorA, HrpA, PorB, and NadA (Merz and So 2000; Hill and Virji 2012). Proposed or demonstrated receptors include platelet-activating factor, CD46, CEACAM1, vitronectin and α-actinin/integrins, complement receptor 3, laminin, and the GP96 scavenger receptor (Hill and Virji 2012).

Initial contact of meningococci with nasopharyngeal epithelial cells is mediated by type IV pili, the receptor for which may be the I-domain of integrin α-chains or possibly CD46 (Bourdoulous and Nassif 2006; Doulet et al. 2006). Meningococci proceed to proliferate on the surface of human nonciliated epithelial cells, forming small microcolonies at the site of initial attachment. Capsule blocks close adhesins other than pili and thus may aid meningococcal transmission from mucosal surfaces. Attachment can activate two-component regulatory systems, leading to loss or downregulation of capsule. Close adherence of meningococci to the host epithelial cells results in the formation of cortical plaques and leads to the recruitment of factors ultimately responsible for the formation and extension of epithelial cell pseudopodia that engulf the meningococcus (Doulet et al. 2006). Intimate association is mediated by the bacterial opacity proteins, Opa and Opc with CD66/CEACAMs and integrins, respectively, on the surface of the epithelial

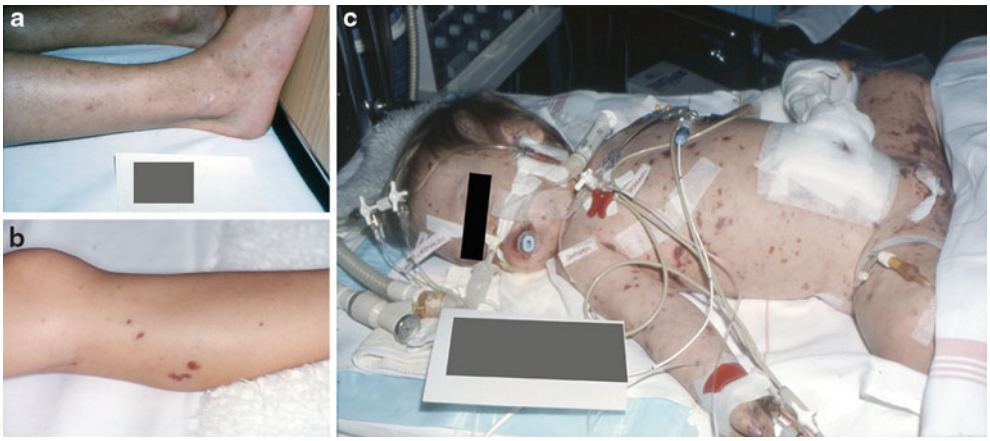

Fig. 18.2
(a) Petechiae on a man with mild systemic meningococcemia caused by *Neisseria meningitidis* serogroup B. (b) Large petechiae and small ecchymoses in a patient with bacteriologically confirmed *N. meningitidis* infection. (c) An 18-month-old boy with lethal cardiovascular collapse caused by *N. meningitidis* serogroup B (Petter Brandtzæg)

cell and is one trigger of meningococcal internalization (Gray-Owen and Blumberg 2006). However, the roles of meningococcal adhesins NadA and LOS are less well defined (Doulet et al. 2006). In this complex process, large molecular complexes involving the molecular linkers ezrin and moesin (known as ERM [ezrin–radixin–moesin] proteins) cluster with integral membrane proteins, including CD44 and intracellular adhesion molecule (ICAM) 1, followed by formation of cortical actin polymers (Hoffmann et al. 2001; Lambotin et al. 2005), which ultimately lead to cortical plaques and cell membrane protrusions. The latter step requires phosphorylated cortactin. Consistent with this, some meningococcal mutants that lack functional LOS demonstrate reduced invasiveness and aberrant actin polymers/polymerization and fail to recruit and/or phosphorylate cortactin (Hoffmann et al. 2001).

The next steps of a meningococcal infection include internalization, intracellular survival, transcytosis through the basolateral tissues, and dissemination into the bloodstream; these processes are not yet thoroughly studied or understood. Intracellular meningococci reside within a membranous vacuole and are capable of translocating through the epithelial layers within 18–40 h after internalization. Intracellular survival requires IgA1 protease, which degrades lysosome-associated membrane proteins (LAMPs), thus preventing phagosomal maturation. IgA1 protease induces a dose-dependent T-cell response, which is mainly a Th1-based proinflammatory immune response (Tsirpouchtsidis et al. 2002). Meningococci can replicate intracellularly, by a process that requires sequestration and utilization of cellular iron through specialized transport systems. This process involves host factors such as the hemoglobin-binding receptor (HmbR), transferrin-binding protein (TbpAB), and lactoferrin-binding protein (LbpAB) (Perkins-Balding et al. 2004) (*Fig. 18.2*).

Pneumococcal Adhesion and Cell Invasion. Pneumococci are encased by a capsular polysaccharide which has been recognized as a sine qua non of virulence. However, several studies have indicated that high amounts of capsular polysaccharide prevent attachment to host cells, probably by masking underlying virulence determinants. Interestingly, the amount of capsule is substantially reduced upon contact with epithelial cells (Hammerschmidt et al. 2005). In addition, the virulence factor pneumolysin plays a significant role in pathogenesis (Paterson and Mitchell 2006). The pneumococcal outer cell wall is composed of peptidoglycan, teichoic acid, and lipoteichoic acid, which differ only in their attachment to the pneumococcal cell wall, as well as phosphorylcholine. Phosphorylcholine is not only targeted by the choline-binding domain of choline-binding proteins but functions itself as an adhesin by recognizing the platelet-activating factor receptor of host cells (Cundell et al. 1995). Through genome mining, it has been predicted that *S. pneumoniae* has approximately 200 proteins with a leader peptide (Bergmann and Hammerschmidt 2006). The leader peptide is recognized by complex secretion machineries known as translocons and is required for protein traversal across the membranes. SecA is the main factor of the general secretory pathway, and the ATPase activity of this protein is the molecular motor of protein translocation across the membranes. Three clusters of pneumococcal surface proteins can be distinguished by genome analysis: lipoproteins, the choline-binding protein family, and proteins with lipoteichoic acid motifs that are covalently anchored in the cell wall after cleavage by a transpeptidase, which is a sortase. Bioinformatics analysis of the pneumococcal genomes also indicates the presence of incomplete biosynthetic pathways, which is consistent with the inability of this pathogen to carry out respiratory metabolism, and also explains the high number of ATP-binding cassette (ABC) transporters produced by *S. pneumoniae*. In addition to these predicted surface proteins, nonclassical surface proteins that lack a classical leader peptide and membrane-anchoring motifs have been identified on the pneumococcal surface, contributing to the virulence of pneumococci and other pathogenic bacteria.

Invasive Meningococcal and Pneumococcal Disease

Invasive Meningococcal Disease and Meningitis. Once infection is established, organisms enter the bloodstream, cause bacteremia, cross the blood–brain barrier, and, ultimately, lead to meningitis. In some instances, untreated meningococcal infections progress rapidly, leading to death within 12–24 h. As discussed above, the prognosis after a non-immunized individual is infected is highly variable, ranging from healthy colonization to serious or fatal clinical disease, and the exact outcome depends on characteristics of both the infectious agent and the host. Some of the most important factors that influence disease outcome are discussed below.

Meningococcal Cell Structure and Virulence Factors

N. meningitidis, like other Gram-negative bacteria, has a cell wall that consists of two membranes separated by a thin peptidoglycan layer. The inner cytoplasmic membrane consists of proteins embedded in a phospholipid bilayer that is impermeable to hydrophilic compounds. The outer membrane is an asymmetrical bilayer composed of phospholipids in the inner leaflet and lipooligosaccharide (LOS) in the outer leaflet. The LOS renders the outer membrane relatively resistant to detergents and is semipermeable due to the presence of protein channels, called porins. Other surface-exposed outer membrane proteins and extracellular appendages such as capsular structures and type IV pili particularly contribute to neisserial survival and virulence (Meyer et al. 1994; Merz and So 2000). The neisserial outer membrane continuously sheds vesicles (blebs) that contain DNA, protein/peptides, and high levels of LOS.

Capsules. Neisseria meningitidis produces a polysaccharide capsule (text box). On the basis of structural differences in capsule, meningococci are divided into at least 13 serogroups (A, B, C, D, 29E, H, I, K, L, W-135, X, Y, and Z). Serogroups A, B, C, Y, and W-135 cause more than 90 % of meningococcal disease. Capsular types are normally stable, but strains can acquire variant alleles of capsule gene (Vogel et al. 2000). For example, serogroup B can switch to C and vice versa. The serogroup A capsule contains N-acetyl-mannosamine-1-phosphate. The capsules of serogroups B, C, Y, and W-135 consist of polymers of N-acetylneuraminic (sialic) acid. The B-polysaccharide resembles structures present in human neural tissues, limiting its immunogenicity and vaccine potential. The carbohydrates can be variably O-acetylated. The capsule polymers are anchored in the outer membrane through a 1,2-dipalmitoyl glycerol moiety. Capsule biosynthesis can vary and is subject to regulation. Isolates from healthy carriers are frequently unencapsulated due to lack of capsule gene expression. A substantial proportion of meningococcal isolates from carriers carry inactivating mutations in or deletions of capsule genes. Isolates from the bloodstream or CSF are invariably encapsulated. In addition to capsule, meningococci are covered with a loosely adherent capsular-like structure containing high-molecular-weight polyphosphate. This layer protects against environmental stress (Zhang et al. 2010).

Pili. Pili are filamentous hairlike fibers consisting of thousands of protein subunits (pilin, 16–20 kDa) (Tonjum and Koomey 1997). Meningococci express long (up to 4,300 nm in length) type IV pili that protrude from the bacterial surface (*Fig. 18.1*). Type IV pili confer bacterial cell-to-cell interactions and twitching motility—a form of locomotion that requires extension and retraction of the pilus filament (Henrichsen 1983). Pili are essential for adhesion to epithelial and endothelial cells adherence of bacteria to human cells and for DNA transformation (Swanson 1973; Stephens and McGee 1981) and impart tissue tropism (Meyer et al. 1994; Merz and So 2000). Expression of type IV pili is also required for efficient DNA uptake in transformation (Jyssum and Lie 1965; Sparling 1966; Davidsen and Tonjum 2006; Hamilton and Dillard 2006). Pili as well as capsule and PorA are expressed during human infection as documented by skin biopsies (Harrison et al. 2002).

Several proteins required for the assembly, extrusion, and retraction of meningococcal type IV pili have been identified (Carbonnelle et al. 2006). These include PilE (Parge et al. 1995), ComP (Wolfgang et al. 1999), PilQ (Tonjum et al. 1998; Collins et al. 2004; Assalkhou et al. 2007), lipoproteins PilP (Balasingham et al. 2007) and PilW (Trindade et al. 2008), the prepilin peptidase PilD (Strom et al. 1993), the ATPase PilT (driving pilus retraction) (Wolfgang et al. 1998; Forest et al. 2004), and the adhesin PilC (Rudel et al. 1995).

The main structural constituent of the type IV pilus fiber is pilin subunit, PilE. During infection, pilins undergo rapid phase shifts and antigenic variation. PilE is encoded by a single gene; however, expression of the PilE gene requires unidirectional donation of coding sequences from multiple silent partial pilS genes in a process similar to gene conversion. During this process, an extensive repertoire of antigenic variants of PilE is generated (Tonjum and Koomey 1997). The frequency of antigenic pili variation can be as high as 10^{-3}. Pilin is also posttranslationally modified with phosphorylcholine, phosphoethanolamine, and variable acetylated O-linked glycans (Power and Jennings 2003; Aas et al. 2006). *N. meningitidis* expresses class I or class II pili, which are antigenically and structurally distinct. Class II pili are encoded by a different *pilE* gene that has no silent cassette counterparts.

Surface Proteins. The repertoire of the meningococcal surface proteins is substantial (Meyer et al. 1994; Merz and So 2000). Trimeric protein channels (porins) transport low-molecular-weight nutrients across the outer membrane. *N. meningitidis* can express two types of porins simultaneously: PorA and PorB. PorA (class 1 protein, 44–47 kDa) is variably expressed, and in some patients, the level of expression is below the detection limit, implying that the gene is highly repressed, inactivated by mutation, or that the protein is expressed but sequestered and/or modified. Antigenic differences in PorA (and PorB) are used to classify meningococci into serological subtypes. Meningococcal PorB is equivalent to the gonococcal

PorB protein and is represented by one of two isoforms, PorB-IA (class 2 protein, 40–42 kDa) or PorB-IB (class 3 protein, 37–39 kDa) (Meyer et al. 1994; Merz and So 2000). Meningococci fail to survive unless they express PorA or PorB.

The neisserial RmpM protein (formerly protein III or class 4 protein) forms a complex with and likely stabilizes outer membrane protein complexes including porins. The protein is stably expressed by gonococci and meningococci. Its C-terminal periplasmic region resembles the analogous protein domain in *E. coli* OmpA, whose role involves binding peptidoglycan. RmpM-specific antibodies interfere with the bactericidal activity of antibodies against other surface antigens, thereby increasing the risk of infection (Plummer et al. 1993).

Meningococci express opacity (Opa) proteins (20–28 kDa) (Meyer et al. 1994; Merz and So 2000). Opa proteins are structurally similar to one another but display considerable intra- and interstrain variation in surface-exposed regions and in level of expression. Intrastrain antigenic variation arises by intragenomic recombination and horizontal gene transfer, and variants are subject to selective pressure during infection. High-frequency phase variation is due to translational frame-shifting involving a pentameric repeat in the opa genes. By this mechanism, Opa proteins are switched on and off, independent of one another, enabling simultaneous expression of multiple proteins (Meyer et al. 1994). The meningococcal genome contains 3–4 *opa* genes. Opa proteins play an important role in promoting adherence to and invasion of eukaryotic cells. Because Opa proteins are subject to a high degree of phase and antigenic variation, they have limited usefulness as targets for vaccine-based interventions. Approximately 70 % of meningococcal strains express the Opc protein. This protein also confers colonial opacity and is functionally similar to Opa.

In addition to the aforementioned major outer membrane proteins, meningococci express >80 other outer membrane proteins. Among these, the pilus-related secretin complex PilQ is one of the most abundant, representing approximately 10 % of the total mass of the outer membrane protein fraction (Tonjum et al. 1998). The level of expression of PilQ varies under different growth conditions. Iron-regulated proteins are also expressed on the surface of meningococci in vivo. Among these, transferrin-binding proteins (Tbp-1 and Tbp-2) and the lactoferrin-binding proteins (Lbp) facilitate transport and internalization of iron, an essential nutrient for sustained infection. Additional conserved proteins that provide exposed antigenic targets on the bacterial cell surface include OMP85, NspA, NadA, GNA1870 (factor H-binding protein), and GNA2132 (hypothetical lipoprotein); these are considered to be viable targets for vaccine development.

Lipooligosaccharide. Approximately 50 % of the neisserial surface is covered by lipid-anchored oligosaccharide (LOS). LOS lack repeating carbohydrate units (O-chain) of enterobacterial lipopolysaccharide (LPS). *Neisseria*l LOS is composed of hexa-acylated lipid A, two KDO molecules, and one or more carbohydrate chains of 8–12 saccharide units, the core oligosaccharide. The lipid A anchors LOS in the outer membrane and is one of the most potent bacterial endotoxins.

The core oligosaccharide of neisserial LOS is divided into an inner and outer core region. The composition of the inner core is heterogeneous due to variable substitutions (phosphoethanolamine, glycine, glucose, *O*-acetyl groups) (Kahler et al. 2005), which occur in response to environmental cues. The outer core is also variable and undergoes high-frequency phase and antigenic variation due to frequent targeted mutagenesis during replication of LOS biosynthesis genes as well as horizontal gene transfer (Kahler and Stephens 1998). A single strain can simultaneously express up to six related LOS. The terminal structure of neisserial LOS is sialylated by bacterial sialyltransferase. Gonococci modify LOS using host sialic acid (CMP-NeuNAc). In contrast, meningococci use an endogenous source of CMP-NeuNAc. The terminal LOS of the pathogenic *Neisseria* spp. often shares epitopes with host gly*coli*pids (Kahler and Stephens 1998). In this manner, molecular mimicry is exploited by the pathogens, which gain the ability to bind to host cell lectin receptors. This limits the usefulness of LOS as a vaccine target.

Meningococcal LOS initially react with CD14 and subsequently with TLR4 receptor which is critical to the innate immune responses to bacterial endotoxins including meningococcal LOS (Akira and Takeda 2004). Activation of TLR4 by endotoxin requires association with the accessory protein MD-2, an N-glycosylated (Viriyakosol et al. 2001) 19–27-kDa protein that is expressed in both a soluble and a membrane-bound form. Binding of endotoxin LOS to MD-2 in association with TLR4 can lead to dimerization or oligomerization of TLR4 receptors and subsequent cellular activation. MD-2 directly interacts with lipid A of meningococcal endotoxin.

*Peptidoglycan. Neisseria*l peptidoglycan consists of long chains of repeated disaccharide units cross-linked via peptide bridges. In *E. coli*, peptidoglycans are found covalently linked to lipoproteins, but this is not thought to occur in meningococci. Peptidoglycan metabolism involves both lytic and synthetic enzymes. Initial synthesis is carried out using four penicillin-binding proteins (PBPs) (Dillard and Hackett 2005), followed by O-acetylation, which protects against autolysis by endogenous lytic transglycosylates and host lysozymes. After release, peptidoglycan fragments activate the innate immune response through the intracellular NOD1 and NOD2 receptors and contribute to inflammatory response.

Secreted Factors. The meningococcal genome is predicted to encode autotransporter, two-partner, and type I and type II secretion mechanisms (van Ulsen and Tommassen 2006). The pathogenic *Neisseria* spp. secrete immunoglobulin A1 (IgA1) protease. This serine protease directs its own transport across the outer membrane into the environment. The enzyme cleaves IgA1 in the hinge region, separating Fab and Fc; this inactivates IgA function. IgA protease also cleaves other proteins such as endosomal Lamp1, important for intracellular vesicle trafficking. The functions of other secreted proteins including the filamentous hemagglutinin (FHA)-like protein TpsA and FrpA/C are largely unknown. A subset (~80 %) of meningococcal and gonococcal strains

secrete DNA via a type IV secretion system (Hamilton et al. 2005). The genes encoding this system are located on the gonococcal genetic island, a DNA region that is acquired by horizontal gene transfer. Unlike many other bacterial pathogens, *Neisseria* spp. lack a type III secretion mechanism.

Meningococcal Genome Characteristics and Dynamics

*Neisseria*l chromosomes are 2.2–2.3 Mb in length with an average G+C content of 48–56 mol%. Approximately 95 % of the genic material, excluding intergenic regions, is shared between *N. meningitidis* and *N. gonorrhoeae* (Claus et al. 2007). The vast majority of genes are also present in nonpathogenic *N. lactamica*, but the same gene may be differentially regulated in pathogenic and commensal strains. The meningococcal and gonococcal genomes are considered to be hyperdynamic (Davidsen and Tonjum 2006), and the high level of genomic plasticity/instability is thought to contribute to pathogenicity and development of hypervirulence. The most important sources of neisserial genome instability are:

1. Phase variation, reflecting slip mispairing in homopolymer nucleotide runs at or near the promoter or in open reading frames (affect translation)
2. Recombination, integration, or rearrangement of DNA from external or internal sources
3. Horizontal gene transfer via uptake of exogenous or "foreign" DNA, with subsequent RecA-mediated integration into homologous region of the genome
4. Hypermutation due to error-prone DNA repair, replication infidelity, or overexpression of error-prone translesion DNA polymerases

Meningococci are naturally competent for DNA uptake throughout their growth cycle (Jyssum and Lie 1965; Sparling 1966; Hamilton and Dillard 2006; Ambur et al. 2009). The pathogenic *Neisseria* spp. share several genomic regions including up to nine prophage and eight genetic islands that are absent from *N. lactamica* (Snyder et al. 2005). In contrast to many other bacterial species, there are no classical pathogenicity islands in *N. meningitidis*. *N. meningitidis*-specific DNA sequences include the *cps* locus encoding the polysaccharide capsule, genes that encode the RTX family of toxins, and an ortholog of the filamentous hemagglutinin of *Bordetella pertussis*. The genomes of disease and carriage isolates show no consistent differences. Certain hypervirulent lineages contain the filamentous prophage Nf1 (Bille et al. 2005). *N. meningitidis* strains of serogroups W-135, H, and Z contain the "gonococcal genetic island" (GGI, 57 kb) (Snyder and Saunders 2006). This is an often chromosomally integrated conjugative plasmid that encodes a type IV secretion system involved in DNA secretion. The genome of *Neisseria* spp. has a variable number of noncoding repeat arrays and insertion (IS) elements among which IS1655 appears to be unique to *N. meningitidis*.

Most isolates of *N. gonorrhoeae*, but not of *N. meningitidis*, carry plasmids (Roberts 1989). Nearly all gonococcal strains carry a 4.2-kb cryptic plasmid of unknown function, and many strains carry plasmids encoding β-lactamase, which confers resistance to penicillin. The conjugative plasmid TetM confers tetracycline resistance.

Genome-based phylogenetic reconstruction indicates that pathogenic *N. meningitidis* emerged from a common unencapsulated ancestor by acquisition of capsule genes has several hundred years ago, probably from members of the family *Pasteurellaceae* (Schoen et al. 2008).

Pathogenesis

Pathogenesis of Meningococcal Meningitis

Humans are the only host for the meningococcus and the pneumococcus. *N. meningitidis* frequently colonizes the human pharynx as well as buccal mucosa, rectum, urethra, urogenital tract, and dental plaque. The most common natural habitat of the meningococcus is the epithelial cells of the naso- and posterior pharynx and the tonsils. *N. meningitidis* is carried in the pharynx by 4–25 % of the human population. Thus, there are hundreds of millions of carriers worldwide, and adolescents are a principal reservoir (Rosenstein et al. 2001).

Invasive strains of meningococci express capsules, and pathological meningococcal strains were originally distinguished from nonpathological strains by differences in capsular polysaccharide structure (Kim 2003). Virulence determinants include the polysaccharide capsule, outer membrane proteins including pili, the porins (PorA and PorB), the adhesion molecule, Opc, iron sequestration mechanisms, and endotoxin (lipooligosaccharide) (de Louvois et al. 2005).

N. meningitidis is classified into 13 serogroups based on the immunogenicity and structure of the polysaccharide capsule. Further classification into serosubtype, serotype, and immunotype is based on class 1 outer membrane proteins (PorA), class 2 or 3 (PorB) outer membrane proteins, and lipopoly[oligo]saccharide structure, respectively (Chang et al. 2004; Jolley et al. 2012). PorA is an important target for bactericidal antibodies. In addition to these specific virulence factors, *N. meningitidis* has evolved and exploits genetic mechanisms that result in high-frequency phase and antigenic variation and molecular mimicry. Capsule switching, due to allelic exchange of capsule biosynthesis genes by transformation, is one mechanism by which meningococci evade immune detection (Fothergill and Wright 1933).

Infection by *N. meningitidis* commonly develops in asymptomatic individuals carrying bacteria in the oronasopharyngeal cavity (Yazdankhah and Caugant 2004). Type IV pili facilitate initial adherence and opacity-associated proteins (Opa and Opc) and PorB trigger uptake of the bacteria into the cells, largely by similar types of receptors (CEACAM, HSPG). Opa variants are found in hyperinvasive meningococcal lineages. Opa and Opc bind to heparin and interact with vitronectin and

fibronectin, promoting transport into cells via integrin receptors (Duensing et al. 1999). This is accompanied by a downregulation of pili and capsule enabling optimal contact between bacterial adhesins and the host mucosa. Transferrin (TbpA, TbpB) and hemoglobin (Hbp) bind and sequester iron to support bacterial growth (Perkins-Balding et al. 2004). Ciliated mucosal cells can be damaged by released peptidoglycan fragments and LOS. However, the oropharyngeal region has a relatively tolerance for foreign matter and is relatively refractory to the typical inflammatory response characteristic of more sterile anatomical niches such as the urethra. This may explain why meningococcal (and gonococcal) colonization of the oropharynx is rarely associated with clinical disease.

The few phylogenetic groups of *N. meningitidis* that cause meningococcal disease often carry a filamentous prophage in their genome that is secreted from the bacteria via the type IV pilin secretin (Bille et al. 2005). The prophage may promote the development of new epidemic clones. The mechanism by which meningococci penetrate and pass through the mucosa is only partially understood (Merz and So 2000). Meningococci survive and multiply during epithelial cell traversal. The IgA1 protease and PorB may promote survival inside epithelial cells. Meningococci isolated from the bloodstream invariably produce polysaccharide capsule. The capsule protects the bacterium from phagocytosis and complement-mediated lysis by preventing insertion of the terminal complement attack complex. Invasive meningococci express sialylated LOS which influences binding of C4b, while the proteins PorA and GNA1870 recruit the negative regulators of complement activation C4BP and factor H (Schneider et al. 2007). Individuals with inherited deficiencies in the late complement components (C5–C9) have a high risk in developing meningococcal disease. Intriguingly, they acquire the infection at a much later age and have frequent recurrences, and the case fatality rate is much lower than for normocomplementemic individuals.

In the blood, *N. meningitidis* replicates to high levels (up to 10^8/mL plasma) and sheds outer membrane vesicles (blebs) (Stephens and Greenwood 2007). The blebs may subvert the complement system, and high levels of circulating LOS overactivate the innate immune system. Circulating levels of proinflammatory mediators (TNF-α, IL-1, and IL-6) strongly correlate with development of lethal septic shock (Stephens et al. 2007; Brandtzaeg and van Deuren 2012).

Meningococci and pneumococci most often enter the CSF likely by the hematogenous route via the capillaries and veins in the subarachnoid space (the blood–CSF barrier) and the choroid plexi rather than through the brain parenchyma (blood–brain barrier). Encapsulated *N. meningitidis* invade the CSF probably via the transcellular route (Nikulin et al. 2006). The absence of non-opsonophagocytosis in CSF initially enables uncontrolled bacterial growth and inflammation of the leptomeninges and subarachnoid space. In the CSF, *N. meningitidis* produce polysaccharide capsule and pili and stimulate proinflammatory cytokine (Il-6, IL-8, MCP-1) and chemokine (RANTES, GM-CSF) production in meningeal cells (Christodoulides et al. 2002). Attracted polymorphonuclear cells aggravate the inflammatory response and release cytotoxic mediators.

Immunity to invasive meningococcal disease depends upon the presence of bactericidal IgG antibodies directed against capsule (except serogroup B), PorA, PorB, Opa, LOS, iron-regulated proteins, and minor surface proteins. Carriage of nonpathogenic *Neisseria* spp. (e.g., *N. lactamica*) in the nasopharynx elicits cross-reactive antibodies that contribute to development of immunity against *N. meningitidis*. In vivo phase and antigen variation of meningococcal surface antigens indicate selective immunological pressure during natural infection.

Pathogenesis of Pneumococcal Meningitis

The mechanisms underlying pneumococcal meningitis are not fully understood, but involve bacterial, host, as well as environmental factors. The route from the initial site of infection to the meninges is believed to occur via a bacteremic phase and subsequent traversal of the organism from the circulation across the blood–brain barrier (BBB) into the subarachnoid space. However, animal experiments suggest direct axonal retrograde transport to the brain without detectable bacteremia, in a process requiring pneumococcal interactions with gangliosides (van Ginkel et al. 2003). There is some epidemiological support for this, in that pneumococcal CSF isolates belong to a multitude of serotypes more reflective of the commensal nasopharyngeal flora than to blood isolates (Henriques Normark et al. 2001).

It is believed that pneumococci exploit selective tropism towards brain endothelial cells to invade into the CSF. The main pathogens causing bacterial meningitis, *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* all interact with the laminin receptor on rodent as well as HBMECs. In pneumococci, this interaction is mediated by choline-binding protein CbpA (PspC) on the pneumococcal surface. The binding site on CbpA for the laminin receptor was localized to a highly conserved, surface-exposed loop not involved in other known CbpA–host interactions (Orihuela et al. 2009).

All pneumococcal isolates possess the *nanA* gene encoding a cell-wall-anchored neuraminidase that cleaves sialic acid from host cells and proteins. The lectin moiety of NanA rather than the sialidase region promotes pneumococcal adherence to and entry into HBMECs, suggesting that bacterial binding to sialylated glycoconjugates might represent an early step in the BBB translocation process (Uchiyama et al. 2009). In addition, lectin-bound NanA activates brain endothelial chemokine expression and recruits neutrophils, promoting a local inflammatory response at the site of infection even the absence of invasion (Banerjee et al. 2010). This proinflammatory response of pneumococcal NanA requires unmasking of an inhibitory sialic acid-binding receptor on the surface of immune cells (Chang et al. 2012). Other data also suggest that local proinflammatory events that trigger endocytosis and/or locally damage HBMECs facilitate transport across the BBB. Cell surface phosphorylcholine on pneumococci can bind the human platelet-activating factor receptor, activating

beta-arrestin-mediated uptake of pneumococci into cells within the BBB (Radin et al. 2005). Pneumococcal-induced death of brain endothelial cells can be mediated by cell-wall components via TLR2 signaling and/or via the pore-forming cytotoxin pneumolysin and the unusually high levels of hydrogen peroxide produced by this catalase-negative organism (Bermpohl et al. 2005). TLR stimulation mediated by pneumococci is sufficient to promote translocation of the organism as well as its inflammatory components across the epithelium by downregulation of genes involved in the formation of tight junctions (Clarke et al. 2011). It is, however, not known whether innate immune activation by pneumococci opens tight junctions in the brain endothelium, even though early work demonstrated that intracisternal infection in rats by *S. pneumoniae* serotype 3 caused morphological changes in cerebral endothelium including completely separated cell junctions (Quagliarello et al. 1986).

Once pneumococci have entered the CSF, they proliferate easily and release large quantities of proinflammatory components that are recognized by resident immune cells via cell surface and intracellular pattern recognition receptors such as TLRs. This leads to high production of cytokines and chemokines that accumulate in CSF, contributing to inflammation-mediated brain damage. Several cytokines increase in CSF from meningitis patients including IL-1beta. In CSF, IL-1beta concentration correlates with CSF leukocyte count and clinical outcome (Mustafa et al. 1989). IL-1beta activation results from induction of the precursor pro-IL-1beta typically mediated by TLR signaling and inflammasome-controlled activation of caspase 1 cleaving the pro-IL-1beta into active IL-1beta. It has been demonstrated that the ASC and NLRP3 components of the inflammasome promote inflammatory damage in a murine pneumococcal meningitis model and that pneumococcal pneumolysin is the main inducer of IL-1beta expression and inflammasome activation upon pneumococcal challenge (Hoegen et al. 2011). However, infection of human dendritic cells revealed that pneumolysin inhibits human dendritic cell maturation, induction of proinflammatory cytokines, and activation of the inflammasome testifying to important differences between human and murine immune cells in their responses to pneumolysin (Littmann et al. 2009).

Pneumolysin and other cytolysins can also have a direct toxic effect on cortical neurons independent on leukocyte infiltration and caspase activation as demonstrated in an infant rat meningitis model. The damaging effect on cortical neurons due to pore-forming cytolysins was shown to be age dependent and more pronounced in 7- as compared to 11-day-old rats (Reiss et al. 2011). The cytolytic effects of pneumolysin on brain tissue astroglia were significantly enhanced by reducing the calcium concentration (Wippel et al. 2011).

The large quantities of H_2O_2 produced by the catalase-negative pneumococci besides causing oxidative damage have also been shown to affect brain tissue in other ways. Thus, H_2O_2 produced by pneumococci contributed to regional hyperemia in an experimental meningitis model (Hoffmann et al. 2007). Pneumococcal production of H_2O_2 was also responsible for the observed transcriptional activation of brain thrombopoietin and its receptor (c-Mpl) after intrathecal infection. Thrombopoietin is known to exhibit proapoptotic effects on neurons, and its upregulation upon pneumococcal infection may therefore have neurotoxic effects (Hoffmann et al. 2011). The high expression of H_2O_2 is normally attributed to the pneumococcal *spxB* gene encoding a pyruvate oxidase. In vivo transcriptomic analyses of mouse brain tissue, after induction of pneumococcal meningitis, revealed an upregulation of the *glpO* gene encoding an alpha-glycerophosphate oxidase that was cytotoxic for HBMECs via generation of H_2O_2. A *glpO* deletion mutant was defective in the progression from the blood to the brain during in vivo infection, and mutant bacteria caused a significantly lower meningeal inflammation and brain pathology compared with wild type. Interestingly, GlpO immunization protected against pneumococcal invasive disease (Mahdi et al. 2012).

Even though brain lesions appear to result from local meningeal infection, experimental infection demonstrates that the systemic bacteremic component in pneumococcal meningitis significantly contributes to clinical disease presentation and the pathophysiology of BBB breakdown and ventricle expansion (Brandt et al. 2008).

By comparing host response proteins in the CSF from survivors with non-survivors with pneumococcal invasive disease, it was found that complement C3 levels were fivefold lower in non-survivors, suggesting that C3 depletion in CSF is a major factor contributing to death in pneumococcal meningitis. Also, transferrin levels in CSF were higher in the group of non- survivors suggesting a more extensive damage of the blood–brain barrier. There were however no differences in the level of cortical necrosis in the two patient groups as monitored by the CSF levels of creatinine kinase BB (Goonetilleke et al. 2012). The central role played by the complement system in protecting the CNS against pneumococcal growth has also been demonstrated in a murine meningitis model. Thus, 24 h after intracisternal infection, bacterial titers in the CNS were almost 12- and 20-fold higher in C1q- and C3-deficient mice, respectively, than in wild-type mice (Rupprecht et al. 2007).

The cell-wall component lipoteichoic acid has been suggested to be a pattern recognition molecule and inflammatory mediator; however, the receptor for this interaction remains controversial. Interestingly, intrathecal treatment with antibodies against phosphorylcholine recognizing teichoic and lipoteichoic acids decreases neuronal damage in experimental pneumococcal meningitis.

Clinical Features

Severity of illness on presentation, infection with antimicrobial-resistant organisms, and incomplete knowledge of the pathogenesis of meningitis are factors that contribute significantly to mortality and morbidity associated with bacterial meningitis (Chang et al. 2004; Davidsen et al. 2007). When bacterial meningitis is suspected, immediate action is imperative to establish a definitive diagnosis, and antimicrobial treatment

must be initiated as soon as possible as a precautionary measure, because the mortality rate for untreated bacterial meningitis approaches 100 %; even with optimal treatment, mortality and morbidity remain high.

However, especially in children, the symptoms and signs depend on the age of the child, the duration of illness, and the host response to infection (Tonjum et al. 1983). Notably, the clinical features of bacterial meningitis in infants and children can be subtle, variable, nonspecific, or even absent. In infants, they might include fever, hypothermia, lethargy, irritability, poor feeding, vomiting, diarrhea, respiratory distress, seizures, or bulging fontanelles. However, at a certain stage, even infants develop nuchal rigidity. In older children, clinical features include fever, headaches, photophobia, nausea, vomiting, confusion, lethargy, or irritability. Other signs of bacterial meningitis on physical examination include neck and back rigidity, Kernig's sign (flexing the hip and extending the knee to elicit pain in the back and legs), Brudzinski's sign (passive flexion of the neck elicits flexion of the hips), focal neurological findings, and increased intracranial pressure. Signs of meningeal irritation are present in 75 % of children with bacterial meningitis at the time of presentation (Levy et al. 1990; Borchsenius et al. 1991). Absence of meningeal irritation in children with bacterial meningitis is substantially more common in those younger than 12 months. The constellation of systemic hypertension, bradycardia, and respiratory depression (Cushing's triad) is a late sign of increased intracranial pressure. Neurological sequelae are relatively common in survivors of meningitis, especially in individuals infected by a pneumococcal microorganism (Arditi et al. 1998; Roine et al. 2008).

Meningococcal Meningitis. The clinical spectrum of systemic meningococcal disease includes meningitis/meningoencephalitis, fulminant septic shock, the combination of the two, or mild meningococcemia without clinically distinct meningitis. Occasionally, the bacteremia leads to localized joint infection, pericarditis, panophthalmitis, and subchronic or chronic meningococcemia (Rosenstein et al. 2001; Stephens et al. 2007; Brandtzaeg and van Deuren 2012).

The most frequent form of meningococcal infection is acute pyogenic meningitis due to inflammation of the meninges. Based on distinct clinical symptoms of 862 patients in three prospective studies with documented meningococcal disease, 37–49 % had meningitis without shock, 13–20 % meningitis with shock, 10–18 % shock without meningitis, and 18–33 % mild meningococcemia without meningitis or shock (Brandtzaeg and van Deuren 2012). Using the same classification of meningococcal patients admitted to a tertiary academic hospital, the blood culture was positive in 50 % of patients with meningitis without shock, 87 % of patients with meningitis and shock, 93 % of patients with shock without clinically distinct meningitis, and 77 % of patients without meningitis or shock. Interestingly, the CSF culture was positive in 84 % with meningitis without shock, 83 % in those with meningitis and shock, 59 % in shock without clinically distinct meningitis, and in 47 % in those without clinically distinct meningitis or shock (Brandtzaeg 2006).

N. meningitidis has the propensity to invade the meninges and will do so in most cases left untreated. Even in the most fulminant cases of septic shock reaching the hospital in Europe within median 12 h, 59 % had a positive CSF culture. Invasive meningococcal infections lead to compartmentalized bacterial proliferation. In patients with clinically distinct meningitis, the levels of meningococci and inflammatory mediators are several logs higher in the CSF than the blood. Conversely, in patients presenting with septic shock, the bacterial proliferation and the inflammatory response mainly occur in the circulation with bacterial load and inflammatory response several logs higher than the subarachnoid space. Thus, the clinical presentation depends on the velocity of proliferation in the blood. Low-graded proliferation leads gradually to meningitis within median 24 h (van Deuren 2001; Brandtzaeg 2006; Stephens 2007; Brandtzaeg 2012). Bacterial load and level of LOS in the circulation are below the shock (10 endotoxin units/mL) threshold. In those developing septic shock, the proliferation is very rapid, the bacterial load massive with up to 10^8/mL, and LOS activity as high as 2150 endotoxin unites/mL (van Deuren 2001; Brandtzaeg 2006; Stephens and Greenwood 2007; Brandtzaeg 2012).

For unknown reasons, patients in the large meningococcal epidemics in sub-Saharan Africa usually develop meningitis without shock and severe DIC, leading to large hemorrhagic skin lesions and thrombosis and subsequent gangrene of peripheral extremities.

On admission, 60 % of cases have experienced symptoms for less than 24 h and 12–20 % for less than 2 days (Tønjum et al. 1986). These symptoms can occur discretely or can blend into one another during clinical disease progression. The disease usually begins abruptly with headache, meningeal signs including stiffness of the neck, and fever. However, classic signs of meningitis (i.e., confusion, headache, fever, and nuchal rigidity) are seen in only about one-half of infected patients. Very young children often have only nonspecific signs including fever, abdominal pain, and vomiting. Other signs include reduced consciousness and photophobia. Mortality approaches 100 % in untreated cases but is around 10 % when appropriate antibiotic therapy is instituted. The incidence of neurological sequelae is low, with hearing deficits, epilepsy, and arthritis most commonly noted. These sequelae are most probably underreported.

In CSF, the number of bacteria is higher than in plasma, leading to a large compartmentalized inflammatory response in the subarachnoid space, with pronounced increase in the concentration of endotoxin, tumor necrosis factor-α (TNF-α), interleukins (IL-1β, IL-6, IL-8, and IL-10), chemokines, and other mediators. The overall inflammatory response in the systemic vasculature, as indicated by activation of cytokines and complement, is modest (Turner et al. 1990; Latorre et al. 2000; Chang et al. 2004; Dubos et al. 2008). Meningococcemia can manifest as pink maculopapular petechial eruptions (Stephens et al. 2007). Rapidly progressive infections can be accompanied by in purpuric/petechial or ecchymotic skin lesions that are hemorrhagic and necrotic. However, skin lesions can be atypical, evanescent, or even entirely absent in patients

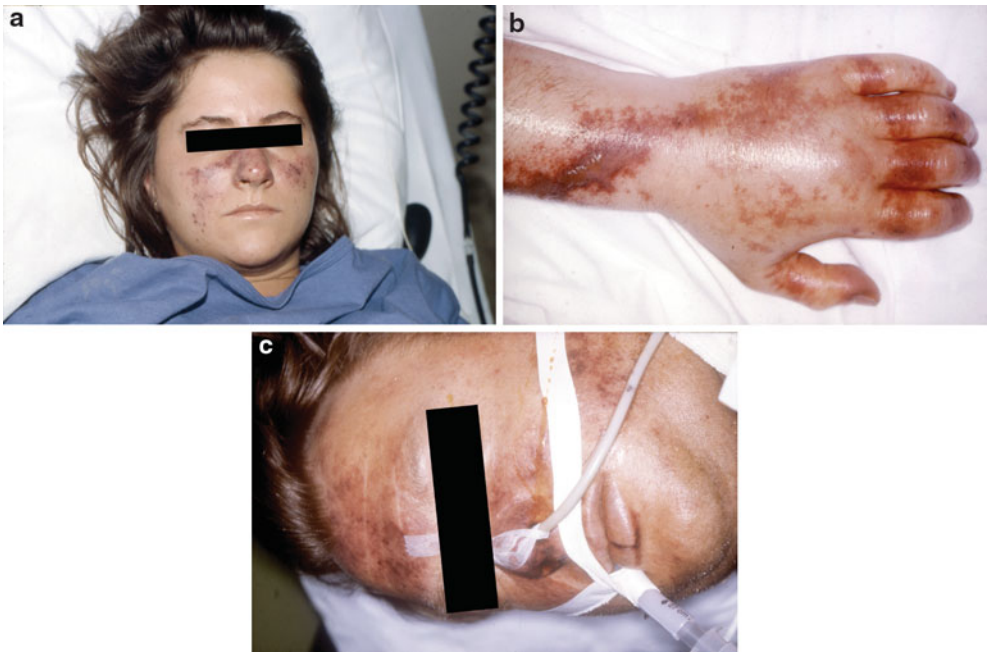

Fig. 18.3
(a) A young woman with sepsis and hemorrhagic skin lesions in the face caused by *S. pneumoniae*. She also had ecchymosis on the extremities. (b) Hemorrhagic skin lesions in a man with fulminant *S. pneumoniae* sepsis. (c) Hemorrhagic skin lesions in the face of a man with fulminant *S. pneumoniae* sepsis. He also had similar lesions on the body and extremities (Petter Brandtzæg)

who have blood culture-positive meningococcal sepsis. Fulminant shock can dominate the clinical picture in patients with acute meningococcal sepsis (Stephens et al. 2007; Brandtzaeg and van Deuren 2012). Sepsis can progress to disseminated intravascular coagulation (DIC) characterized by increasing petechiae or purpura fulminans, resulting in extensive areas of tissue destruction secondary to coagulopathy, rapid onset of hypotension, and adrenal hemorrhage (Waterhouse–Friderichsen syndrome). Gangrenous cases in the extremities can occur due to thrombosis, and death is usually caused by cardiovascular collapse (*Fig. 18.3*).

Diagnosis

The clinical diagnosis of meningococcal meningitis begins with recognition of fever, petechial rash, meningeal signs, and altered mental status and is confirmed by pleocytosis, Gram stain with or without culture of CSF, or blood or skin lesions. The early diagnosis of meningococcemia is difficult when rash and meningeal signs are not present. General symptoms of sepsis (leg pains, cold hands and feet, abnormal skin color) develop first in patients with severe meningococcemia (Borchsenius et al. 1991; Tonjum et al. 1983). However, these symptoms are not specific to meningococcal and pneumococcal disease. Parents and relatives should be instructed to undress and inspect a febrile child, adolescent, or young adult for rash, and physicians and health-care providers should heed the concern of parents or relatives, if and when they describe abrupt or rapid deterioration of a patient.

Laboratory Diagnosis of Meningitis

It is of paramount importance to examine the CSF in order to properly diagnose all forms of meningitis. Cerebrospinal fluid (CSF), blood, skin biopsies, nasopharyngeal swabs, and aspirates are relevant specimens for the diagnosis of meningococcal and pneumococcal disease. Synovial fluid, sputum, and conjunctival swabs can also be cultured, if clinically indicated. Because meningococci and pneumococci are susceptible to desiccation and temperature extremes, specimens should be cultured as soon as possible after collection.

For presumptive diagnosis, specimens are examined by Gram and acridine orange stain. Gram- and acridine orange-stained smears are made directly from CSF, if the CSF is cloudy or after centrifugation when the CSF is clear. The majority of the smears will show Gram-negative diplococci inside and outside polymorphonuclear cells when the CSF bacterial count is $>10^5$/mL. Approximately 25 % of smears will stain positively with Gram stain when the bacterial density in the CSF is $<10^3$ mL; on average, 60–90 % of CSF specimens that are culture positive are stain positive. Gram-stained smears combined with culture from disease-related petechial skin lesions detect meningococci in 62 % of cases (Stephens and Greenwood 2007).

Meningococcal capsular polysaccharides are detected directly in CSF by performing latex agglutination and coagglutination with polyclonal antibodies for serogroups A, B, C, Y, and W-135 (Chanteau et al. 2007). These methods can detect 0.02–0.05 mg of antigen per mL, with a sensitivity of approximately 50 %, compared to 82–90 % for direct detection of meningococci in CSF and

blood by NAATs/PCR (Taha and Fox 2007). The latter tests are also useful for confirming the diagnosis in patients treated with antibiotic prior to sample collection or who tests negative in all prior testing (i.e., Gram stain, antigen test, and culture).

Positive Gram stain is observed in approximately 90 % of children with pneumococcal meningitis, 80 % of children with meningococcal meningitis, half of patients with Gram-negative bacillary meningitis, and a third of patients with *Listeria meningitis* (La Scolea and Dryja 1984). When CSF is first clarified by Cytospin centrifugation, the fraction of samples that stain positively increases (Shanholtzer et al. 1982). CSF cell count and differential, and concentrations of protein and glucose often help with differential diagnosis of various forms of meningitis. The proportion of polymorphonuclear cells in CSF from patients who have meningitis ranges from 49 % to 98 % (mean of 86 %). The prognosis is poor when patients present with low white blood cell count and positive Gram staining in CSF. CSF culture can be negative in children who receive antibiotic treatment before CSF examination. During a course of antibiotic treatment, the CSF leukocyte count, glucose and protein concentration, and antigen tests are abnormal for several days, even though bacteria might not be evident on smear or by CSF culture. Blood cultures test positive in only 50 % of the patients with meningococcal and pneumococcal disease. In those who have received antibiotics prior to the collection of blood for culture, blood cultures are sterile. A nasopharyngeal swab from young children will provide valuable information in cases of suspected meningococcal and pneumococcal disease.

For isolation of *N. meningitidis*, the clinical specimen should be inoculated on selective and nonselective growth media (Tonjum 2005). Appropriate nonselective media are 5 % sheep or human blood agar and chocolate agar. Meningococci and pneumococci are grown on agar media in a 5–10 % carbon dioxide-enriched atmosphere with rather high humidity at 35–37 °C (95–98.6 °F). After 18–24 h, flat, gray–brown, translucent, smooth, 1–3 mm colonies of *N. meningitidis* or *S. pneumoniae* are present which can be analyzed by Gram stain (Tonjum 2005). The finding of oxidase- and catalase-positive Gram-negative diplococci is sufficient to support a tentative diagnosis of meningococcal disease. Differentiation characteristics are the production of acid from glucose and maltose. Optochin-sensitive bacteria with a characteristic central umbilicus indicate *S. pneumoniae*. Isolation of *N. meningitidis* and *S. pneumoniae* can also be finally confirmed by nucleic acid amplification technique (NAATs/PCR), DNA sequence, or MALDI/TOF analysis.

Non-culture Methods. Non-culture tests are particularly important for patients who need rapid identification of pathogens or have previously received antibiotics, or whose initial CSF Gram stain is negative with negative culture at 72-h incubation. Such tests include latex agglutination, PCR, loop-mediated isothermal amplification method, microarray or biochip, and immunochromatography. Latex agglutination uses latex beads adsorbed with microbe-specific antibodies. In the presence of homologous antigen, there is visible agglutination of the antibody-coated latex beads (Gray and Fedorko 1992). In a multicenter pneumococcal meningitis surveillance study, latex agglutination was positive in 49 (66 %) of 74 CSF samples that grew *S. pneumoniae* and in four of 14 CSF samples that were culture negative. The use of standard or sequential-multiplex PCR has been shown to be useful in identification of infecting pathogens in patients who have previously received antibiotics or in resource-poor settings (Corless et al. 2001; Schuurman et al. 2004; Saha et al. 2008; Chiba et al. 2009). Multiplex real-time PCR or broad-range PCR aimed at the 16S ribosomal RNA gene of eubacteria is promising for the detection of pathogens from CSF. The detection rate was substantially higher with PCR than with cultures in patients who had previously received antibiotics (Chiba et al. 2009). However, the limit of detection differs between assays. Real-time PCR has been shown to detect as few as two copies of *N. meningitidis*, *S. pneumoniae*, *and E. coli*, 16 copies of *L. monocytogenes*, and 28 copies of group B streptococcus, whereas the sensitivity for broad-range 16S ribosomal DNA PCR was about 10–200 organisms per mL CSF (Lu et al. 2000; Schuurman et al. 2004). The time needed for the whole process from DNA extraction to the end of real-time PCR was 1.5 h (Chiba et al. 2009), an attractive timeframe for its application in clinical practice. A Gram-stain-specific probe-based real-time PCR using 16S ribosomal RNA has been shown to allow simultaneous detection and discrimination of clinically relevant Gram-positive and Gram-negative bacteria directly from blood samples (Wu et al. 2008), which might provide more rapid and accurate diagnosis of bacterial meningitis. In addition, sequential PCR-based serotyping of *S. pneumoniae* using serotype-specific primers could improve ascertainment of pneumococcal serotype distribution in settings in which prior use of antibiotics is high (Saha et al. 2008). A recently developed NAAT, loop-mediated isothermal amplification, which amplifies DNA under isothermal conditions (63 °C), is a promising tool, particularly in resource-poor settings, because it does not require a thermocycling apparatus and the results can be read with the naked eye (based on turbidity or color development by SYBR Green dye for staining nucleic acids) (Seki et al. 2005). The assay detected ten or more copies of *S. pneumoniae* in oral mucosa swab samples (Seki et al. 2005), but its use in the diagnosis of bacterial meningitis has not been tested. Identification of pathogens by use of a microarray or biochip involves extraction of genomic DNA from CSF, amplification of targeted DNA, and hybridization of labeled DNA with oligonucleotide probes (pathogen-specific or virulence genes) immobilized on a microarray. A rapid immunochromatographic test for *S. pneumoniae* was evaluated in 122 children with pneumococcal meningitis (Saha et al. 2005). Compared with CSF culture (sensitivity of 71 %) and latex agglutination (86 %), immunochromatography was 100 % sensitive for the diagnosis of pneumococcal meningitis, suggesting that immunochromatography might be useful in the diagnosis of pneumococcal meningitis.

PCR or other non-culture tests can be especially useful for diagnosis in regions where patients frequently receive antibiotics before reaching the hospital. Recent WHO reports may have underestimated the real disease burden, because bacteremia and

severe (fatal) disease are often not reported. Further underestimation may be due to limited resources for establishing a diagnosis. In many, but not all, developing countries, at least one laboratory is available for the surveillance of meningococcal disease, but limitations in the availability of diagnostic and typing methods may further result in underestimation of disease burden.

Molecular Typing of *N. meningitidis*

Phenotypic classification of *N. meningitidis* is based upon antigenic differences of the major surface antigens which provides information about the serogroup (capsule, e.g., B), serotype (PorB porin, e.g., 15), serosubtype (PorA porin, e.g., P1.7), and LOS immunotype (e.g., L3) of a particular strain. This results in the classification: B, 15, P1.7, and L3. Multiple epitopes can be recognized depending upon the presence of phase or antigen variants in the bacterial population. Antigen-based typing is currently only relevant for vaccine efficacy studies.

A genetic typing system based upon polymorphisms in multiple housekeeping genes (multilocus sequence typing or MLST) is the gold standard for molecular typing and has defined hypervirulent meningococcal lineages (Maiden et al. 1998). Why hypervirulent meningococcal lineages are more pathogenic has been a subject of considerable interest. Based on sequencing of eight genomes, the chromosome is between 2.0 and 2.1 Mb in size and contains about 2,000 genes (Parkhill et al. 2000; Tettelin et al. 2000; Schoen et al. 2008). Each new strain sequenced has identified 40–50 new genes, and the meningococcus shares about 90 % homology at the nucleotide level with either *N. gonorrhoeae* or *N. lactamica*. Mobile genetic elements including IS elements and prophage sequences make up ~10 % of the genome (Parkhill et al. 2000). Other than the capsule locus, no core pathogenome has been identified, suggesting that virulence can be clonal group dependent. Given that transformation is an efficient mechanism of genetic exchange and that meningococci have acquired DNA from commensal *Neisseria* spp. and other bacteria (e.g., *Haemophilus*) as well as phages, the gene pool for adaptation and evolution is quite large. Genome plasticity and phenotype diversity through gain and loss of DNA or, for example, through DNA repeats are characteristics of meningococcal evolution. This is in contrast to the relatively conserved genomes of, for example, *Bacillus anthracis*. The acquisition of the capsule locus by horizontal transfer possibly from *Pasteurella multocida* or *P. haemolytica* (Schoen et al. 2008) appears to be a major event in the evolution of the pathogenicity of the meningococcus.

Many molecular methods are being used to characterize the structure and evolution of the *N. meningitidis* genome. These include multilocus enzyme electrophoresis (ET) typing, DNA restriction analysis, randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and ribotyping (Yazdankhah and Caugant 2004). The current strain typing method, MLST, shows that epidemics are often caused by specific complexes of related hypervirulent lineages (Maiden et al. 1998; Yazdankhah and Caugant 2004). MLST is presently the best method for global typing of meningococci and for understanding the impact of vaccination. As has been the case for the W-135 epidemic in Africa, surveillance can direct the preparation of vaccines against new clonal strains or specific serogroups (Khatami and Pollard 2011). However, the robustness of MLST in predicting antigenic profile needs further analysis. Especially in the context of new protein-based meningococcal vaccines, the expansion of current typing protocols needs consideration. Because MLST uses conserved core sequences, it may not necessarily predict associations between many vaccine antigens because of the lack of concordance introduced by recombination. Targeted and complete genome sequencing is now the method of choice for genotyping (Jolley et al. 2012).

Molecular Typing of *S. pneumoniae*

S. pneumoniae is highly diverse genetically, due to an efficient transformation system. Some of the many clonal lineages in the genus cause severe pneumonia with invasive disease or meningitis. On the other hand, other lineages are colonizers that are less virulent and being less lethal, and they instead tend to promote the spread of antibiotic resistance. Comparative genomic analyses and functional studies reveal that a significant fraction of the pneumococcal genome is variable and not conserved in all strains. These genomic regions encode nonessential "accessory" gene products, which, at least in mouse models, alter virulence (Blomberg et al. 2009). A number of pneumococcal genomes have been sequenced completely, revealing that the core pneumococcal genome, the portion that is conserved between strains, comprises approximately 50 % of all pneumococcal genes (Hiller et al. 2007). Hence, the accessory genome is of considerable size and may provide different pneumococcal strains (clones) with different properties. Molecular tools such as pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (partial sequencing of seven housekeeping genes) have allowed direct strain comparisons from the same or different geographic areas (Blomberg et al. 2009). Together, these studies show that the capsule and its accessory components are the major virulence attribute of the pneumococcus. Based on difference in capsular components, at least 93 capsular serotypes have been identified. Epidemiological studies that compared carrier and invasive isolates from the same geographic area over the same time period revealed that different serotypes have different odds ratios (ORs) of causing invasive disease (Brueggemann et al. 2003; Sandgren et al. 2004). However, strains of different MLST (ST) but with the same serotype display different degrees of virulence in mice (Sandgren et al. 2005). Thus, the OR for risk of invasive disease varies between strains of the same serotype and between strains of the same ST type as well as by some host characteristics including age and health status, as discussed above (Sjostrom et al. 2006). Therefore, disease severity and disease outcome are affected by the capsular type as well as by other pneumococcal

properties, especially those encoded by the variable accessory genome, and by characteristics of the host.

The serotype and clonal distribution for pneumococcal meningitis is broader than for cases of septicemia caused by the same organism (Henriques et al. 2000; Henriques Normark et al. 2001; Darenberg and Henriques Normark 2009). Clinical presentation and outcome of pneumococcal meningitis also vary for pneumococcal strains of different genotypes (van Hoek et al. 2012). For example, Burckhardt et al. found in a prospective population-based study that serotype 23F was an independent risk factor for pneumococcal meningitis (Burckhardt et al. 2010). In France et al. showed that despite a high vaccination rate, the incidence of pneumococcal meningitis did not decline between 2001–2002 and 2007–2008, due to a wide diversity of serotypes causing pneumococcal meningitis after vaccine introduction (see below) (Levy et al. 2011). However, they observed a decline in cases in children younger than 2 years of age, which was offset by an increase in older children.

Host Susceptibility to Meningococcal and Pneumococcal Meningitis

Genetic Cofactors. Absence of protective bactericidal antibodies is the most important single predisposing factor for systemic meningococcal and pneumococcal disease, but genetic polymorphisms and other host cofactors contribute to disease (Sanders et al. 2011). Complement deficiency and polymorphisms in other innate host determinants, such as the FcII receptor, play important roles as risk factors for meningococcal disease. Complement is required both for meningococcal bactericidal activity and for opsonophagocytosis (Fijen et al. 2000). Individuals deficient in both the early and late components of the complement system are at increased risk for meningococcal disease (Sjoholm et al. 1982; Fijen et al. 1999; Emonts et al. 2003). Compared to the general population, these patients usually experience less severe, more recurrent disease at an older age with less common serogroups. Ten to 20 % of invasive meningococcal disease in adults is associated with a complement defect. The mannose-binding lectin (MBL) pathway of complement activation can be genetically variable and is associated with difference in susceptibility to meningococcal disease in one third of all cases (Hibberd et al. 1999). Other genetic polymorphisms affecting the risk of acquiring meningococcal disease have also been described (TNF, FcγRIIA, FcγRIII, PAI-1, ACE-1, IL-1Ra, IL-1β, TLR4) (Stephens et al. 2007).

It is well known that genetic variation in innate immune response genes contributes to interindividual differences in meningococcal and pneumococcal disease manifestations (Sanders et al. 2011). Disappearance of antibody acquired from the mother increases the risk for infants and young children. For example, homozygotes for codon variants in the mannose-binding lectin, an important mediator of host innate immunity, representing about 5 % of north Europeans and North Americans and larger proportions of populations in many developing countries, have a substantially increased risk of invasive pneumococcal disease (Roy et al. 2002). In contrast, little is known about genetic variation affecting susceptibility to pneumococcal meningitis, but polymorphisms in Toll-like receptors 2, 4, and 9 were recently linked to hearing loss in patients who survived meningococcal or pneumococcal meningitis (van Well et al. 2012).

Polymorphisms in genes coding for the Fcγ-receptor II (CD32), Fcγ-receptor III (CD16), mannose-binding lectin, and TLR4 are associated with increased risk (Hibberd et al. 1999; Fijen et al. 2000; Read et al. 2001; Smirnova et al. 2003; Faber et al. 2006; Tully et al. 2006). Mannose-binding lectin is a plasma opsonin that initiates complement activation; specific polymorphisms in this gene are enriched in children with meningococcal disease relative to controls (Hibberd et al. 1999). Expression of plasminogen activator inhibitor (PAI-1) affects severity and mortality of meningococcal sepsis, suggesting that impaired fibrinolysis is an important factor in the pathophysiology of meningococcal sepsis (Emonts et al. 2003). Meningococcal disease is occasionally linked to immune suppressive disorders such as the nephrotic syndrome, hypogammaglobulinemia, splenectomy, and HIV/AIDS (≈10-fold increased risk for sporadic disease vs ≈100-fold increased risk for infection with the pneumococcus or meningococcus in HIV/AIDS). However, the risk of an epidemic outbreak of meningococcal disease in countries with high rates of HIV does not appear to be elevated.

Congenital and acquired antibody deficiencies, and possibly blocking of IgA immunoglobulin, also increase risk. Opsonization and phagocytic function are important host defense mechanisms that influence disease incidence as well; for example, pp[as shown by disease reduction after polysaccharide vaccination in individuals with complement deficiencies. Rapidly progressive, fatal meningococcemia can arise in patients deficient in properdin (Sjoholm et al. 1982), and there is a significant increased risk of recurrent meningococcal infections for those with defects in the terminal complement pathway (C5–C9) (Fijen et al. 1999).

Epigenetic Cofactors. There is limited knowledge concerning the cofactors that influence the spread and severity of meningococcal and pneumococcal disease. Low absolute humidity can damage the nasopharyngeal mucosa, allowing meningococci to pass the mucosal barrier more easily or be transmitted by coughing. In countries with a temperate climate, susceptibility to meningococcal and pneumococcal disease is highest in the winter when absolute humidity is low. There is also evidence that viral (influenza) and mycoplasma respiratory infections can predispose to meningococcal and pneumococcal disease, perhaps by damaging mucosal surfaces; altering dynamics of adherence, colonization, and spread; and impairing mucosal immunity. The role of mucosal immunity in preventing or enhancing meningococcal disease requires more analysis in the future.

The association between HIV and meningococcal disease is not well studied. Population-based studies in the USA indicate an increased risk (~7-fold) (Stephens et al. 1995), but this has not been noted for African outbreaks. However, the high prevalence of HIV in African and Asian countries might influence

carriage or susceptibility to and severity of meningococcal disease, as demonstrated for pneumococcal infections.

Therapy and Management

Antimicrobial Treatment of Meningococcal and Pneumococcal Meningitis

Eradication of the infecting organism from the CSF is entirely dependent on antibiotics, and bactericidal antibiotics should be administered intravenously at the highest clinically validated doses to patients with suspected bacterial meningitis (Tunkel et al. 2004). Several retrospective and prospective studies showed that delay in antibiotic treatment was associated with adverse outcomes (Miner et al. 2001; Auburtin et al. 2006). In patients with suspected bacterial meningitis for whom immediate lumbar puncture is delayed due to pending brain imaging study or the presence of disseminated intravascular coagulation, blood cultures must be obtained, and antimicrobial treatment should be initiated immediately. Selection of empirical antimicrobial regimens is designed to cover the likely pathogens, based on age of the patient and specific risk factors, with modifications if CSF Gram stain is positive.

The recommended treatment for patients who have meningococcal or pneumococcal meningitis is benzylpenicillin or a third-generation cephalosporin (e.g., ceftriaxone) (Stephens et al. 2007). For most cases of uncomplicated bacterial meningitis, 7-day treatment is adequate. When the etiology is not known at admission, ceftriaxone or cefotaxime is used for the first 24–48 h to cover the possibility of other bacterial pathogens (Stephens et al. 2007). Beta-lactamase-producing strains have occasionally been recovered, harboring a penicillinase-encoding plasmid. In addition, there are *N. meningitidis* strains that are not β-lactamase positive, but have decreased sensitivity to penicillin due to reduced affinity of penicillin to penicillin-binding proteins 2 and 3, resulting from an altered *penA* gene (Spratt et al. 1989; Bowler et al. 1994). Studies have reported isolates of *S. pneumoniae* with penicillin MICs of 0.12–1.0 μg/mL that had mutations in the target penicillin-binding proteins (Spratt et al. 1989; Bowler et al. 1994; Dowson et al. 1989). Similarly, penicillin has been the standard treatment for meningococcal meningitis, but penicillin resistance has evolved, with an implication of treatment failures. An increased incidence in penicillin non-susceptible strains of *N. meningitidis* (e.g., MICs 0.1–0.5 μg/mL) from 9.1 % in 1986 to 81.4 % in Spain and South America (Latorre et al. 2000; Ibarz-Pavon et al. 2012). By contrast, relative resistance to penicillin (MIC 0.1 μg/mL) has been shown to occur in 3–4 % of the meningococcal isolates in the USA and in 2 % of the 137 isolates recovered between 2000 and 2006 from equatorial sub-Saharan Africa (Brigham and Sandora 2009). These findings support the use of a third-generation cephalosporin for meningococcal meningitis in areas where penicillin resistance is prevalent, at least until penicillin susceptibility is known. Although the frequency of relatively penicillin-resistant meningococci is low, continued surveillance is necessary. Cefotaxime or ceftriaxone is used when relatively penicillin-resistant strains are isolated.

The ability of an antimicrobial agent to penetrate the blood–brain barrier is the most important factor that determines whether efficient bacterial killing in the CSF will happen. Blood–brain barrier penetration is affected by lipophilic property, molecular weight, and protein-binding ability of drugs, inflammation of the meninges, water transport, and efflux transporters (Loscher and Potschka 2005). Lipophilic agents (i.e., fluoroquinolones and rifampicin) penetrate relatively well into the CSF even if the meninges are not inflamed, whereas hydrophilic agents (i.e., β-lactams and vancomycin) have decreased penetration into CSF in the absence of meningeal inflammation (Ahmed et al. 1999). An important factor in the choice of empirical antimicrobial agents is the emergence of antimicrobial-resistant organisms, including *N. meningitidis* and *S. pneumoniae* that is resistant to penicillin or third-generation cephalosporins, and Gram-negative bacilli that are resistant to many β-lactam drugs. For example, the prevalence of *S. pneumoniae* strains that are relatively resistant to penicillin (minimum inhibitory concentration [MIC] 0.1–1.0 μg/mL) or highly resistant to penicillin (MIC greater than 1.0 μg/mL) is increasing, and many of the penicillin-resistant pneumococci have reduced susceptibility to third-generation cephalosporins (i.e., cefotaxime and ceftriaxone) (Tunkel et al. 2004). Treatment failures in bacterial meningitis as a result of multiresistant organisms have been reported (John 1994). Therefore, empirical treatment for patients with bacterial meningitis in areas where resistant *S. pneumoniae* strains are prevalent must include the addition of vancomycin. However, penetration of vancomycin into the CSF can be reduced in the absence of meningeal inflammation and also in patients who receive adjunctive dexamethasone treatment.

Before passive immune or antibiotic treatment was available, the mortality of systemic meningococcal and pneumococcal disease was 70–90 %. The case fatality rate is now around 10 % in many countries. However, early recognition by parents and health professionals of the importance of fever and headache with a non-blanching rash (the glass test), prehospital antibiotic treatment, rapid transportation to a local hospital, and stabilization in an intensive care unit has substantially reduced the case fatality rate in children (Levy et al. 1990; Borchsenius et al. 1991). For patients in intensive care, recognition of the different pathophysiological processes associated with meningococcal meningitis (which causes death predominantly by cerebral edema) and meningococcal septic shock (which causes death predominantly through hypovolemia, capillary leak, myocardial dysfunction, and multiorgan failure) has led to major changes in treatment and management strategies for these two different forms of disease (Stephens et al. 2007; Brandtzaeg and van Deuren 2012). Aggressive management of raised intracranial pressure reduces mortality.

Prehospital antibiotic treatment is advocated in many countries, to reduce the case fatality rate for patients with fulminant meningococcal or pneumococcal disease. If antibiotic treatment is initiated before admission, benzylpenicillin, ceftriaxone, or

another effective antibiotic should be injected intravenously or intramuscularly in adults and intramuscularly in children (Stephens et al. 2007). During epidemics in developing countries, a single injection of long-acting chloramphenicol injected intramuscularly can be sufficient treatment for patients with meningitis, and this simple treatment has saved many thousands of lives. A single injection of ceftriaxone is equally effective and could become the preferred treatment for epidemic meningitis.

Pneumococcal infections have been treated with penicillin since decades. However, an emerging increase in resistance rates to penicillin and to other common antibiotics is now being observed, affecting treatment outcome. So far resistance has been found to most antibiotic drugs except for vancomycin. Resistance rates to penicillin and macrolides can be high and above 50 % in some areas (Prymula et al. 2011). Also, pneumococci with reduced susceptibility to penicillin often carry other resistance determinants. In countries with low antibiotic resistance rates such as Norway and Sweden, pneumococcal isolates with reduced susceptibility to penicillin are multiresistant (resistant to more than two antibiotic classes) in 30–60 % of cases. The spread of antibiotic resistance is mainly due to the spread of successful international clones carrying resistance traits. The wide use of antibiotics also influences resistance rates. Milder infections caused by pneumococci with low MIC values to penicillin can usually be treated with a higher dose of penicillin. However, pneumococcal meningitis caused by pneumococci with a reduced susceptibility to penicillin needs to be treated with other antibiotics than penicillin.

Adjunctive Treatment

Neurological sequelae are common in survivors of meningitis and include hearing loss, cognitive impairment, and developmental delay. For example, bacterial meningitis has been identified as the leading postnatal cause of developmental disabilities, including cerebral palsy and mental retardation (Kim 2012). Hearing loss happens in 22–30 % of survivors of pneumococcal meningitis compared to 1–8 % after meningococcal meningitis (Andersen et al. 1997). In a recent meta-analysis, adjunctive treatment with dexamethasone was associated with lower case mortality, and lower rates of severe hearing loss and long-term neurological sequelae (van de Beek et al. 2010). The beneficial effect of adjunctive dexamethasone treatment was also evident in adults with bacterial meningitis.

The outcome of bacterial meningitis has been suggested to be related to inflammation of the subarachnoid space. Hence, it has also been suggested that in addition to antibiotics, meningococcal and pneumococcal meningitis can be treated with corticosteroids. Data in the literature are controversial where some studies show an effect on sequelae such as hearing loss and mortality, while others do not.

Dexamethasone given shortly before or when antibiotics were first given has been shown to reduce the rate of hearing loss in children with *H. influenzae* type b meningitis, but its beneficial effects on hearing and other neurological sequelae are not as clear against meningitis caused by other organisms (van de Beek et al. 2010). Dexamethasone treatment might be considered for infants and children older than 6 weeks with pneumococcal meningitis after considering the potential benefits and possible risks. There is, however, no evidence from randomized controlled clinical trials that dexamethasone reduces death caused by brain edema, which can take place in patients with meningococcal meningitis. The widespread use of dexamethasone in children with bacterial meningitis needs careful monitoring of clinical (e.g., fever curve, resolution of symptoms and signs) and bacteriological responses to antimicrobial treatment, particularly for patients with meningitis caused by pneumococci that are resistant to third-generation antibiotics, in whom bacteriological killing in the CSF depends on vancomycin. Monitoring of the clinical response (e.g., fever curve) can be complicated by the use of dexamethasone. In addition, concomitantly given dexamethasone and vancomycin can reduce penetration of vancomycin into the CSF by virtue of the anti-inflammatory activity of dexamethasone, resulting in treatment failure. However, CSF bactericidal activity has been shown in children who have meningitis due to cephalosporin-resistant pneumococci, and such cases should be treated with dexamethasone as well as vancomycin and ceftriaxone (Klugman et al. 1995). Another issue with adjunctive dexamethasone treatment is the possibility of neuronal injury, including hippocampal apoptosis in experimental animals with pneumococcal meningitis who received dexamethasone (Leib et al. 2003). Long-term follow-up studies are thus needed to address the effect of dexamethasone treatment on any cognitive and neuropsychological outcomes in patients with bacterial meningitis.

Prevention of Meningococcal and Pneumococcal Disease

Prevention of meningococcal and pneumococcal disease is based on chemoprophylaxis and vaccination (Rosenstein et al. 2001). The advancement of vaccine design in enhancing immunogenicity has been shown to be important in preventing meningitis caused by *N. meningitidis* and *S. pneumoniae.* Protein-conjugated capsular polysaccharide vaccines have almost completely eliminated meningitis caused by vaccine serotypes.

Meningococcal Capsular Vaccines. Meningococcal polysaccharide vaccines reduce the incidence of infection among military recruits, reduce the progress of epidemics of serogroup A disease, and protect susceptible complement-factor-deficient individuals (Stephens et al. 2007). Capsule polysaccharide vaccines are available for the pathogenic meningococcal serogroups A, C, Y, and W-135. These vaccines are safe with mild local adverse events and have good efficacy (>85 %) in older children and adults. However, due to lack of a T-helper response, the vaccines are poorly immunogenic below 2 years of age, fail to induce immunological memory, and provide protection for only 3–5 years. Polysaccharide vaccines are used by travelers visiting countries with a high incidence of meningococcal disease.

A polysaccharide vaccine against serogroup B meningococci is not available due to carbohydrate mimicry and poor immunogenicity.

Capsular polysaccharide vaccines to decrease A, C, Y, and W-135 meningococcal disease were introduced in the 1970s and 1980s on the basis of Gotschlich, Gold, Goldschneider, and Artenstein's classic studies (Snape and Pollard 2005). These vaccines are safe with mild local adverse events, are effective (>85 %) in children (older than 2 years) and adults, but are less immunogenic (C less than A) in children younger than 24 months. Immunity to the polysaccharide vaccines is limited to 3–5 years of protection, and immunological hyporesponsiveness is induced by repeated doses of the group C and possibly group A polysaccharides. Polysaccharide vaccines do not induce immunological memory and have little or no effect on nasopharyngeal carriage. Despite their limitations, meningococcal polysaccharide vaccines have been used extensively to control epidemics in countries of the African meningitis belt, and they have saved many lives. However, they have often been deployed too late in the course of an outbreak to achieve maximum effect.

A major advance in the prevention of meningococcal disease has been the development of meningococcal polysaccharide and protein conjugate vaccines and their introduction into the UK, other parts of Europe, Canada, and the USA (Snape and Pollard 2005; Borrow 2012). These vaccines are safe and immunogenic in young children, induce immunological memory, and decrease nasopharyngeal carriage of meningococci. In the UK, introduction of the C conjugate meningococcal vaccines in 2000 to all children and young adults greatly reduced the rate of serogroup C disease (90 % vaccine effectiveness at 3 years for patients aged 11–18 years) (Vipond et al. 2012). A major protective effect of the C conjugate vaccine is mediated through herd immunity. Rates of serogroup C carriage and disease in non-vaccinated individuals are reduced by more than 50 %. However, the three-dose schedule of group C immunization in infancy originally used in the UK provided only transitory protection, and a booster dose or alternative immunization schedule was needed. Thus, the UK has changed to a schedule of two doses of meningococcal C conjugate vaccine given at 3 and 4 months of age, followed by a booster at 12 months. In the Netherlands, meningococcal C conjugate vaccination is not started until the second year of life. A serogroup A, C, Y, and W-135 polysaccharide-conjugate meningococcal vaccine has been introduced in the USA for adolescents (Vipond et al. 2012). In addition to children and adolescents, populations who should benefit from the new conjugate vaccines are military recruits, patients with complement or other immune deficiencies, microbiologists who are routinely exposed to isolates of *N. meningitidis*, and people who travel to or reside in countries where *N. meningitidis* is epidemic.

The immunogenicity of polysaccharide vaccines is greatly improved by chemical conjugation to a protein carrier. The resulting polysaccharide-conjugate vaccines are safe and immunogenic in young infants and induce long-term memory. Conjugate polysaccharide vaccines against serogroups A, C, W-135, and Y are now available. These vaccines are so far safe and immunogenic, are anticipated to provide long duration of protection (as they induce a T-cell-dependent response), and are effective in young children (Bilukha and Rosenstein 2005). Introduction of the C conjugate meningococcal vaccines in 2000 markedly reduced the incidence of serogroup C disease in the UK with estimated vaccine efficacies of 88 % in young children and 95 % in young adolescents. Immunization also decreased nasopharyngeal carriage by 66 % and transmission of the pathogen (herd immunity) (Snape and Pollard 2005; Vipond et al. 2012). However, widespread use of monovalent serogroup conjugate vaccines can become ineffective when the capsule types switch due to genetic exchange or strains arise that show reduced capsule expression.

Additional research on meningococcal conjugate vaccines holds great potential for control of meningococcal disease in areas (e.g., sub-Saharan Africa) where epidemics are frequent. A serogroup A conjugate vaccine has been carried out by a nonprofit organization, the Meningitis Vaccine Program, which is supported by the Bill & Melinda Gates Foundation (Borrow 2012; Caugant et al. 2012). The results of the first trials of this vaccine in Africa are promising, and researchers hope that this vaccine shortly will be ready for widespread deployment. A combined pediatric vaccine that contains serogroup A and C meningococcal conjugates has also been tested in Africa (Borrow 2012).

Pneumococcal Capsular Vaccines. Year 2000, a pneumococcal vaccine (PCV7) based on 7 out of the 93 capsular serotypes, associated to a protein in a so-called conjugated vaccine, was introduced in the United States. The 7 capsular types (types 4, 6B, 9V, 14, 18C, 19F, and 23F) were chosen because they were the most prominent causing invasive pneumococcal disease in the United States. The vaccine introduction led to a decrease of invasive pneumococcal disease among infants and children younger than 5 years and also a herd immunity effect in the adult population (Hsu et al. 2009). Here, Hsu et al. showed a decline of pneumococcal meningitis in eight sites in the Unites States between 1998–1999 and 2005 from 1.13 cases to 0.79 cases per 100, 000. In children younger than 2 years of age and in those 65 years of age or older, the incidence of pneumococcal meningitis decreased by 64 % and 54 % respectively during the study period. Furthermore, importantly since pneumococcal carriage is a prerequisite for a pneumococcal invasive disease, a reduction of vaccine type carriage was observed. Use of these protein-conjugated vaccines has also reduced *H. influenzae* type b and pneumococcal meningitis among unvaccinated populations through herd immunity. At present, limitations with PCV7 conjugate vaccines include an apparent increase in the incidence of invasive pneumococcal disease, including meningitis caused by non-PCV7 serotypes, such as serotype 19A (a penicillin and third-generation cephalosporin-resistant non-PCV7 serotype), and an apparent decline in bactericidal antibody against *N. meningitidis* in infants, requiring a booster immunization in the second year of life (Borrow 2012). The conjugated pneumococcal vaccines have now been introduced into the childhood vaccination program in several countries worldwide, and a decrease of invasive disease has been noticed in most countries.

However, recently, it was shown that serotypes not included in the vaccine are increasing as well as certain lineages harboring antibiotic resistance determinants (serotype replacement and serotype shift) (Grijalva and Pelton 2011). An increase has been seen, for example, of serotype 19A causing invasive disease and carrying antibiotic resistance markers creating treatment problems (McGee 2007). In some countries, the decline in invasive disease has been hampered because of an increase of non-vaccine types. Moreover, some studies show that the decline in colonization has been hampered by an increase of non-vaccine-type carriage (Tocheva et al. 2011). Recently, second-generation conjugated vaccines including 10 (PCV10 including also serotypes 1, 5, and 7F) or 13 (PCV13 including in addition types 3, 6A, and 19A) serotypes have been launched.

Outer Membrane Protein Vaccines. The development of vaccines for serogroup B *N. meningitidis* remains a challenge (Borrow 2012). The serogroup B capsule has an identical structure to polysialic structures expressed in fetal neural tissue and does not induce a protective IgG response. Thus, strategies have focused on non-capsular antigens such as outer membrane porins and vesicles and lipooligosaccharides. The diversity of major outer membrane structures in meningococci has, however, limited these approaches (Snape and Pollard 2005; Bjune et al. 1991).

Complement-mediated killing of encapsulated strains is also achieved with cross-reactive antibodies directed against outer membrane components. Developed outer membrane vesicle (OMV) vaccines with a low LOS composition show efficacies of 50–80 % in clinical trials, but do not protect young children and are in general too strain specific; that is, the vaccines can be used against clonal disease outbreaks but not for prevention of sporadic diseases caused by diverse strains. Multivalent vaccine strains based on common variants of PorA (a major inducer and target of bactericidal antibodies) may provide protection against multiple subtypes of *N. meningitidis*. Recently, novel conserved candidate vaccine antigens have been identified using a "reverse vaccinology" approach (Rappuoli 2000; Giuliani et al. 2006; Palumbo et al. 2012). First, the lipoproteins GNA1870 and GNA2132; the conserved surface proteins OMP85, NspA, and NadA; but also PorA, pilin, and LOS conjugates were evaluated for their vaccine potential. The resulting vaccine, referred to as the four-component MenB (4CMenB) vaccine, currently contains the OMV (PorA) from the New Zealand vaccine along with three recombinant proteins identified by reverse vaccinology: factor H-binding protein (fHbp), neisserial adhesin A (NadA), and *Neisseria* heparin-binding antigen (NHBA) (Major et al. 2011).

Chemoprophylaxis

The aim of chemoprophylaxis is to reduce secondary cases of meningococcal and pneumococcal disease and to arrest outbreaks. The risk of a secondary case among close contacts in the household setting is 150–1,000 times higher than that in the general population. Children are at greatest risk, but secondary disease can occur at all ages. Risk is maximal in the week following recognition of the index case but extends for several weeks.

Many antibiotics used for therapy do not effectively eradicate or prevent carriage of meningococci because of inadequate levels in oropharyngeal secretions (Deghmane et al. 2009). Rifampicin, ceftriaxone, azithromycin, and the quinolones are effective against meningococci in the naso- and oropharynx (Stephens et al. 2007). However, rifampicin resistance can develop rapidly, and quinolone resistance in meningococci has recently been reported. Ceftriaxone as a single intramuscular dose is 97 % effective in household contacts 1–2 weeks after infection. The advantage of ceftriaxone is that it can be used in pregnancy and in small children.

Chemoprophylaxis can be helpful in the control of localized outbreaks in residential schools, barracks, etc., but is generally not recommended for the control of epidemics because of cost and drug resistance. For example, widespread distribution of rifampicin would be unwise in communities in which tuberculosis is prevalent. Many meningococcal strains are sulfur resistant (folP mutation), and so sulfur drugs, once highly effective, can no longer be used for chemoprophylaxis. Rifampicin, ceftriaxone, azithromycin, and quinolones all have activity against meningococci in the nasopharynx. However, resistance to rifampicin can develop rapidly, and quinolone resistance in meningococci has been reported (Gorla et al. 2011). Chemoprophylaxis is sometimes recommended for patients given penicillin or chloramphenicol for treatment since pharyngeal carriage may not be eliminated by intravenous administration of these antibiotics and the patient could remain colonized with a virulent strain.

Future Challenges and Opportunities

Bacterial meningitis continues to be an important cause of mortality and morbidity throughout the world, with differential risk for disease among small children, individuals living in low-income countries, and due to infection with antimicrobial- or multidrug-resistant pathogen. Vaccination with protein-conjugated *H. influenzae* type b, *S. pneumococcus* PCV, and *N. meningitidis* Mencevax ACWY has successfully reduced worldwide incidence of meningitis; this raises the hope that other conserved bactericidal epitopes exist and can be identified and exploited in a similar manner. Unfortunately, conjugated vaccines have so far been introduced only in the developed world, even though the highest incidence of meningococcal and pneumococcal invasive diseases occurs in less affluent countries.

Host receptors and signal transduction pathways involved in the microbial invasion of the BBB might represent potential targets for novel therapeutic approaches for meningococcal and pneumococcal disease (Huang and Jong 2009). Using a model system that analyzed penetration of the BBB by *E. coli*, a proof-of-concept study suggested that the HBMEC receptor for CNF1 (RPSA) and cytosolic phospholipase A2α might represent such "druggable" targets, at least for *E. coli*, but potentially also for meningococci and pneumococci

(Plant et al. 2006; Orihuela et al. 2009). Other studies suggest that the cell-wall component lipoteichoic acid is a pattern recognition molecule and inflammatory mediator that plays a role in pneumococcal disease; however, the identity of the receptor for lipoteichoic acid remains controversial. Interestingly, intrathecal treatment with antibodies that recognize phosphorylcholine, teichoic acid, and lipoteichoic acid was effective in reducing neuronal damage in experimental pneumococcal meningitis (Gerber et al. 2012).

Basic understanding of the molecular mechanisms of meningococcal and pneumococcal pathogenesis is still lacking and is urgently needed to support advances on novel therapeutic and preventive approaches for meningitis. Such basic research will enhance our understanding of bacterial emergence, pathogenic genome structure, horizontal genetic exchange, and innate and adaptive immune responses. *N. meningitidis* and *S. pneumoniae* are valuable low-complexity model organisms for such studies, at least in part because they only colonize and infect human beings. Further studies of the genetics and pathogenicity of *N. meningitidis* and *S. pneumoniae* should reveal much about how they evolved, spread worldwide, and how and why they cause disease only in humans. This research is important in the quest to define fundamental mechanisms of microbial pathogenesis and to facilitate design of novel strategies for managing emerging or reemerging microbial threats.

In summary, earlier clinical recognition and more effective treatment of meningococcal and pneumococcal disease will be critical to further reduce morbidity and mortality associated with these diseases. Improved understanding of the pathophysiology of infection by meningitis-causing bacteria will undoubtedly lead to innovative new approaches for the management also of patients with meningococcal and pneumococcal septicemia, which could further reduce case fatality and/or case morbidity. The ultimate control of meningococcal and pneumococcal disease will require widespread and expanded use of effective vaccines. Worldwide surveillance, the expanded use of polysaccharide-conjugate vaccines, and the development of broadly effective serogroup B vaccines could eliminate *N. meningitidis* and *S. pneumoniae* as major threats to human health in industrialized countries within the next decade. However, strong, sustained, and coordinated support will be needed from the international community, if meningococcal and pneumococcal disease is to be controlled in Africa and other developing areas, where the infection poses the greatest persistent threat.

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19 Rickettsiia Diseases

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Abstract
The genus *Rickettsia* includes lethal pathogens such as *R. rickettsii*, the etiologic agent of Rocky Mountain spotted fever, and *R. prowazekii*, the etiologic agent of epidemic typhus. All the members of this genus are obligately intracellular bacteria, and those that are pathogenic to humans preferentially target the vascular endothelium and are transmitted by arthropod vectors. The diseases they cause are systemic and difficult to diagnose because of initial nonspecific signs and symptoms, and the lack of appropriate diagnostic tests. These small bacteria have a gram-negative wall structure and have lost many genes that became unnecessary once they evolved to an intracellular lifestyle. Much remains to be learned about these fascinating intracellular parasites. Factors that have limited their investigation include the required biosafety level 3 to work with most of the pathogenic rickettsiae, the lack of tools for genetic manipulation, and their intracellular nature, which adds a layer of complexity for experimental work.

Introduction

Rickettsia are notable bacteria because they are the etiologic agents of two of the most lethal infections known to man, namely Rocky Mountain spotted fever (*Rickettsia rickettsii*) and epidemic typhus (*R. prowazekii*). Furthermore, one of them, epidemic typhus, has shaped history due to the massive epidemics that it produced during times of war until World War I (Raoult et al. 2004; Zinsser 2007). Both agents are select agents because of their potential use as bioweapons (Azad 2007). On the other hand, several new pathogenic *Rickettsia* have been discovered in the last few decades; new rickettsioses are certainly emerging and old rickettsioses are reemerging (Parola et al. 2005).

Members of the genus *Rickettsia* (family *Rickettsiaceae*, order *Rickettsiales*) are α-proteobacteria that share the following general characteristics:

- They have closely related A/T-rich small genomes, a consequence of evolutionary loss of genes encoding proteins that participate in various biosynthetic pathways (Fuxelius et al. 2007; Darby et al. 2007; Blanc et al. 2007b).
- They can only survive in the cytoplasm of eukaryotic cells where they obtain needed metabolic substrates that they cannot synthetize themselves. Thus, they are strict obligate intracellular parasites.
- Most of the well-known rickettsiae reside within arthropods. Indeed, hematophagous insects and ticks transmit rickettsiae that are pathogenic to humans and other vertebrates (they are zoonoses). However, new *Rickettsia* or their DNA has been identified in amoebas, ciliates, leeches, hydrozoa, and environmental samples with unknown host (Weinert et al. 2009b).
- In humans, rickettsiae preferentially target endothelial cells (*Fig. 19.1*), the cells that line vascular and lymphatic vessels (except for *Rickettsia akari*, the agent of rickettsialpox, which specially targets monocytes and macrophages). This is a characteristic shared only by two other bacteria, *Orientia tsutsugamushi* (the other member of the family *Rickettsiaceae*) and *Ehrlichia ruminantium* (family *Anaplasmataceae*, order *Rickettsiales*) (Valbuena and Walker 2009).

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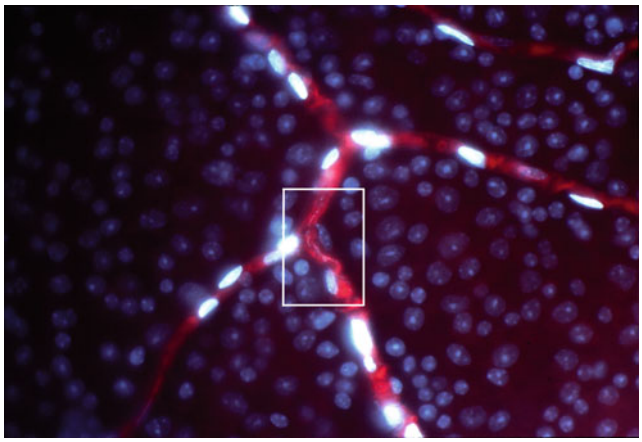

Fig. 19.1
Wide-field fluorescence microscopy of a whole-mount retinal preparation from a C3H/HeN mouse infected with *Rickettsia conorii* 4 days earlier (original magnification: 400×). The mouse was injected with Evans blue (*red fluorescence*) to highlight the intraluminal content of microvessels, and Hoechst 33258 (*white-blue fluorescence*) to highlight DNA in host nuclei and rickettsiae (seen as *small white dots* inside the *white box*)

History

The transmission of *Rickettsia* by hematophagous arthropod vectors was established early in the twentieth century, a decade before the nature of the agent was first suggested. In 1906, in two almost simultaneous but separate reports, WW King (King 1906) and HT Ricketts (Ricketts 1906) described their experiments with guinea pigs in which they demonstrated that ticks transmit Rocky Mountain spotted fever (RMSF). At the time, Ricketts and others recognized that the clinical presentation of Rocky Mountain spotted fever closely resembled that of epidemic typhus; however, it was not yet known that closely related organisms caused the two diseases. What was clear then was that the human body louse was the vector of typhus (Gross 1996). Charles Nicolle received the 1928 Nobel Prize for this discovery.

H. Plotz, a pathologist from New York, reported in 1914 the identification of a gram-positive bacillus in the blood of patients with typhus as well as their lice (Plotz 1914). H. da Rocha-Lima confirmed these findings in 1916 (da Rocha-Lima 1968); he named the organism *Rickettsia prowazekii* in honor of Ricketts and Stanislaus von Prowazek, both of whom died of typhus acquired in the course of their investigations.

In 1916, SB Wolbach, another pathologist, studied samples from guinea pigs with Rocky Mountain spotted fever and identified very small gram-negative organisms in vascular vessels (Wolbach 1916a, b). Subsequently, in 1917, he confirmed this finding as well as the vascular nature of the infection in autopsies of human patients with Rocky Mountain spotted fever (Wolbach 1917). However, the observation of the parasites in the nuclei of tick cells made him propose that the organism was not bacterial; he introduced the name *Dermacentroxenus rickettsii* for a parasite that he thought was intermediate between bacteria and protozoa (Wolbach 1919). We now know that the presence of spotted fever group (SFG) rickettsiae in the nucleus of host cells (Burgdorfer et al. 1968) is due to their ability move directionally using host cell actin polymerization. The integration into a single genus, *Rickettsia*, would not be proposed until 1943 (Philip 1943).

By the early 1920s, there was still no agreement about the nature of the etiologic agents of typhus and the spotted fevers because of the inconsistent staining with the Gram method and because the organism could not be cultivated. The discovery by E. Weil and A. Felix that sera from typhus patients agglutinate *Proteus* (particularly the strain OX-19) did not help (Wilson 1922; Sievers 1945). The inability to cultivate the organism and its filterability suggested to many that the group of typhus-like diseases were caused by "filterable viruses." Of course, the modern concept of viruses only began to form after WM Stanley's description of the tobacco mosaic virus in 1935.

The difficulties in characterizing these intracellular bacteria contributed to much confusion during the first half of the twentieth century. By the late 1960s and early 1970s, a more modern conception began to be synthetized (Ormsbee 1969; Weiss 1973).

Clinical Presentation and Treatment

Rickettsioses are systemic febrile diseases that affect individuals of any age independently of their immune status (Centers for Disease Control and Prevention (CDC) 2004; Paddock et al. 1999, 2002; Lee et al. 2008, 2009). Although the pathogenetic mechanisms are shared, not all rickettsioses are equally severe, which is explained by differences in virulence of the individual species- and vector-related factors (*Table 19.1*).

The main target cells of most *Rickettsia*, with the exception of *R. akari*, are endothelial cells, the cells that line all vascular vessels in the body (*Fig. 19.2*). These cells have important regulatory functions in angiogenesis, hemostasis, permeability and solute exchange, vascular tone, and inflammation (Michiels September 2003; Danese et al. 2007; Pober et al. 2009). Thus, their targeting by rickettsiae explains many of the clinical features of the diseases including systemic involvement and leakage of intravascular fluid. Rickettsial infection of endothelial cells induces cellular damage leading to detachment. Those infected endothelial cells circulate in the blood (George et al. 1993; La Scola and Raoult 1996) and are likely to be the source of new foci of infection once they lodge in distal capillaries.

Several mechanisms are likely to contribute to the increased vascular permeability observed in clinical cases. These include production of vasoactive prostaglandins as a consequence of increased expression of COX-2 (Rydkina et al. 2006), endothelial production of nitric oxide (Woods et al. 2005), effects of inflammatory cells and their mediators (Woods and Olano 2008), and endothelial detachment and denudation of vessels. Such damage may be caused by phospholipase activity (Walker et al. 1983),

Table 19.1
Confirmed pathogenic rickettsiae

Rickettsiae		Vector	Disease
Typhus group	*R. prowazekii*	Human body louse	Epidemic typhus and Brill-Zinsser disease
	R. typhi	Fleas	Murine or endemic typhus
Spotted fever group	*R. rickettsii*	Ticks	Rocky Mountain spotted fever, Brazilian spotted fever
	R. conorii	Ticks	Mediterranean spotted fever, Israel tick typhus, Astrakham fever, Indian tick typhus
	R. sibirica	Ticks	Siberian tick typhus
	R. heilongjiangensis	Ticks	Far-eastern spotted fever
	R. japonica	Ticks	Japanese spotted fever
	R. honei	Ticks	Flinders Island spotted fever
	R. marmionii	Ticks	Australian spotted fever
	R. slovaca	Ticks	Tick-borne lymphadenopathy (TIBOLA) and Dermacentor-borne-necrosis-erythema-lymphadenopathy (DEBONEL)
	R. parkeri	Ticks	Spotted fever with eschar and lymphadenitis
	R. massiliae	Ticks	Spotted fever
	R. africae	Ticks	African tick bite fever
	R. raoultii	Ticks	Spotted fever similar to TIBOLA/DEBONEL
Transitional group	*R. australis*	Ticks	Queensland tick typhus
	R. felis	Fleas	Flea-borne spotted fever
	R. akari	Mites	Rickettsialpox

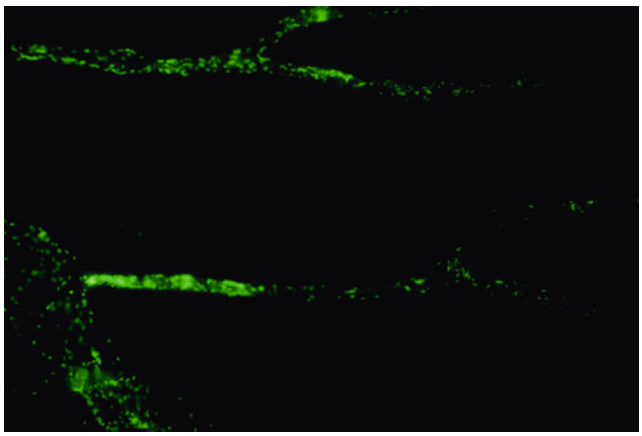

Fig. 19.2
Wide-field fluorescence microscopy of a whole-mount retinal preparation from a C3H/HeN mouse infected with *Rickettsia conorii* 5 days earlier (original magnification: 400×). The retina was stained with a rabbit polyclonal serum against *R. conorii* and a secondary goat antirabbit IgG labeled with Alexa 488. The specific staining of rickettsiae highlights the microanatomy of retinal microvessels, thus confirming their preferential endothelial localization

mechanical damage to the membrane caused by exiting rickettsiae under actin propulsion (Walker and Cain 1980), or lipid peroxidation of the cell membrane (Silverman 1984; Silverman and Santucci 1988; Hong et al. 1998; Devamanoharan et al. 1994). The most severe clinical presentations are a consequence of endothelial damage in the lungs (*Fig. 19.3*) and brain and include noncardiogenic pulmonary edema, interstitial pneumonia, adult respiratory distress syndrome, meningoencephalitis (*Fig. 19.4*), seizures, and coma (Walker et al. 2003; Rizzo et al. 2004; Bechah et al. 2008; Chen and Sexton 2008; Demeester et al. 2010); involvement of these organs explains the majority of the mortality, which is observed particularly with Rocky Mountain spotted fever and epidemic typhus (the reported mortality without antibiotics ranges from 10 % to 60 %). However, it should be emphasized that reliance on serological methods for diagnostic confirmation may lead to underestimation the actual case-fatality rate. This was well illustrated in a recent report of nine fatal cases with negative serological results that were confirmed by immunohistochemical demonstration of the antigen in tissues (Paddock et al. 1999). At the other end of the clinical spectrum are several rickettsioses; murine typhus, with a mortality of less than 2 %, is the most important of them because of its global distribution (Civen and Ngo 2008).

Although multiple coagulation abnormalities have been described during the course of clinical and experimental rickettsioses (Sahni 2007), disseminated intravascular coagulation occurs only rarely in lethal cases and is not a common feature of rickettsioses (Schmaier et al. 2001).

The cells that are infected immediately after inoculation have not been identified. Many of the rickettsiae that result in less

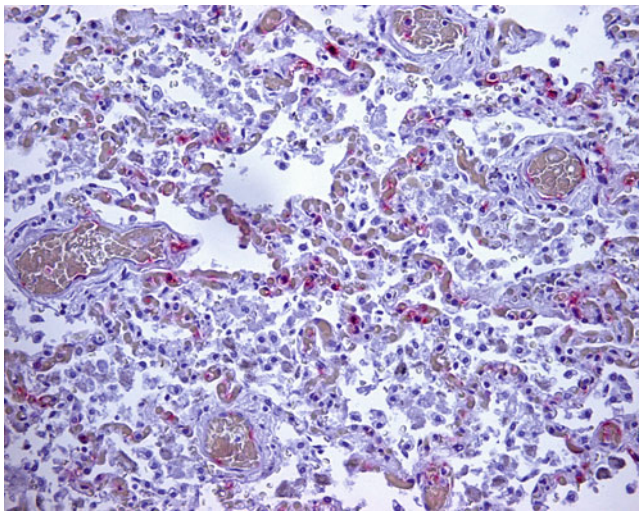

Fig. 19.3
Immunohistochemical analysis of a lung section from a fatal human case of Rocky Mountain spotted fever (original magnification: 200×). The section was stained with a rabbit polyclonal serum against spotted fever group rickettsiae and a goat antirabbit IgG labeled with alkaline phosphatase. The product of the enzymatic reaction shows the rickettsiae in red. The section was counterstained with hematoxylin. Rickettsiae can be observed in microvessels as well as the endothelium of larger vessels

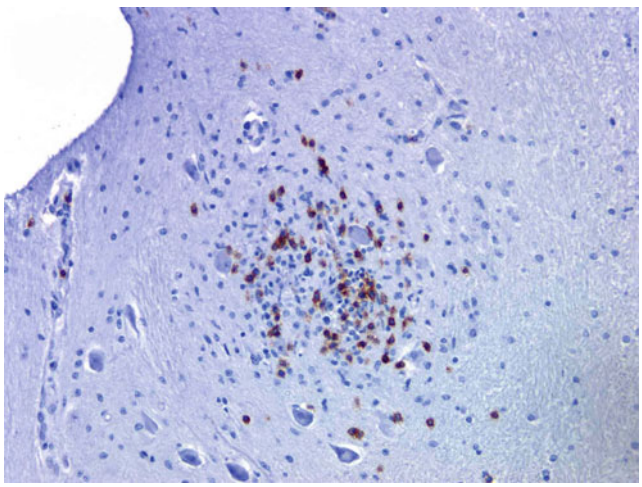

Fig. 19.4
Immunohistochemical analysis of a brain section from a fatal human case of Rocky Mountain spotted fever (original magnification: 200×). The section was stained with a mouse antihuman CD8 monoclonal antibody and donkey antimouse IgG labeled with horseradish peroxidase. The product of the enzymatic reaction shows the $CD8^+$ T cells in *brown*; the *blue* contrast is given by hematoxylin. Notice the nodular infiltration by other leukocytes in addition to $CD8^+$ T cells (this lesion is known as typhus nodule)

severe disease also produce an eschar (area of necrosis with a rich inflammatory infiltrate and local rickettsial proliferation) at the bite site (Walker et al. 1988). When an eschar is present, another frequent clinical finding is local lymphadenitis, suggesting initial spread through lymphatics. Rocky Mountain spotted fever, the most severe of the spotted fever rickettsioses, does not manifest with an eschar or local lymphadenitis. This could be due to a more rapid hematogenous dissemination; however, whether clinical outcomes are determined by the initial route of dissemination (lymphatic vs. vascular) or not remains to be tested experimentally.

The recommended antibiotic treatment for all rickettsioses is doxycycline (Chapman et al. 2006). This antibiotic has the advantage of covering other tick-borne bacterial infections. Rickettsiae are resistant to many antibiotics (Rolain et al. 1998). Other antibiotics, including chloramphenicol and fluoroquinolones may be effective, although there is evidence that they may have deleterious effects (Holman et al. 2001; Botelho-Nevers et al. 2011). A molecular explanation for this observation was presented for chloramphenicol. *Rickettsia* of the spotted fever group (SFG), unlike the two *Rickettsia* of the typhus group (*R. typhi* and *R. prowazekii*), induce early apoptotic death of host cells (with marked downregulation of host *bcl2*) when treated with this antibiotic. This effect appears to be due to the increased expression of the rickettsial toxin VapC (Audoly et al. 2011).

The antibiotic resistance of *Rickettsia* combined with the nonspecific initial clinical presentation and lack of commercially available laboratory tests for confirmatory diagnosis during the acute presentation, lead to delayed diagnosis and inappropriate treatment; the consequence is excessive mortality (Holman et al. 2001). The clinical presentation can indeed be confusing and some assumptions are misleading (Masters et al. 2003). For example, not all patients present with rash and few refer a history of tick bite (the tick bite is not painful and ticks tend to bite in places of the body that are not very visible). Also, in the Americas, including the United States, RMSF can present during any season and in any geographical location. The best recommendation is to treat with doxycycline on clinical suspicion.

Epidemiology

The epidemiology of rickettsioses is determined by the complex interactions between the arthropod hosts, the environment, amplifying hosts, and humans that invade the ecological niches where the arthropod hosts exist. In some cases pets, particularly dogs, can bring tick vectors into the human habitat. This is true in the case of *Dermacentor variabilis* and *Rhipicephalus sanguineus* (Dumler and Walker 2005).

In temperate regions of the globe, the seasonality of SFG rickettsioses is explained by the activity of the tick vectors, particularly the adults, which are more active during the spring and early summer. There is also a periodicity in a timeframe of decades that has not been appropriately explained yet. It is

possible that climate change may affect the behavior of tick vectors (Parola et al. 2008). One of the recent peaks of reporting of Rocky Mountain spotted fever (RMSF) occurred during the early 2000s (Openshaw et al. 2010). This may be related to increased disease activity but also to renewed interest not only in the United States but also throughout the Americas (RMSF occurs only in the Americas). The disease has now been documented in almost all countries of Latin America (Peacock et al. 1971; Ripoll et al. 1999; Zavala-Velazquez et al. 1999; de Lemos et al. 2001; Blair et al. 2004; Hidalgo et al. 2007; Paddock et al. 2008b; Zavala-Castro et al. 2008). Even more importantly, new SFG rickettsioses have been discovered. For instance, *R. parkeri*, which was considered a nonpathogenic *Rickettsia* for a very long time, was recently shown to produce a mild spotted fever with an eschar and local lymphadenopathy (Paddock et al. 2004, 2008a; Paddock 2005; Whitman et al. 2007). Other recently described *Rickettsia* associated with eschars and relatively mild disease include *Rickettsia* 364D (Shapiro et al. 2010) and *R. massiliae* (Vitale et al. 2006; García-García et al. 2010).

One of the consequences of the nonspecific initial febrile syndrome and the lack of commercially available diagnostic methods that are sensitive and specific during the acute presentation of the rickettsioses is that the disease is frequently underreported (Paddock et al. 2002) and diagnosed as a viral illness. In Latin America, the umbrella diagnosis of dengue is frequently applied to cases of rickettsioses (Zavala-Velazquez et al. 1996).

Ultimately, humans are only accidental hosts. The exception is epidemic typhus since it is transmitted by the human body louse, which parasitizes only humans. Epidemic typhus is also the only known rickettsiosis that can present in a recrudescent form years after the primary infection (Zinsser and Castaneda 1933); this form of the infection is called Brill-Zinsser disease and is less severe than acute primary epidemic typhus. Recrudescent epidemic typhus is a potential source of new epidemics if it occurs at a place and time with deteriorated living and hygiene conditions that would favor the presence of the human body louse. The mechanisms of persistence and reactivation are not understood; recent experimental evidence from a mouse model suggests that adipose tissue may provide a reservoir where dormant rickettsiae may reside (Bechah et al. 2010a).

Phylogeny and Taxonomy

The genus *Rickettsia* includes the bacteria most closely related to mitochondria (Andersson et al. 1998; Kitada et al. 2007). The genus began its separate evolution approximately 150 million years ago (Weinert et al. 2009b). It is estimated that the transition to the intracellular life style led to the loss of 2,135 genes from the 3,000 to 4,000 genes calculated to be present in the α-proteobacterial ancestor (Georgiades et al. 2011). Of the currently sequenced rickettsiae, *Rickettsia bellii* (1,395 genes) does not appear to have lost genes after the initial adaptation of the proteorickettsiae and, in fact, it has genes that may have been horizontally transferred from protists; this is consistent with the idea that amoebae might have been an early host of the proteorickettsiae (Blanc et al. 2007b). On the opposite end of the spectrum, *R. prowazekii* and *R. typhi* lost the largest number of genes; they currently have around 830 genes. As a consequence, rickettsial genomes have a high content of noncoding DNA (Andersson and Andersson 2001).

The recent comparison of the genomes of two strains of *R. rickettsii*, one pathogenic (Sheila Smith) and the other nonpathogenic (Iowa), provides a clear snapshot of the process of evolution by genomic reduction (Ellison et al. 2008). For instance, there is a ~10 kb deletion in *R. rickettsii* Sheila Smith that includes several degraded genes in *R. rickettsii* Iowa. Some of the these genes appear to be involved in biosynthetic pathways that evidently are not needed for survival in arthropod or vertebrate hosts. *Rickettsia* has a larger proportion of noncoding DNA than most known bacteria; 24 % of the genome in the case of *R. prowazekii* (Andersson et al. 1998). Many pseudogenes are present in the noncoding DNA. There are also areas consisting of consecutive ORFs with internal stop codons. Since there is some evidence that at least some of them are actually expressed, they are called split genes (Ogata et al. 2001).

It has been suggested that one critical event for the adaptation to the intracellular environment was the acquisition of a type IV secretion system (Gillespie et al. 2010a), although we do not know anything about the substrates for transport. The current version in most rickettsiae lacks the *virB5* component (which would encode a T-pilus-like structure), has duplications of other *virB* genes and is fragmented across the genome instead of being an operon (Gillespie et al. 2009b). The pilus structure is probably not needed because rickettsiae are intracytoplasmic and do not need to secrete components across a membrane. Another possibly important element for the evolution of *Rickettsia* is the presence of conjugation genes (Weinert et al. 2009b). However, they do not play a role in pathogenicity since they are present only in nonpathogenic rickettsiae. Finally, several *Rickettsia* share the same arthropod host; this shared location allows sympatric evolution through horizontal gene transfer. Evidence of this process was obtained from the analysis of two rickettsiae found in fleas, *R. felis* and *R. typhi*; *R. felis* has formed chimeric genes after recombining genetic material received from *R. typhi* (Merhej et al. 2011).

At the present moment, there are 22 entries for *Rickettsia* genomes in the database of NCBI. They are *R. rickettsii*, *R. prowazekii* (Andersson et al. 1998), *R. conorii* (Ogata et al. 2001), *R. typhi*, *R. massiliae*, *R. canadensis*, *R. slovaca*, *R. bellii*, *R. africae*, *R. sibirica*, *R. peacockii*, *R. akari*, *R. felis*, *R. montanensis*, *R. rhipicephali*, *R. australis*, *R. parkeri*, *R. philipii*, *R. japonica*, *R. heilongjiangensis*, *Candidatus Rickettsia amblyommii*, and *Rickettsia endosymbiont of Ixodes scapularis*. Based on the analysis of a subset of these data (Gillespie et al. 2007, 2008), new phylogenetic relationships were proposed. Accordingly, there are four groups: (1) the nonpathogenic ancestral group (*R. bellii* and *R. canadensis*), which diverged earlier; (2) typhus group (*R. typhi* and *R. prowazekii*); (3) spotted fever

group (*R. rickettsii, R. parkeri, R. conorii*, and several others); and (4) transitional group (*R. akari, R. australis*, and *R. felis*). A more recent analysis proposes to split the ancestral group in two with one *Rickettsia* in each group (i.e., *R. bellii* and *R. canadensis*) and to include the transitional group within the spotted fever group (SFG) (Merhej and Raoult 2011). According to this new scheme, the SFG group is divided in four subgroups: (1) the *R. rickettsii* subgroup (*R. rickettsii*, *R. conorii*, *R. africae*, *R. parkeri*, *R. sibirica*, *R. slovaca*, *R. honei*, *R. japonica*, *R. heilongjiangensis*, and a few others), (2) *R. massiliae* subgroup (*R. massiliae*, *R. montanensis*, *R. aeschlimannii* and *R. rhipicephali*, *R. raoultii*, and others), (3) *R. helvetica* subgroup (*R. helvetica, R. asiatica, R. tamurae, R. monacensis*), and (4) *R. akari* subgroup (*R. akari, R. australis, and R. felis*). A phenotypic characteristic of the *R. rickettsii* subgroup is its susceptibility to rifampin, while the *R. massiliae* subgroup is resistant to this antibiotic (Blanc et al. 2007a). For a long time, the serological response was the main criterion used to classify rickettsiae in only two groups (Philip et al. 1978; Ormsbee et al. 1978), spotted fever and typhus; using those criteria, *R. canadensis* was included in the typhus group at that time. Also, until 1995 (Tamura et al. 1995), *Orientia tsutsugamushi*, the etiologic agent of scrub typhus, was included in the genus *Rickettsia* (i.e., *Rickettsia tsutsugamushi*) and considered a third group.

Based on early genomic information from the most pathogenic rickettsiae, plasmids were not thought to be present in *Rickettsia*. However, the sequence of *R. felis* provided the first evidence of the presence of plasmids (some conjugative) as well as transposases (Ogata et al. 2005), both drivers of the important evolutionary event of horizontal gene transfer (Gillespie et al. 2007). Since then, many other low-copy-number plasmids have been identified in several members of the genus *Rickettsia* in all of the current phylogenetic groups except for the typhus group (Blanc et al. 2007b; Baldridge et al. 2008, 2010; Fournier et al. 2009).

Structure

The rickettsiae divide by transverse binary fission (Weiss 1973). They are some of the smallest known bacteria (0.3–0.5 μm in width and 0.7–2 μm in length). Their wall structure is that of gram-negatives (Wood and Wisseman 1967; Perkins and Allison 1963; Anacker et al. 1967; Silverman and Wisseman 1978; Pang and Winkler 1994). However, note that they will not stain with the Gram stain. One stain that works consistently well for *Rickettsia* is the Gimenez stain (Gimenez 1964). Rickettsiae produce peptidoglycan and a minimally endotoxic lipopolysaccharide (LPS) (Schramek et al. 1977). The rickettsial LPS cross-reacts with LPS from *Proteus*, and this probably is the basis for the Weil-Felix test (Amano et al. 1993). Outside the outer envelope, there is a microcapsular layer and a slime layer that requires special handling, fixation, and staining for its demonstration (Silverman et al. 1978). Based on its staining with ruthenium red and silver methenamine, it was thought to consist of polysaccharides.

Vectors

Insects are the vectors of typhus group rickettsiae. *Rickettsia prowazekii* is transmitted in the excrement of the human body louse (*Pediculus humanus corporis*), which is autoinoculated into small open wounds created by scratching. *R. prowazekii* is not transmitted through direct inoculation into the infected person's bloodstream because the salivary glands of lice do not become infected. *Rickettsia prowazekii* grows in the midgut epithelial cells of the human body louse until they burst (Silverman et al. 1974). The rickettsiae are eventually excreted in the feces but the process is ultimately fatal for the lice, which die within two weeks after the initial infection (Fuller et al. 1949). For a long time, *R. prowazekii* was believed to be confined to the louse and the human host until its presence was documented in flying squirrels (*Glaucomys volans*) and their ectoparasites (lice and fleas) in North America (Bozeman et al. 1975; Sonenshine et al. 1978; McDade et al. 1980; Duma et al. 1981). Even ticks have been suggested as a natural niche for *R. prowazekii*, although the significance of this finding has not been addressed further (Medina-Sanchez et al. 2005). Lice become infected with *R. prowazekii* after feeding on persons suffering from epidemic typhus. Lice feed four to six times a day, resting in between feedings on parts of clothing that are not in direct contact with the skin where temperature is closer to the ideal 20 °C. Once the afflicted person develops fever, the body louse seeks another host (Gillespie et al. 2009b).

The other typhus group *Rickettsia*, *R. typhi*, exists in nature in an enzootic cycle involving rodents and their parasitic fleas and lice. *R. typhi* is transmitted to humans mainly by the excrement of the oriental rat flea (*Xenopsylla cheopis*), although other species of fleas, as well as lice and mites, have been implicated (Traub and Wisseman 1978; Azad 1988). Murine typhus is transmitted to humans by inoculation of infected flea feces into skin abraded by scratching; however, transmission via the bite of *X. cheopis* has been documented under experimental conditions (Azad and Traub 1985). Fleas acquire *R. typhi* upon feeding on an infected host (rats, opossums, and other small mammals), and it remains there for the life of the flea without affecting its survival. *R. typhi* proliferates almost exclusively in the epithelial cells of the flea's gut and then is excreted in the feces. Nevertheless, there is experimental evidence of transovarial transmission (Farhang-Azad et al. 1985).

Spotted fever group (SFG) rickettsiae are transmitted by the bite of any developmental stage (larva, nymph, and adult) of hard ticks (family *Ixodidae*), particularly the genera *Amblyomma*, *Dermacentor*, and *Rhipicephalus*. SFG *Rickettsia* can be maintained by transovarial (adult female to egg) and transstadial (egg to larva to nymph to adult) transmission because the ovaries become infected (Burgdorfer and Brinton 1975). Ticks puncture the skin with their chelicerae and anchor themselves using the hypostome, which is then reinforced though salivary secretion of cement. The chelicerae rupture superficial small vessels in order to create a small pool of blood, a cavity from which female ticks feed for several days. *R. akari* is the only SFG *Rickettsia* not transmitted by ticks. It is

transmitted by the house mouse mite *Liponyssoides sanguineus* (Huebner et al. 1946).

In the United States, *R. rickettsii* is found in the wood tick, *Dermacentor andersoni* (mainly in the Rocky Mountain region), the American dog tick, *D. variabilis* (eastern and southern regions), and *Rhipicephalus sanguineus* (southwest region) (Demma et al. 2005). In Latin America, *Amblyomma cajennense* and other species of *Amblyomma* are the main vectors of RMSF (Labruna 2009). Like other SFG *Rickettsia, R. rickettsii* can be maintained in nature through transovarial transmission in addition to horizontal acquisition from infected hosts. However, there is evidence that the infection can reduce the reproductive fitness of *R. rickettsii*-infected female ticks, even causing death in some cases because of excessive rickettsial growth during feeding (Burgdorfer and Brinton 1975; Niebylski et al. 1999). This phenomenon might explain the observation that only less than 0.1 % of ticks are infected with *R. rickettsii* in nature. Further contributing to this statistic is the observation that nonpathogenic SFG *Rickettsia* prevents transovarial transmission of *R. rickettsii* (Socolovschi et al. 2009).

Life Cycle and Physiology

The entry of *Rickettsia* into host cells is an active process that requires energy from both the host and the rickettsiae (Walker and Winkler 1978). There is evidence that rickettsiae use surface cell antigen 0 (sca0 or rOmpA) (Li and Walker 1998) and sca 1 (Riley et al. 2010) to attach to target cells (these and the other rickettsial sca proteins are autotransporters). Subsequent to attachment, which is mostly a passive process, endocytosis of Rickettsia is actively triggered when the rickettsial outer membrane protein B (rOmpB or sca5) binds to the host cell membrane form of Ku70 (Martinez et al. 2005). Since blocking of this interaction only inhibits about 50 % of rickettsial entry, other ligands and receptors must be present; sca2 (Cardwell and Martinez 2009) and adr2 (Vellaiswamy et al. 2011) appear to be some of those bacterial ligands.

The necessary cytoskeletal rearrangements that produce the zipper-like entry mechanism of *Rickettsia spp.* involve multiple host pathways that activate the Arp2/3 complex (Martinez and Cossart 2004) with the participation of Cdc42, cofilin, c-Cbl, clathrin, and caveolin 2 (Chan et al. 2009). *Rickettsia* may also enter phagocytic cells such as monocytes and macrophages (which are a secondary target of most *Rickettsia*) by antibody-mediated opsonization (Feng et al. 2004). In regard to entry of rickettsia to arthropod cells, all that has been published is that histone H2B interacts with *R. felis* sca5 (Thepparit et al. 2010).

Within a short period of time after endocytosis, rickettsia escapes into the cytosol. The rickettsial genes *pld*, which encodes an enzyme with phospholipase D activity (Renesto et al. 2003), and *tlyc*, which encodes a hemolysin (Radulovic et al. 1999), are believed to be effectors of this function. This conclusion is based on the ability of the normally vacuolar *Salmonella enterica* to escape into the cytosol when it expresses rickettsial *tlyc* or *pld* (Whitworth et al. 2005). In addition, rickettsial proteins with phospholipase A activity were confirmed (Housley et al. 2011; Rahman et al. 2010) but only in the typhus group *Rickettsia*. That activity underlies the phenomenon of hemolysis produced by these rickettsiae in vitro (Clarke and Fox 1948; Ramm and Winkler 1973).

Once *Rickettsia* escapes the phagocytic vacuole, it acquires multiple metabolic substrates from the host cytoplasm. The availability of those substrates allowed genome reduction through loss of many genes including, among many others, those for nucleotide synthesis and enzymes for sugar metabolism (Walker and Yu 2005). Multiple transporters of substrates from the host cytoplasm, including ATP (Winkler 1976), compensated for these gene losses (McLeod et al. 2004). The mechanisms of transport are active and include the use of the transmembrane electrical potential (Zahorchak and Winkler 1983).

Typhus group *Rickettsia* grow until they burst the host cell (Wisseman et al. 1976b) while spotted fever group *Rickettsia* rapidly spread from cell to cell (Wisseman et al. 1976a) due to their actin propulsion (*Figs. 19.5* and *19.6*). Of course, host cells are damaged in the process (Silverman 1984); the mechanisms may involve the production of free radicals (Silverman and Santucci 1988; Eremeeva and Silverman 1998) and phospholipase activity (Walker et al. 1983). On the other hand, there is experimental evidence that rickettsiae can maintain their cellular niche through inhibition of apoptosis (Bechelli et al. 2009) and that pathogenic *Rickettsia* can inhibit autophagy (Uchiyama et al. 2011).

The spotted fever group (SFG) *Rickettsia* differ from the typhus group *Rickettsia* in a lipopolysaccharide (LPS) that contains antigens specific for each group (Vishwanath 1991), the absence of the rickettsial outer membrane protein OmpA (Sca0) in typhus group rickettsiae, and their capacity to stimulate host cell actin polymerization for directional cell-to-cell movement (Heinzen et al. 1993). *R. prowazekii* does not show any evidence of actin polymerization; however, *R. typhi* does produce short actin tails that result in nondirectional movement.

Most rickettsiae of the spotted fever group polymerize actin and move directionally in the host cell cytoplasm (Schaechter et al. 1957; Teysseire et al. 1992; Heinzen et al., 1993). The only known SFG *Rickettsia* that does not polymerize actin is the nonpathogenic *R. peacockii*; this defect may be partly explained by the presence of an insertion element disrupting the sequence of *rickA* (Simser et al. 2005). Unlike the tails of other actin-polymerizing bacteria (including *Listeria monocytogenes, Shigella flexneri, Mycobacterium marinum*, and *Burkholderia pseudomallei*), those of *Rickettsia* consist of long unbranched parallel bundles (similar to those of filopodia) instead of branched filaments (like the other actin-polymerizing bacteria). The difference might be partly explained by the unique need for host profilin and fimbrin/T-plastin for formation of rickettsial actin tails (Serio et al. 2010). A screening with transposon-mediated mutagenesis identified rickettsial sca2 as an important protein involved in actin polymerization since its inactivation completely disrupted the formation of actin tails (Kleba et al. 2010). Sca2 has an intrinsic actin nucleating activity that, unlike

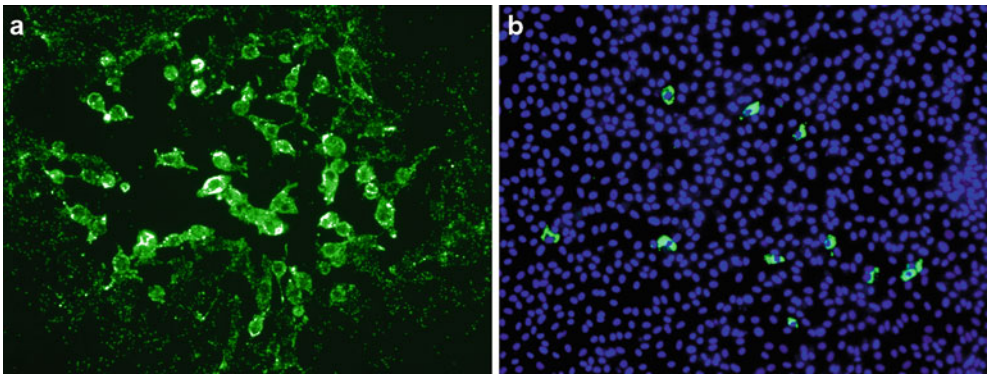

Fig. 19.5
Immunofluorescence analysis of monolayers of Vero cells infected with *Rickettsia conorii* (a) or *Rickettsia typhi* (b). The original magnification is 400× for (a) and (b). The rickettsial antigens were detected with group-specific rabbit polyclonal antisera and goat antirabbit IgG labeled with Alexa 488. Nuclei were stained with DAPI in (b). Notice rickettsial spreading away from heavily infected cells in the center of (a) in contrast to the pattern observed in typhus group rickettsiae (b), which do not polymerize actin in a directional manner (typhus group rickettsiae grow inside cells until they burst)

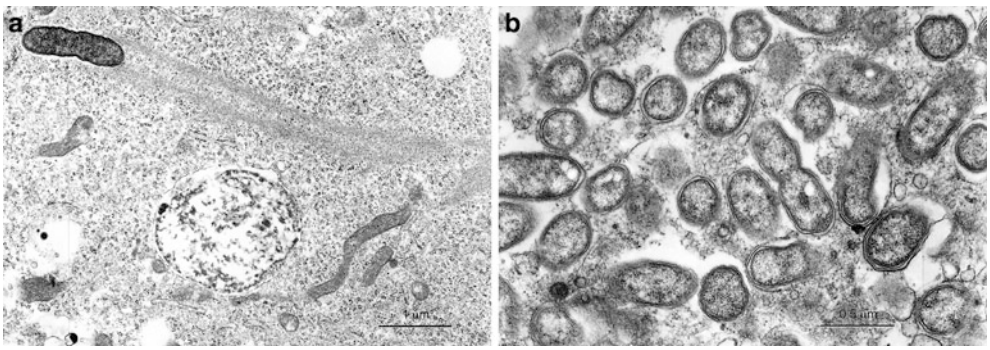

Fig. 19.6
Transmission electron microscopy of Vero cells infected with *Rickettsia rickettsii* (a) or *R. prowazekii* (b). Notice the characteristic actin tails of spotted fever group rickettsiae in (a) and their absence in (b) Courtesy of V. Popov. University of Texas Medical Branch

rickA (a nucleation-promoting factor identified earlier) (Jeng et al. 2004; Gouin et al. 2004), is independent of the host Arp2/3 complex. The nucleation activity is mediated by functioning in a manner analogous to eukaryotic formin (Haglund et al. 2010). In addition, sca4 probably participates in rickettsial motility since it directly activates vinculin, a host cell protein that is abundant in focal adhesions, by displacing its association with talin (Park et al. 2011); this displacement leads to vinculin binding to F-actin.

Similar to other bacteria, rickettsial gene expression is regulated by promoters, and genes are organized into operons (Shaw et al. 1997). Interestingly, even when predicted terminators are present, specific termination sites cannot be empirically identified in several genes (Woodard et al. 2011). This is suggested by the lack of a specific band in a ribonuclease protection assay (RPA) with a probe that hybridizes to a region downstream of the translational stop codon. In the case of convergent genes, this means that transcription of one gene will be extended into the coding region of the adjacent gene to generate antisense RNA; this phenomenon could have a role in gene expression regulation. In some cases, under the stress conditions of in vitro cell culture, studied transcripts terminated at the predicted site, but there was read-through in rickettsiae cultivated in yolk sacs (in vivo). This fact implies that transcription termination can be regulated in *Rickettsia*.

Different strains of pathogenic rickettsiae from the same species can present different degrees of virulence (Turco and Winkler 1994; Bechah et al. 2010b). In the case of *R. prowazekii*, an attenuated strain (Madrid E) was even used as an effective live vaccine (Fox et al. 1954). The attenuation correlates with a reduced lysine methylation profile (Turco and Winkler 1994). Proteomic and transcriptomic investigations comparing *R. prowazekii* strains of different virulence lend further support to the important role of posttranslational modifications in regulating virulence (Bechah et al. 2010b). However, other mechanisms clearly participate in the attenuation of *R. prowazekii* Madrid E; for instance, the adhesin adr1 has a deletion in this strain.

R. rickettsii in ticks kept at a low temperature (in the laboratory or in nature) are in an inactive or dormant state. Extracts from those ticks can immunize guinea pigs but do not cause evident disease (Spencer and Parker 1923). However, if the ticks are allowed to feed for at least 10 h, extracts from those ticks produce a febrile illness in guinea pigs. The term "reactivation" was introduced by Spencer and Parker to describe this observation. Such a phenomenon may be partly explained by active multiplication of SFG rickettsiae during blood feeding (Wike and Burgdorfer 1972). However, active regulation of gene expression can also play a role (Policastro et al. 1997). Such active regulation of the rickettsial phenotype is suggested by ultrastructural changes including a transition from well-defined microcapsular and slime layers in engorged ticks to compromised layers in starved ticks (Hayes and Burgdorfer 1982). A proteomic corroboration of active gene regulation was recently published (Tucker et al. 2011); *R. prowazekii* differentially expressed some proteins when cultivated in a mammalian cell line, chicken yolk sacs, or arthropod cell lines. Interestingly, in the more physiological of these models, the yolk sac (because it is in vivo), there were larger quantities of several proteins that, taken together, suggest increased metabolism. On the other hand, rickettsiae in cell culture expressed higher levels of proteins associated with a stress response.

Rickettsiae have genes that are very similar to the *spoT* genes. Those genes encode proteins that hydrolyze the alarmone nucleotide (p)ppGpp to mediate the stringent response in other gram-negative bacteria in response to nutritional challenges; rickettsiae are confronted with such a situation during the long periods of fasting inherent to tick biology or in insect feces. It is believed that the rickettsial *spoT* genes are functional based on the high degree of conservation, which implies selective pressure (McLeod et al. 2004), and the fact that the expression of some of these genes is differentially regulated in *R. conorii* grown in mammalian vs. arthropod cells in vitro (as well as in ticks); furthermore, *spoT1* is responsive to nutritional and temperature changes (Rovery et al. 2005).

In another series of experiments using microarray analysis, the transcriptional response of *R. rickettsii* under different conditions was compared (Ellison et al. 2009). The parameters studied intended to mimic conditions that may underlie the quiescence and reactivation phenomena; they included different temperatures, cold shock, limiting iron, mammalian cells, and insect cells. They found that temperature changes typical of the transition between the tick and mammalian host did not induce significant changes (more than threefold); however, many genes had significant changes in expression when rickettsiae were exposed to 4 °C (simulating overwintering ticks). Only five genes had a significant change in response to iron chelation and seven were differentially regulated in two types of host cells. This limited transcriptional response to environmental changes was attributed to the loss of many regulatory genes during the adaptation to a constant intracellular environment (whether in arthropods or vertebrates). A similar analysis performed with *R. typhi*, in which the investigators compared the transcriptome when rickettsiae grew at 37 °C vs. 25 °C, showed that the expression of 60 genes is downregulated (at least 1.5-fold) with the shift to lower temperature; many of this genes code for proteins involved in metabolism. With the shift to 25 °C, 70 genes were upregulated; the function of half of them is not known or cannot be predicted (Dreher-Lesnick et al. 2008). Almost simultaneously, another microarray study was published. The investigators of this other study analyzed *R. prowazekii* exposed to 42 °C for 30 min (a model of heat shock); they found 23 rickettsial genes that changed their expression more than twofold (Audia et al. 2008). Several of those corresponded, not surprisingly, to annotated chaperones.

Another mechanism of regulation, adaptive mutation, may be operational in *Rickettsia*. It may involve reversible gene inactivation by splitting in areas of poly(A) tracts. For instance, the attenuated Madrid E strain of *R. prowazekii* has a mutation in the *recO* gene, which encodes a protein that participates in DNA repair. When this gene mutates back to a functional one, other genes mutated by splitting (including sequences annotated as pseudogenes) are repaired (Bechah et al. 2010b). This phenomenon may underlie a change in phenotype from avirulent to virulent.

Virulence

Many rickettsial genes have been predicted to participate in virulence based on bioinformatics analyses (McLeod et al., 2004); several toxin-antitoxin systems are examples. One of them, encoded by the *vapB/C* genes, was shown to be functional; *E. coli* transformed with rickettsial *vapC* significantly decrease their growth, while VapB formed a complex with VapC to inhibit its RNase activity (Audoly et al. 2011). More importantly, microinjection of VapC to mammalian cells induced apoptotic death.

A large number of intracellular bacteria use type IV secretion systems to inject proteins into the host in order to produce a favorable niche. Interestingly, genomic analysis showed that multiple genes with the potential to encode a reduced type IV secretion system are conserved in *Rickettsia* (Gillespie et al. 2009a). Whether the system is actually functional or not remains to be tested.

The phospholipase D encoded by the gene *pld*, a likely mediator of phagosomal escape, is a virulence factor as suggested by the milder disease produced in guinea pigs infected with *R. prowazekii* with a mutated *pld* (Driskell et al., 2009). This study used homologous recombination for targeted knockout of a rickettsial gene. Previous studies using the difficult techniques of genetic manipulation of *Rickettsia*, including transposon-mediated mutagenesis, indicated that mutation of the open reading frames (ORFs) 243, 294, and 689 of *R. prowazekii* do not produce an observable phenotypic difference (Qin et al. 2004). Thus, these genes may be nonessential genes (at least for growth in a mouse cell line in vitro). Also, *R. rickettsii* mutants lacking expression of sca2, which participates in actin polymerization, do not cause apparent illness in guinea pigs (Kleba et al., 2010).

Loss of regulation due to genome decay has also been proposed as a mechanism of increased virulence (Fournier et al. 2009); however, this argument does not explain why *R. rickettsii* and *R. prowazekii* are almost equally pathogenic and the radical difference in virulence between the two typhus group rickettsiae, *R typhi* and *R. prowazekii*.

In the absence of genetic approaches that work well and consistently for *Rickettsia*, other methods have been introduced to identify virulence factors. One example is the comparison of the genomes of closely related *Rickettsia* with different pathogenicity. The *D. andersoni* endosymbiont *R. peacockii* was compared to virulent *R. rickettsii*; it was found that it had a plasmid, multiple transposons with intact transposase sequences, and many deletions, nonsense mutations, and split genes (Felsheim et al. 2009). The authors proposed that some of the absent or mutated genes in *R. peacockii* might explain the lack of pathogenicity. Those genes include *DsbA* (a catalyzer of disulfide bond formation), *RickA*, *Sca0, Sca1*, a gene encoding protease II, and a gene encoding a putative phosphoethanolamine transferase that could play a role in the formation of the prominent slime layer found in the pathogenic spotted fever-group rickettsiae. Interestingly, the hypothetical protein A1G_05165 of a virulent strain of *R. rickettsii* (strain Sheila Smith) is deleted in *R. peacockii* and it is also not present in other nonpathogenic rickettsiae. This hypothetical protein has ankyrin repeats; similar proteins in other members of this order (i.e., *Anaplasma*) appear to play a role in virulence through binding of host DNA and altered host gene regulation. A1G_05165 is also mutated in a nonpathogenic strain of *R. rickettsii* (strain Iowa). In addition, the genomic study that compared the pathogenic strains R and Sheila Smith with strain Iowa also found 23 deletions within predicted ORFs of *R. rickettsii* Sheila Smith and 24 deletions within predicted ORFs of *R. rickettsii* Iowa (Ellison et al. 2008). One of the genes deleted in *R. rickettsii* Iowa is the adhesin rOmpA (sca0). Also, *rompB* has four single nucleotide polymorphisms (SNPs) that may explain the defective processing of this important membrane protein in strain Iowa (Hackstadt et al. 1992). Finally, it should be emphasized that there is a good opportunity to understand virulence by comparing the genomes, transcriptomes, and proteomes of the two typhus group *Rickettsia* since they have very closely related genomes but very different virulence in humans with *R. prowazekii* producing a much more severe infection (epidemic typhus) than *R. typhi* (murine or endemic typhus).

Another system to study the physiology of *Rickettsia* in the absence of more efficient genetic systems is the use of *E. coli*-based assays. For example, to identify proteins transported out of the rickettsial cytoplasm, bioinformatic tools were used to uncover predicted secreted proteins (based on the presence of N-terminal signal peptides). The signal peptides of those proteins from *R. typhi* were then fused to the *E. coli* alkaline phosphatase *phoA* gene (lacking an intrinsic signal peptide sequence) to test if those signal peptides provided information to translocate PhoA into the periplasm of *E. coli* (Ammerman et al. 2008). Eighty four functional signal peptides were identified suggesting that those rickettsial proteins might be secreted using the rickettsial Sec system. Those proteins include sca1-3, sca5, Pld, and proteins that are believed to be part of a type IV secretion system.

Antigens, Vaccines, and Immune Response

An often overlooked but critical factor in the pathogenesis of rickettsial diseases is the transmission by arthropod vectors because their saliva is not a passive vehicle for transmission (Brossard and Wikel 2004; Wikel 1999; Francischetti et al. 2009). In fact, the tick saliva modifies the host environment in order to successfully complete the blood feeding, which occurs during extended periods (several days for nymph and adult ticks). Proteins in the tick saliva modulate host hemostasis, innate and adaptive immunity, complement activation (Tyson et al. 2008), angiogenesis, and extracellular matrix regulation (Steen et al. 2006; Ribeiro and Francischetti 2003). Evidently, all of those factors could determine the final outcome of the infection. Furthermore, tick saliva can modulate the physiology of endothelial cells, the main target cells of *Rickettsia*. For example, salivary gland extracts from *D. andersoni* reduces the upregulation of ICAM-1 induced by TNF-α on a mouse endothelial cell line (Maxwell et al. 2005). This change could contribute to reduce the migration of leukocytes into tick bite sites. Much research in this area remains to be done.

Endothelial cells are not passive actors in the antirickettsial immune response. Upon rickettsial infection, the transcription factor NFκB (a critical transcription factor of the immune system) becomes activated in endothelial cells (Sporn et al. 1997; Sahni et al. 1998, 2003). Other critical signaling mediators become activated as well. They include STAT1, STAT3 (Sahni et al. 2009), and p38 MAPK (Rydkina et al. 2005, 2008, 2010). As a consequence of the activation of these various signaling systems, endothelial cells respond by expressing a variety of chemokines (Valbuena et al. 2003; Valbuena et al. 2004); cytokines such as IL-1α and IL-6 (Kaplanski et al., 1995; Sporn and Marder 1996); adhesion molecules such as E-selectin, VCAM-1, ICAM-1 (Sporn et al. 1993; Dignat-George et al., 1997; Damås et al., 2009) (*Fig. 19.7*), and αVβ3 integrin (Bechah et al., 2009); and secretion of prostanoids (Rydkina et al. 2006, 2009).

NK cells are early producers of IFN-γ after infection with *Rickettsia* (Billings et al., 2001; Jordan et al. 2009). This cytokine is important because, together with TNF-α, it activates the bactericidal functions of the endothelium (Feng et al. 1994; Walker et al. 1997). Those functions are performed in part through expression of indoleamine-2,3-dioxygenase (IDO), which leads to tryptophan starvation (Feng and Walker 2000). Animal studies have demonstrated the importance of a T helper 1 (Th1) response in effective immunity against rickettsiae (Mansueto et al. 2008) with a particularly important role for $CD8^+$ T cells (Feng et al. 1997; Walker et al. 2001). In fact, T cells are sufficient to mediate protection against a lethal rickettsial challenge, even in the context of a heterologous challenge where

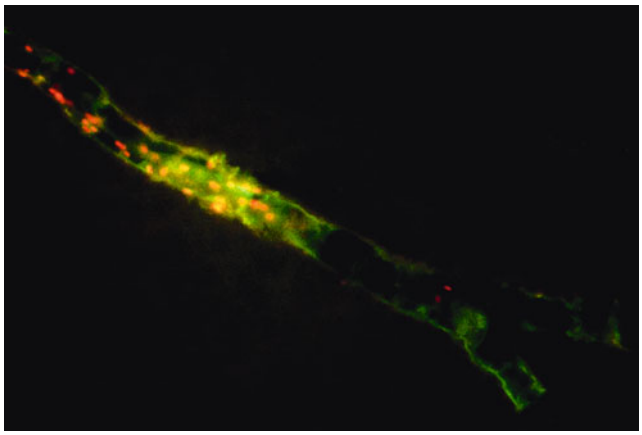

Fig. 19.7
Wide-field fluorescence microscopy of a whole-mount retinal preparation from a C3H/HeN mouse infected with *Rickettsia conorii* 5 days earlier (original magnification: 400×). The retina was stained with a rabbit polyclonal serum against *R. conorii* and a secondary goat antirabbit IgG labeled with rhodamine (*red signal*). The retina was also stained with a rat anti-ICAM1 antibody and a goat anti-rat IgG labeled with Alexa 488 (*green signal*).

anti-typhus group T cells protect against a lethal challenge with SFG *Rickettsia* and vice versa (Valbuena et al. 2004).

Despite the fact that rickettsiae are intracellular parasites and that cellular adaptive immunity is critical during a primary infection, there is clear evidence that the humoral immune response is very important in preventing the development of disease during secondary infections or after a lethal challenge following passive serum transfer. In fact, it was Ricketts himself who demonstrated this fact (Ricketts and Gomez 1908). The antirickettsial humoral immune response is cross-reactive within rickettsiae of the same group but not across groups (e.g., between typhus and SFG groups) (Shirai et al. 1975; Vishwanath 1991). The most abundant surface protein of *Rickettsia* is rOmpB (Sca5), which is an autotransporter. It is an immunodominant protein and antibodies against it are protective (Anacker et al. 1987).

It will be possible to produce vaccines that cover more than one species of *Rickettsia* given the evidence of cross-protective immunity within the typhus or spotted fever groups (Zinsser and Castaneda 1933; Zinsser 1937; Feng and Waner 1980; Eisemann et al. 1984; Jerrells et al. 1986; Gage and Jerrells 1992; Feng and Walker 2003) or even across groups (Parker 1939; Parker 1940; Valbuena 2004a). The production of an effective anti-Rickettsia vaccine is a public health priority for several reasons. Firstly, some rickettsioses are highly lethal not only to humans but also to companion animals (i.e., dogs). Secondly, clinical diagnosis of rickettsioses is very difficult due to the nonspecific initial clinical presentation. Thirdly, there are no commercially available diagnostic tests that can be used during the acute stage when antibiotic intervention is helpful. Additionally, two rickettsiae (*R. prowazekii* and *R. rickettsii*) could potentially be used as bioweapons and are included in CDC's list of select agents (Walker 2003).

Inactivated vaccines for *R. rickettsii* and *R. prowazekii* were produced early from a variety of sources including their vectors but they were very reactogenic and protection was incomplete. Later on, inactivated vaccines were produced from *Rickettsia* cultivated in eggs but antigenicity was variable and protection was poor (Mason et al. 1976; DuPont et al. 1973; Clements et al., 1983; Woodward 1986). In the 1950s, a very effective vaccine for epidemic typhus was produced. It was an attenuated strain denominated Madrid E (Fox et al. 1954); however, spontaneous reversion to a virulent phenotype precluded further development and testing (Balayeva and Nikolskaya 1973; Nikolskaya and Balayeva 1973). We now know that the attenuation is explained, at least in part, by a point mutation in the gene encoding an S-adenosulmethionine-dependent methyltransferase (Zhang et al. 2006). Given the nature of the mutation, it is not surprising that reversion was not an uncommon occurrence. Deletion of the entire gene would permit the production of a safer vaccine. Alternatively, strains with multiple genetic differences could prove to be safe vaccines. In this regard, it is interesting to note that the strain Iowa of *R. rickettsii*, which is attenuated and has multiple genetic differences when compared with virulent strains, can protect guinea pigs against a challenge with virulent *R. rickettsii* (Ellison et al. 2008).

Other recent efforts have focused on the production of a subunit vaccine. Fragments of rickettsial proteins that may trigger protective immunity were tested. They included rOmpA (Crocquet-Valdes et al. 2001; Sumner et al. 1995) and rOmpB (Churilla et al. 1990; Li et al. 2003; Chan et al. 2011) and results were encouraging; however, these approaches are limited and biased because of their focus on proteins that elicit a strong humoral response. A major effort for identification of immunogenic antigens is clearly needed; fortunately signs of this effort are starting to appear. For instance, immunoblot analysis from a 2D gel of *Rickettsia parkeri* using serum from a confirmed human case and proteomic analysis of corresponding spots showed new immunoreactive proteins including translation initiation factor IF-2, cell division protein FrsZ, and cysteinyl-tRNA synthase (Pornwiroon et al. 2009). This example is still biased toward the humoral immune response. Thus, the antigen discovery effort will need new tools to identify relevant conserved antigens recognized by T cells.

Laboratory Methods and Animal Models

Cultivation of *Rickettsia* in chicken embryonated eggs was first described in 1938, and it is still the most efficient method (Cox 1938, 1941). Similarly, the plaque assay, first described in 1966, remains one of the best quantification methods (Kordová 1966; McDade et al. 1969; Weinberg et al. 1969; Wike et al. 1972), particularly for SFG *Rickettsia*. One of the simplest media to preserve frozen rickettsial stocks (SPG, consisting of sucrose, potassium, and glutamate) was described

in 1950 (Bovarnick et al. 1950); glutamate is an essential component of this buffer because it provides a metabolic substrate. Procedures with live pathogenic *Rickettsia* must be performed in a biosafety level 3 (BSL-3) environment because the infection can be acquired through aerosols.

Presently, there are no commercially available methods for the diagnosis of rickettsioses that are informative during the early stages of the fever. There are commercially available antigen slides for indirect fluorescent assay (IFA); however, this technique is inherently retrospective (since antibody titers are frequently negative during the first week of illness) and confirmation requires a fourfold increase in IgG titers between the acute and convalescent serum samples (◉ *Fig. 19.8*).

Specialized and reference laboratories can perform more sophisticated techniques for diagnosis during the acute stage. They include direct immunofluorescence to detect rickettsial antigen on biopsy specimens obtained from involved skin and PCR-mediated amplification of conserved rickettsial genes from biopsies, buffy coat, or even plasma. Bacterial cultivation can be performed by inoculating monolayers of eukaryotic cells with the clinical sample; this is the essential principle of the shell vial technique (Marrero and Raoult 1989). However, positive results and identification take at least a couple of weeks.

For the spotted fever and typhus group rickettsioses, the best animal model is C3H/HeN mice inoculated intravenously with *R. conorii* or *R. typhi*, respectively (Walker et al. 2000; La et al. 2007). The rickettsial infection of these animals faithfully recapitulates many of the clinical features of human rickettsioses including a disseminated and predominantly endothelial infection (◉ *Figs. 19.1*, ◉ *19.2*, and ◉ *19.6*). The infection is sublethal when a low-dose inoculum is used and lethal when a large number of *Rickettsia* is inoculated. Just like humans, animals that survive develop solid immunity against rechallenge with a lethal dose. Unfortunately, the most virulent rickettsiae for humans, *R. rickettsii* and *R. prowazekii*, do not produce infections in mice that model appropriately the pathology and pathogenesis of humans with Rocky Mountain spotted fever or epidemic typhus. Intravenous inoculation of Balb/C mice with *R. prowazekii* was recently described as a model of epidemic typhus (Bechah et al. 2007). This same strain of mice was also used in a recently described model of SFG rickettsiosis caused by *R. heilongjiangensis* (Duan et al. 2011). However, further validation and characterization of these models are necessary. Guinea pigs remain the most useful models for *R. rickettsii* and *R. prowazekii* infections; however, the limited number of laboratory reagents to study immunology and pathogenesis in guinea pigs (as compared to mice) is a limiting factor.

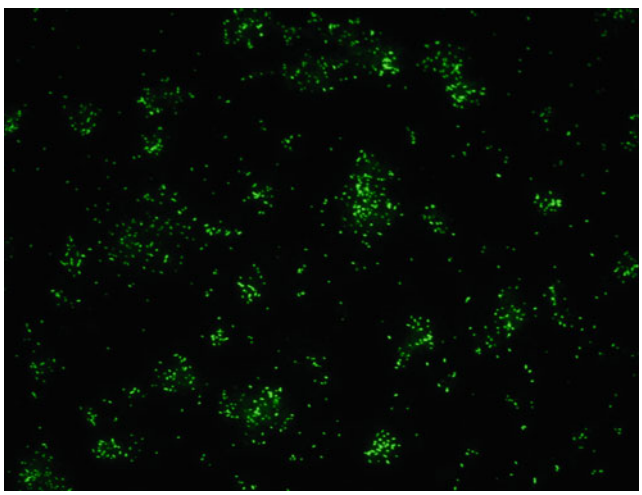

Fig. 19.8
Wide-field fluorescence microscopy of a well from a diagnostic antigen slide prepared with Vero cells infected with *Rickettsia rickettsii*. The antigen slide was stained with the serum from a patient suspected of having Rocky Mountain spotted fever. The secondary antibody was a donkey anti-human IgG (γ chain-specific) labeled with Alexa 488. Notice the characteristic small bacilli

Current Challenges

Consistent genetic systems to work with *Rickettsia* are still not available. The first successful attempt to transform *Rickettsia* was reported in 1998 (Rachek et al. 1998). However, genetic systems for the analysis of rickettsia are still very imperfect due to the difficulties of manipulating an obligate intracellular parasite with a very limited armamentarium of genetic tools. To complicate the matter further, two of the most effective antibiotics for selection of transformants in bacteria, chloramphenicol and tetracycline, are the antibiotics of choice for clinical treatment of rickettsioses; thus, their use is not permitted for genetic manipulation of pathogenic *Rickettsia*. Moreover, rickettsiae are resistant to beta-lactams (probably mediated by the proteins encoded by *AmpG* genes) and aminoglycosides. In the later case, it is because of the intracellular localization of rickettsiae and the poor intracellular penetration of this class of antibiotics. Alternative antibiotics such as rifampin (Rachek et al. 1998) and erythromycin (Rachek et al. 2000) have been used for selection of transformants but spontaneous resistant mutants arise with a high frequency, making selection very difficult. In addition, rickettsial codon usage is different from many other bacteria due to the rich AT composition of the genome (Andersson and Sharp 1996). This fact must be a consideration for rickettsial transformation since some exogenous genes (i.e., those encoding antibiotic resistance) may be poorly expressed due to a different codon usage.

Transposition to produce random mutants of *Rickettsia* was first successfully used for *R. prowazekii* using the EZ-Tn5™ (or EZ::TN™) transposon system(Qin et al. 2004; Baldridge et al. 2005). Further refinement of the system was obtained with the *Himar1* transposase (Liu et al. 2007). In both cases, the transposed element included a gene encoding for rifampin resistance (through ribosylation of the antibiotic), which was used for selection. Limiting dilution cloning was necessary in order to identify clonal populations with transposon insertions and to separate them from spontaneous mutants that

become resistant to rifampin. Expression of the fluorescent protein GFP, whose sequence is in the transposon, helped in this process.

The prospect of developing a natural genetic transformation system for *Rickettsia* is possible since the genes are present in many of the rickettsial endosymbionts of arthropods (Weinert et al. 2009a). In a fact, *tra* genes for a conjugation system and a surface appendage connecting two rickettsiae have been documented (Ogata et al., 2006).

Another challenge in the field of rickettsiology is our lack of understanding of the early events immediately after vector-mediated inoculation, which could be determinants of the final outcome of the infection. Unfortunately, current animal models do not include transmission by appropriate vectors. Finally, from the clinical perspective, a major challenge is the development of sensitive and specific assays for the diagnosis of rickettsioses during the acute stage of the disease. Such assays need to be easy to implement in rural settings where many of the rickettsioses occur.

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20 Syphilis

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Abstract
Syphilis is an infection that has intrigued scientists, baffled clinicians, and terrified patients for centuries. Although an inexpensive and highly effective treatment has been available for 60 years, syphilis continues to be a public health problem in both developed and developing parts of the world. The protean clinical manifestations, long periods of asymptomatic infection, and lifelong persistence of syphilis suggest a highly complex relationship between *Treponema pallidum* and the host's immune response. The extreme fragility of the causative bacterium and the inability to cultivate it in vitro have impeded progress in identifying and understanding the important virulence factors. The absence of an inbred animal model of syphilis and the consequent lack of immunological reagents complicate progress further. Despite these difficulties, the post-genomic era has yielded a new understanding of the molecular interactions of *T. pallidum* and the host. In this chapter, we present our current understanding of the mechanisms of syphilis pathogenesis in the context of a discussion of the clinical stages of syphilis.

History

Few diseases elicit the simultaneous disdain, fear, and fascination as does syphilis. There is much lore surrounding this infection. An unresolved debate continues to be waged over the origin of syphilis—did Columbus really bring the infection back to Europe from the New World? Was the new "plague" that swept through Europe in the late 1490s truly a new disease, or did the increased travel and communication of that era simply expose more people to, or make people more aware of, an already-existing disease? Regardless, it is clear that, for centuries, syphilis was a very common infection in pre-antibiotic Europe and the United States, and many claims (true and untrue) have been made about this infection. Numerous famous literary, artistic, and historical figures have been implicated as having had the "Great Pox," and their creative genius has been credited by some to the effects of syphilis on the brain (Hayden 2003). Syphilis has figured in the writings of Brontë, Voltaire, Cather, Dickens, Keats, and Ibsen. Nationalistic barbs have resulted in the French referring to syphilis as the "mal de Naples" and the English calling it the "French Disease."

The impact of syphilis was so great that many giants of microbiology and medicine studied it during the late nineteenth and early twentieth centuries. Monumental progress in syphilis was made during the first decade of the twentieth century when the infection was successfully transmitted to chimpanzees (Metchnikoff and Roux 1906); the causative agent, then called "Spirochaeta pallida," was first visualized (Schaudinn and Hoffman 1905); the first serological test for syphilis was developed based upon the work of Bordet and Gengou (Wassermann et al. 1906); and Ehrlich described the first effective chemotherapeutic agent for treatment of syphilis (described in Fitzgerald 1911). Ultimately, three of these scientists (as well as Julius Wagner-Jauregg) would receive the Nobel Prize in Medicine, largely, or in part, because of their work on syphilis. The first grant review committee at the National Institutes of Health was called the "Syphilis Study Section" (*Fig. 20.1*) (Mandel 1996).

In the early decades of the twentieth century, syphilis was twice as common as tuberculosis in the USA, and it was estimated that ~10–20% of people in Europe and the United States were infected with syphilis. Dr. Thomas Parran had been fighting for a decade, as Chief of the Venereal Diseases Division of the

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Fig. 20.1
The first review committee at the National Institutes of Health was the Syphilis Study Section, pictured here in 1947. At the first meeting on February 7–8, 1946, this group declared two preparations of penicillin ineffective against syphilis. J. E. Moore, seated in the center on far side of the table, served as chair (Mandel 1996) (The image was provided courtesy of the Office of History, National Institutes of Health)

US Public Health Service, to bring attention to the public health importance of syphilis when, in 1934, the Columbia Broadcasting Company unwittingly launched Parran's national campaign against syphilis by refusing to air an interview with Parran if the words "syphilis" and "gonorrhea" were uttered. Parran countered by issuing his own news release about the incident, which was carried widely by the national newspapers. Subsequently, as US surgeon general, Parran was credited with instituting the "Wasserman dragnet," including widespread serological screening, contact finding, and rapid treatment measures, that resulted in a rapid decline of infectious syphilis in the United States, particularly following the recognition of penicillin as a safe and highly effective treatment (Parran 1937). During World War II, active public information campaigns warned of the dangers of syphilis and gonorrhea, usually placing the blame on "loose women" (*Fig. 20.2*).

Infamous past studies of syphilis have led directly to the development of today's strict Department of Health and Human Services guidelines for the protection of human subjects in research studies. The Tuskegee Study of Untreated Syphilis in African American Men is the best known of these studies (Rockwell et al. 1964; Reverby 2000, 2009), but prospective investigations of untreated syphilis were also conducted on a largely Caucasian population at Stanford University (Barnett and Blum 1948; Blum and Barnett 1948). The most recently discovered unethical syphilis study involved the intentional infection of Guatemalan prisoners to test the effectiveness of the new drug, penicillin, for treatment of syphilis (Semeniuk and Reverby 2010). Interestingly, each of these studies was ongoing while Parran was surgeon general.

Despite the ready availability of penicillin for the past 60 years, syphilis is again epidemic in major cities in the United States, Europe, and China, and continuing high rates of infection exist in sub-Saharan Africa, Southeast Asia, and

Fig. 20.2
Poster, circa 1940, warning soldiers against women who might infect them with syphilis and gonorrhea (Courtesy of the National Library of Medicine, National Institutes of Health)

South America. Recent estimates suggest that 11.5 million new infections occur globally each year. A more detailed discussion of the epidemiology of syphilis is found later in this chapter.

T. pallidum subspecies *pallidum*: The Causative Agent

T. pallidum subsp. *pallidum* is a member of the order Spirochaetales and is closely related to other Treponema species and subspecies including the agents of the human endemic treponematoses (yaws, bejel, and pinta) and the many Treponema species associated with periodontal disease. Additionally, the closely related *Treponema paraluiscuniculi* causes natural venereal infection in rabbits, and other treponemes cause both yaws-like and genital infection of baboons and other nonhuman primates in Africa (Knauf et al. 2012).

Although *T. pallidum* causes a chronic infection that can persist for several decades in the host, it is extremely fragile in

vitro. It cannot tolerate desiccation, extreme temperatures, or atmospheric levels of oxygen. *T. pallidum*'s inability to survive higher temperatures was exploited by Julius Wagner-Jauregg in the pre-penicillin era when he used the fevers induced by intentional malaria infection as a form of "treatment" to stop progression of late neurosyphilis (Wagner-Jauregg and Bruetsch 1946). This discovery led to his receipt of the Nobel Prize in Medicine in 1927, indicating the importance of syphilis to public health in that era.

T. pallidum can undergo several generations of division in vitro in rabbit epithelial cells but cannot be subcultured (Fieldsteel et al. 1982). Thus, the organism must be propagated by passage in rabbits to maintain strains and to obtain organisms for laboratory investigation. The methods for isolation of new strains and propagation of existing strains of *T. pallidum* in rabbits have been described in detail elsewhere (Lukehart and Marra 2007). Because of the inability to culture *T. pallidum* in vitro, genetic manipulation of this organism is not yet possible, making investigation of the organism and disease pathogenesis challenging.

Examination of the *T. pallidum* genome reveals the possible basis for *T. pallidum*'s reliance on host cells, as the genome lacks the genes for the Krebs cycle, electron transport, and de novo synthesis of lipids, nucleotides, and most amino acids. In apparent compensation, a number of predicted transport proteins are encoded (Fraser et al. 1998). When the genome was initially sequenced, it was hoped that the key missing gene would be identified and that successful in vitro cultivation would at last be possible; unfortunately, no simple answer was found. For several decades, multiple laboratories explored factors that affect *T. pallidum's* viability in vitro, and sensitivity to O_2 and reactive oxygen species was explored (Fitzgerald et al. 1977b; Norris et al. 1978; Norris and Edmondson 1986; Cox et al. 1990). The genome revealed a lack of apparent genes for a common bacterial solution to oxygen toxicity, including no genes for superoxide dismutase, catalase, or glutathione peroxidase. Hazlett *et al.* hypothesized a role for another protein, neelaredoxin, in protection of *T. pallidum* from the toxic effects of oxygen species (Hazlett et al. 2002). Superoxide reductase, identified in 1999 as a mechanism used by some bacteria to protect against oxygen toxicity, is present in *T. pallidum,* and the crystal structure of *T. pallidum* superoxide reductase was solved (Santos-Silva et al. 2005).

Most *T. pallidum* research has been performed using the Nichols strain, which was isolated from the cerebrospinal fluid of a patient with neurosyphilis (Nichols and Hough 1913) and has been propagated in rabbits for 100 years. More recently, the advantage of studying additional strains has been recognized, as there is significant diversity among strains and subspecies in the sequences of several genes, many of which are putative outer membrane proteins (OMPs). Thus, comparative genomics can highlight genes of potential import to syphilis immunity for further investigation. The examination of Nichols strains that have been independently propagated in different laboratories for a number of years also provides important information about the evolution of *T. pallidum* and identifies natural mutants that are useful in pathogenesis research. Approximately 130 isolates of *T. pallidum* have been propagated in rabbits and are currently available for study, but the vast majority of these were isolated from a fairly small sexual network in a single city (Seattle, WA) and therefore may represent multiple isolations of the same "strain." New isolates from diverse geographic regions are needed. An important tool for separating individual clones of *T. pallidum* has been developed, in which individual skin lesions that develop following intravenous infection of rabbits arise from a single treponeme seeded to that skin site (Centurion-Lara et al. 2004). Biopsying these lesions is analogous to picking colonies from an agar plate of cultivable bacteria and has yielded isogenic populations that have proved quite useful for studies of antigenic variation discussed later in this chapter.

While *T. pallidum* shares structural similarities with classical Gram-negative bacteria such as *Escherichia coli (E. coli)*—it has both outer and inner membranes and a periplasmic space—notable differences in *T. pallidum* include the presence of periplasmic flagella (endoflagella), and the fact that its outer membrane lacks lipopolysaccharide, a potent proinflammatory glycolipid found in most Gram-negative pathogens. In addition, freeze-fracture electron microscopy and cell fractionation studies have indicated a paucity of integral membrane proteins in the outer membrane of *T. pallidum*, suggesting this relatively naked membrane may be an important factor in the ability of the organisms to evade the immune response during decades of infection (Radolf et al. 1989b; Walker et al. 1989). More recent cryoelectron microscopy has confirmed the low number of surface proteins (Izard et al. 2009; Liu et al. 2010). The identities of the rare outer membrane proteins have been a source of much controversy in the *T. pallidum* field, and candidate outer membrane proteins will be discussed below in the context of their potential roles in the pathogenesis of syphilis.

The Natural History and Pathogenesis of Syphilis

The complicated multistage clinical course of untreated syphilis is shown schematically in ❯ *Fig. 20.3*. The treponeme-laden lesions of primary and secondary syphilis are transient (usually 3–12 weeks) and heal without treatment, yet the bacteria, which disseminate widely very early during the course of infection, are not completely eradicated by the immune system. Latent syphilis, subclinical by definition, can persist for many decades—often for the remainder of the person's life—without development of further clinical manifestations. In a subset of infected persons, the bacteria will again trigger the development of clinical signs of tertiary syphilis—manifest as gummatous tissue destruction, aortic damage, or destructive processes in the central nervous system.

Much of our knowledge of the natural history of untreated syphilis is derived from the retrospective Oslo study of nearly 2,000 persons diagnosed with primary or secondary syphilis at the turn of the twentieth century and reexamined 60 years later (Clark and Danbolt 1955; Gjestland 1955), and from the

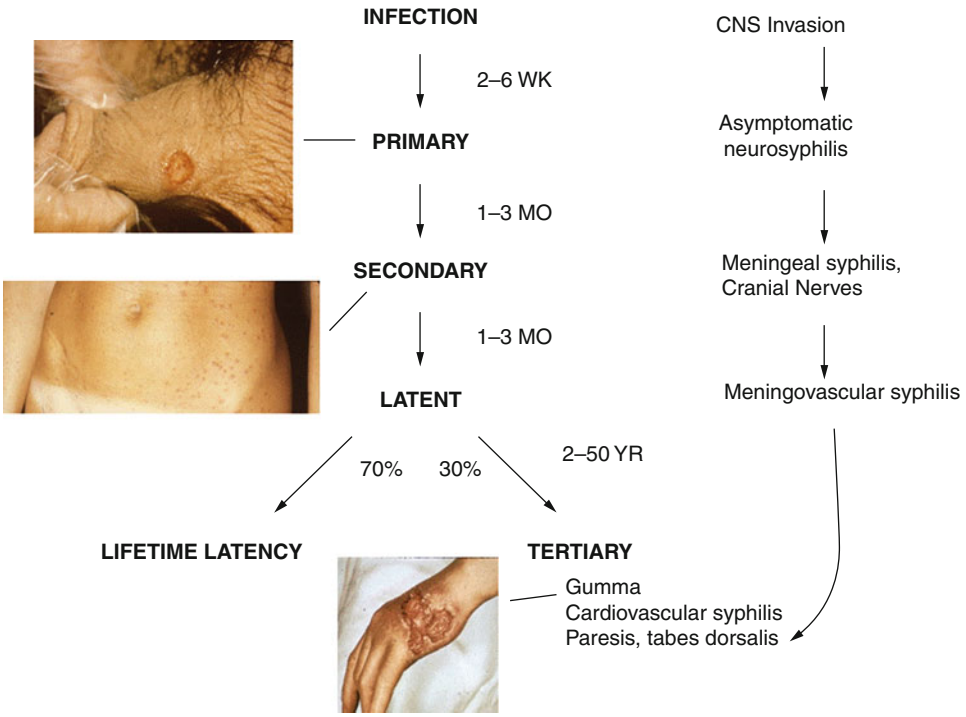

Fig. 20.3
The natural history of untreated syphilis in immunocompetent individuals. Stages of syphilis infection are in *bold*. Percentages of individuals developing specific stages and time intervals are based on information in references Stokes et al. (1944), Merritt et al. (1946), Clark and Danbolt (1955)

prospective study of over 400 African American men in the notorious Tuskegee study conducted from 1932 to 1972 (Rockwell et al. 1964). In the Oslo study, nearly one quarter of patients had recurrent secondary lesions, and 28% developed one or more manifestations of tertiary syphilis. Gummatous syphilis developed in 16% of patients, 7% developed late symptomatic neurosyphilis, and cardiovascular syphilis (typically aortitis or aortic aneurysm) was seen in 10%. Serious late manifestations were nearly twice as common in men as in women. In the Tuskegee study, aortitis was found in 40–60% of patients who underwent autopsy (compared to 15% of control subjects), and neurosyphilis was detected in only 4%. Syphilis was considered to be the direct cause of death in 15% of males and 8% of females in the Oslo study, and the death rate of infected men in the Tuskegee study was 17% higher than for controls.

For each stage of syphilis described in more detail below, the relevant aspects of pathogenesis will be incorporated into the discussion as indicated in the headings for each stage.

Transmission–Attachment

T. pallidum infection is transmitted by direct, usually sexual, contact with an infectious lesion. Thus, people who have the lesions of primary and secondary syphilis are considered to be infectious. Studies have reported that 30–60% of persons who were contacts of patients with primary or secondary syphilis ultimately develop syphilis, and the rate of infection did not differ significantly between contacts of primary versus secondary syphilis (Alexander et al. 1949; Schroeter et al. 1971; Schober et al. 1983). *T. pallidum* invades by penetration of mucous membranes or via dermal microabrasions, and the primary chancre is thought to appear at the site of initial contact. This is consistent with the fact that the most common sites for primary lesions are genital or anorectal regions, although oral lesions are increasingly common, especially among men who have sex with men (MSM); chancres at other sites are rarely seen. The beliefs that primary and secondary syphilis are equally infectious and that primary chancres appear at the site of contact appear to be somewhat contradictory. If, as suggested by the studies referenced above, the rash of secondary syphilis (which occurs largely on the trunk and extremities) is as infectious as primary chancres, individuals who have contracted syphilis by exposure to a person with secondary syphilis would logically be expected to develop primary lesions on their trunk and extremities through microabrasions occurring during the skin-to-skin contact of sexual activity. This is not observed. Further, although swabs of primary lesions are frequently positive for *T. pallidum* DNA by PCR, swabs of nongenital secondary lesions rarely have amplifiable *T. pallidum* DNA, suggesting that direct contact with secondary skin lesions might be less infectious than contact with primary lesions. This discrepancy brings into doubt the infectiousness of secondary skin lesions and raises the question of whether oral or genital mucosal lesions, perhaps unrecognized,

in persons with secondary syphilis might be the actual sources of infection in contacts to secondary syphilis patients.

The number of *T. pallidum* necessary to cause infection by natural contact is not known. The 50% infectious dose (ID50) for intradermal inoculation of the Nichols strain in humans was calculated to be 57 organisms (23 for rabbits). When lesions developed, they appeared at a mean of 20–28 days after inoculation for inoculations of 10–100 *T. pallidum* (Magnuson et al. 1956). The incubation period of naturally acquired primary syphilis in humans ranges from 9 to 90 days, with an average of 2–6 weeks, suggesting that ~10–100 organisms actually penetrate the epithelial layer during natural infection. This estimate is consistent with the recognized 30–60% chance of infection after natural exposure (Alexander et al. 1949; Schroeter et al. 1971; Schober et al. 1983).

Establishment of infection is dependent upon the binding and penetration of epithelial cells of the host by *T. pallidum*. *T. pallidum* can attach to a wide variety of eukaryotic cell types, likely accounting for the many and varied organs and tissues that can be infected by this organism. The in vitro attachment of the bacterium to eukaryotic cells can be inhibited by sera from infected rabbits and humans, suggesting that there are specific surface-exposed proteins or other components that mediate attachment (Fitzgerald et al. 1977a; Hayes et al. 1977). The mechanisms of attachment are not yet clearly defined, although several candidate protein adhesins have been identified. In all cases, the candidate adhesins have been demonstrated to bind to extracellular matrix (ECM) proteins, suggesting that *T. pallidum*'s promiscuous cell-binding ability may be related to the use of ECM components as bridges between the bacterium and the host cell. Fibronectin binding has been best studied (Peterson et al. 1983; Thomas et al. 1985; Cameron et al. 2004; Brinkman et al. 2008), with Tp0155, 0483, and 0136 all having fibronectin-binding ability. Tp0751, now termed "pallilysin," also binds to laminin and fibrinogen (Cameron 2003; Cameron et al. 2005; Houston et al. 2011). No direct adhesins (without ECM-bridging) have yet been identified in *T. pallidum*.

Primary Syphilis: Local Proliferation, Dissemination, and Immune Clearance

It has been widely considered that most of the symptoms and tissue damage observed in syphilis are due to activation of the immune and inflammatory responses of the host, as will be seen below for each stage of disease. In the primary stage, *T. pallidum* replicates at the site of initial inoculation, dividing once every 30–33 h, and triggers a local inflammatory response that is initially manifest as a papule. This lesion subsequently ulcerates to form a painless indurated lesion, the primary chancre, that appears approximately 3 weeks (range 9–90 days) after initial infection. In women, chancres usually occur on the labia or cervix. In heterosexual men, primary chancres occur most commonly on the penis. In men who have sex with men (MSM) and in women, chancres can also be found in the rectum, perianal region, and oral cavity (Hourihan et al. 2004). Chancres can appear, however, at other sites of infection, as evidenced by the development of chancres on the fingers of dentists as a result of contact with oral mucosal lesions in the era prior to universal precautions. The chancre may not be noticeable due to its location and lack of tenderness, making the diagnosis of primary syphilis in MSM and women difficult. Generally, patients have a single chancre, although two to three simultaneous chancres may be seen in a minority of infected persons. In the chancre, numerous spirochetes are surrounded by immune cells, including lymphocytes (CD4+ and CD8+ T cells), macrophages, and plasma cells (Van Voorhis et al. 1996b; Carlson et al. 2011; Juanpere-Rodero et al. 2011). Infiltration by polymorphonuclear leukocytes is scant and usually occurs only very early. Ulceration is thought to be due to a small vessel vasculitis, seen as endothelial proliferation, which reduces or obstructs blood flow to local tissues resulting in local necrosis. Treponemes disseminate to the regional lymph nodes early, and infiltrating immune cells cause a nontender regional lymphadenopathy.

Studies in the rabbit infection model show that, as early as 3 days after initial infection, T cells are sensitized and react to *T. pallidum* lipoproteins and endoflagellar proteins (Lukehart et al. 1980b; Baker-Zander et al. 1988; Arroll et al. 1999). After infection with a large inoculum, the peak T cell infiltration occurs at days 10–13, in correlation with maximum *T. pallidum* numbers. Macrophages first appear on day 6, peaking at day 13 (Lukehart et al. 1980a). The local production of IL-2 and IFN-γ cytokines suggests a TH1-skewed response (Leader et al. 2007). Antibodies directed against surface-exposed antigens lead to opsonization of *T. pallidum* and ingestion by activated macrophages (Baker-Zander and Sell 1980; Lukehart et al. 1980a; Lukehart et al. 1980b; Baker-Zander et al. 1993). Concurrent with infiltration of maximum numbers of macrophages, *T. pallidum* organisms decline dramatically at the site of primary infection (Lukehart et al. 1980a), suggesting a mechanism of clearance similar to that of delayed-type hypersensitivity, in which IFN-γ-activated macrophages ingest and kill *T. pallidum* (Baker-Zander and Lukehart 1992). The TH1 immune response has been confirmed in humans in biopsies of early syphilis skin lesions (Tosca et al. 1988; Van Voorhis et al. 1996a; McBroom et al. 1999). Within 3–8 weeks, the chancre spontaneously heals due to this intense local immune response *Fig. 20.4*.

During the early days of infection, even before appearance of the chancre, *T. pallidum* enters the surrounding tissue and gains access to the bloodstream to disseminate rapidly to distant tissues (Stokes et al. 1944; Turner and Hollander 1957; Salazar et al. 2007b). *T. pallidum* DNA can be identified by PCR in the bloodstream of patients with all stages of syphilis, and the quantity of treponemes in blood is relatively high during early syphilis (Cruz et al. 2010; Tipple et al. 2011a). Early in vitro electron microscopic studies suggest that *T. pallidum* traverses endothelial monolayers by moving between the cells (Thomas et al. 1988; Riley et al. 1992), although recent electron microscopic images of secondary syphilis skin lesions suggest that the bacterium may also use transcytosis to cross the endothelium (Juanpere-Rodero et al. 2011). Surface Tp0751 binds to laminin, which is found in highest concentration in the basement

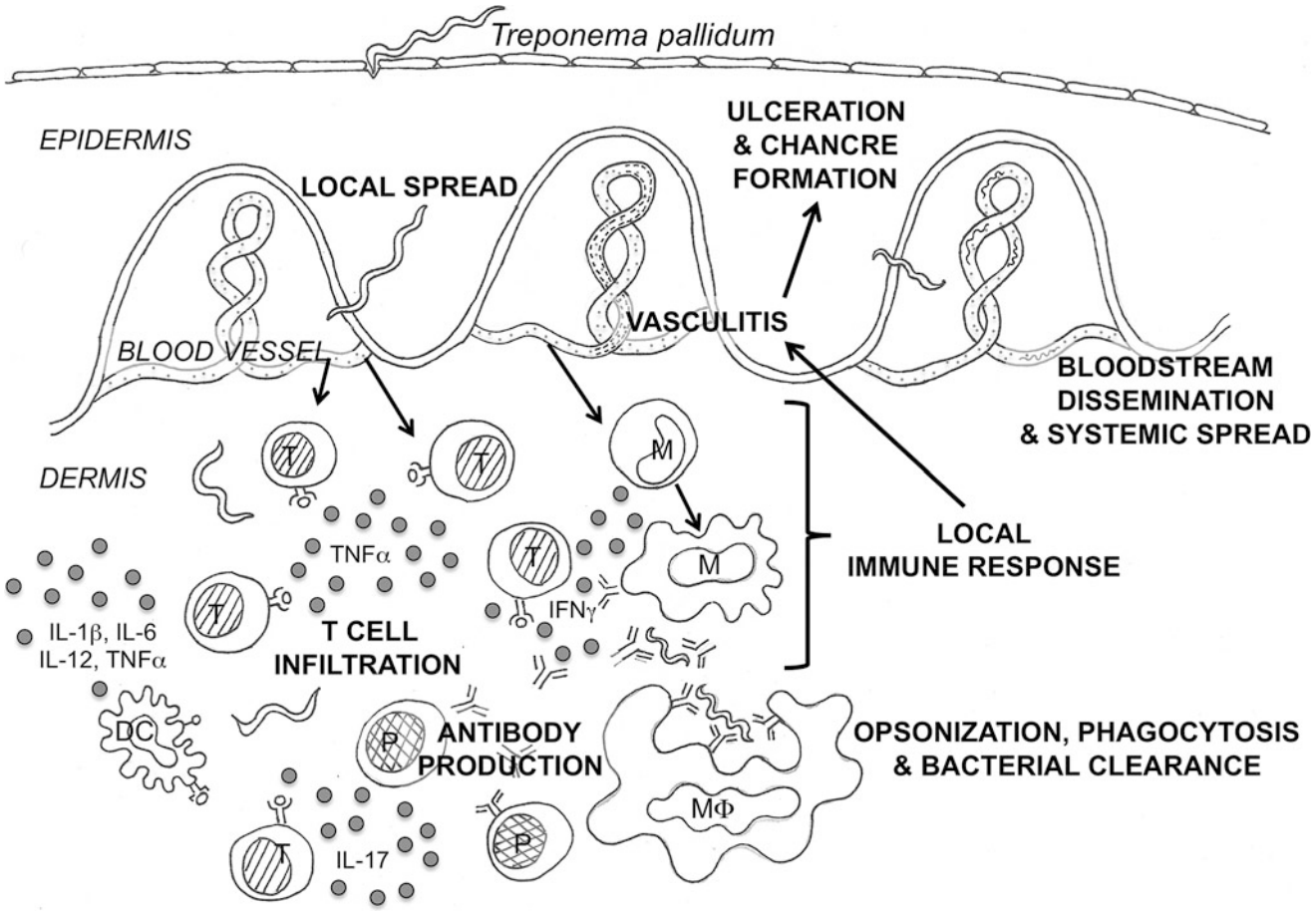

Fig. 20.4

The immune response to early syphilis in the skin. Dendritic cells (*DC*), T cells (*T*), plasma cells (*P*), monocytes (*M*), and macrophages (*MΦ*) are as labeled. Treponemes initially invade through mucosal surfaces or microabrasions in skin, then disseminate locally and, gaining access to the bloodstream, disseminate widely. The cellular infiltration in early lesions is comprised primarily of T lymphocytes (CD4+ and CD8+), macrophages, and scattered plasma cells, in a Th1-focused cytokine milieu. Ulceration of the chancre results from localized vasculitis and resulting necrosis. Bacterial clearance occurs though phagocytosis and killing of opsonized *T. pallidum* by IFN-γ-activated macrophages

membrane, and may thus facilitate movement from the blood to the tissues. Tp0751 also binds to fibrinogen, a blood-clotting protein that functions to contain bacteria, and can degrade both laminin and fibrinogen using its zinc-dependent protease domain further enhancing its role in dissemination (Houston et al. 2011). Additionally, *T. pallidum* induces the production of matrix metalloproteinase-1 (MMP-1) (Chung et al. 2002), which degrades collagen, perhaps allowing *T. pallidum* access to deeper tissues. The spread of *T. pallidum* to distant sites, including the skin, sets the stage for the secondary and later stages of syphilis.

Secondary Syphilis: Motility, Systemic Inflammation, and Host Immune Response

T. pallidum propels itself in a corkscrew-like fashion by rotating around its longitudinal axis using endoflagella located in the periplasmic space (Hovind-Hougen 1976; Izard et al. 2009; Liu et al. 2010). Flagellar fibrils attach at each end of *T. pallidum* and extend longitudinally, toward the center of the cell (Jepsen et al. 1968; Sykes and Miller 1973). Each fibril has a long shaft as well as a hook, collar, and basal knob that make up the insertion (Holt 1978), and each flagellar filament is made of multiple proteins, including core proteins (FlaB1, FlaB2, and FlaB3), sheath protein (FlaA), and motility-related proteins that make up the motor/switch and assembly apparatuses (Holt 1978; Isaacs et al. 1989; Champion et al. 1990b; Hardham et al. 1995, 1997; Limberger et al. 1996; Fraser et al. 1998). In the rabbit model and in infected humans, specific immune responses are mounted against endoflagellar proteins during infection (Hanff et al. 1982; Lukehart et al. 1982; Hanff et al. 1983a,b; Baker-Zander et al. 1985; Arroll et al. 1999; Martin et al. 2010), but immunization with flagellar proteins does not confer complete protection against *T. pallidum*, as these proteins are not surface exposed (Hindersson et al. 1985; Champion et al. 1990a).

The symptoms of secondary syphilis usually appear within 3 months following infection and are typically manifest as a disseminated maculopapular rash. Although the rash is often seen on the trunk and extremities, *T. pallidum's* predilection for temperatures slightly lower than body core may result in the pathognomonic rash that affects the palms and the soles. In most cases, secondary syphilis skin lesions persist for weeks to months, without discomfort, then heal spontaneously, presumably due to similar immune mechanisms as for primary chancres. In rare cases, however, lesions may be dramatic and may ulcerate, called "malignant syphilis."

Syphilis is already well disseminated during the secondary stage, and organ-specific symptoms appear in a minority of patients (Stokes et al. 1944; Chapel 1980; Mindel et al. 1989) and may include malaise (13–24%), weight loss (2–10%), muscle aches (6–8%), generalized lymphadenopathy (63–86%), alopecia (3–4%), mucous patches (superficial lesions on mucosal tissues in the oral cavity and genital sites; 7–17%), clinical hepatitis (0.5%), signs and symptoms of meningitis (including meningismus and headaches; 2–5%), and ocular inflammation (1–2%).

What triggers the development of the secondary lesions? We hypothesize, based upon work in the rabbit model, that each secondary lesion is seeded by a single treponeme that reaches the skin and other sites via hematogenous spread during the incubating or primary stages. The organisms multiply locally, leading to clonal expansion of the founder treponeme, and eventually reach a critical mass large enough to trigger an inflammatory response that results in clinically apparent rash or organ-specific inflammation. *T. pallidum* does not appear to produce any classical toxins, and it lacks the lipopolysaccharide that is responsible for local and systemic inflammation in other Gram-negative infections. Evidence suggests that inflammation is initially triggered by responses to treponemal lipoproteins, and several in vitro studies have shed light on this process. Although most work has been done with the lipoprotein TpN47 (TP0574), analysis of the genome sequence suggests that there are as many as 22 predicted lipoproteins in *T. pallidum* that may trigger inflammatory responses (Fraser et al. 1998).

In cultured endothelial cells, *T. pallidum* and TpN47 induce expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin (Riley et al. 1992; Lee et al. 2000), which are important in adhesion of immune cells to vascular endothelium as they migrate out of the bloodstream into tissue sites. The recent discovery of the proteolytic activity of a putative OMP, Tp0751, for both laminin and fibrinogen (Houston et al. 2011) suggests that this protein may also affect the integrity of the endothelial cells and basement membranes of capillary walls during the secondary stage, leading to local and systemic inflammation.

Early syphilis lesions transiently contain scant polymorphonuclear leukocytes (PMNs) (Bos et al. 1980), similar to that observed in experimental infection of rabbits (Turner and Hollander 1957; Musher et al. 1983; Sell et al. 1985). Transient infiltration by PMNs can be induced experimentally by injection of recombinant *T. pallidum* lipoproteins TpN17 (TP0435) and TpN47 into the dermis (Norgard et al. 1995; Sellati et al. 2001). After binding *T. pallidum* lipoprotein TpN47, Toll-like receptor (TLR) 2, which is expressed by dendritic cells and macrophages, mediates macrophage production of IL-12 (Brightbill et al. 1999). In addition, when dendritic cells are exposed to *T. pallidum* or the lipid moiety of TpN47, they release inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α (Bouis et al. 2001) and express maturation markers including CD54, CD83, and MHC class II (Bouis et al. 2001; Hertz et al. 2001; Sellati et al. 2001; Shin et al. 2004; Salazar et al. 2005). *T. pallidum* lipoproteins may also stimulate macrophages and dendritic cells by binding CD14, transmitting activation signals through the TLR1/TLR2 heterodimer (Aliprantis et al. 1999; Brightbill et al. 1999; Hirschfeld et al. 1999; Lien et al. 1999; Alexopoulou et al. 2002). These cytokines serve to bridge the innate and acquired immune responses and lead ultimately to lymphocyte sensitization, proliferation, and immune activation.

The in vivo response to intradermal injection of *T. pallidum* lipoproteins, TpN17 and TpN47, or the lipid portion of these molecules, in humans shows local enrichment of monocytes, macrophages, memory T cells, and dendritic cells compared to the peripheral blood (Sellati et al. 2001; Salazar et al. 2005). Skin lesions of patients with secondary syphilis showed a similar local immune response in the skin (Salazar et al. 2007a; Stary et al. 2010). Lesional skin samples from HIV-infected and uninfected individuals with early syphilis contain predominantly CD4+ and CD8+ T cells and macrophages, while plasma cells appear later (Van Voorhis et al. 1996b; Fegan et al. 2010; Stary et al. 2010). Immunohistochemistry and RT-PCR reveals that the CD8+ T cells present in the skin produce IFN-γ, perforin, and granzyme B (Van Voorhis et al. 1996b) as well as IL-17, the latter suggesting a new subclass of effector T cells (TC17 cells) that may respond to syphilis infection in the skin (Fegan et al. 2010). The number of plasma cells seen in secondary lesions is variable, and infiltration by plasma cells may occur after lesions have been present for some time (Stary et al. 2010).

As in primary lesions, macrophages play a role in the immune clearance of *T. pallidum* from secondary lesions through phagocytosis and killing of opsonized *T. pallidum*. *T. pallidum* organisms have been seen in the phagocytic vacuoles of macrophages (Sell et al. 1982), and opsonized treponemes are phagocytized by rabbit peritoneal macrophages in vitro (Lukehart and Miller 1978). Opsonization occurs with both IgG and IgM antibodies (Baker-Zander et al. 1993; Shaffer et al. 1993), and monospecific antisera directed against the recombinant variable putative OMP TprK (TP0897) have been reported to opsonize *T. pallidum* (Centurion-Lara et al. 1999), as do antisera raised against a number of other putative OMPs (Cameron et al. 2000). In the rabbit infection model, after most *T. pallidum* have been cleared, a few organisms remain at the site of infection, and these remaining bacterial cells are able to resist ingestion by macrophages even in the presence of immune serum (Lukehart et al. 1992), suggesting that a subpopulation of treponemes can avoid opsonic antibody and may persist to cause the later stages of infection. Mechanisms of immune evasion are discussed in a later section.

The humoral response to *T. pallidum* infection begins to develop during primary syphilis, and antibody titers reach their highest levels during the secondary stage. These high antibody titers can contribute to immune complex deposition in the kidney glomeruli, resulting in glomerulonephritis during secondary syphilis. The functions of anti-*T. pallidum* antibodies include opsonization (Lukehart and Miller 1978) and complement-mediated immobilization or neutralization in vitro (Nelson and Mayer 1949; Bishop and Miller 1976b). Despite the presence of functional antibody, passive immunization of rabbits with immune serum is unable to protect animals against *T. pallidum* infection (Bishop and Miller 1976a), indicating that a local cellular immune response is also required for protection. The functional connection between humoral and cellular immunity in humans is indicated in studies of human PBMCs exposed in vitro to *T. pallidum,* in which internalization of the treponeme by monocytes and dendritic cells occurs only in the presence of opsonic human syphilitic serum (Moore et al. 2007). This ingestion of *T. pallidum* upregulates surface expression of CD40 on monocytes, as well as secretion of TNF-α, IL-6 and IL-1β by PBMCs, and IFN-γ production by natural killer (NK), NK T cells, and T cells (Moore et al. 2007). The lipoproteins of *T. pallidum* are particularly immunogenic and robust antibody and cellular immune responses are mounted to these antigens, as well as the endoflagellar proteins (Hanff et al. 1982; Lukehart et al. 1982; Hanff et al. 1983; Baker-Zander et al. 1985; Baker-Zander et al. 1988) and the *T. pallidum* repeat (Tpr) antigens (Leader et al. 2003; Brinkman et al. 2006; Giacani et al. 2007b). The use of antibody detection for syphilis diagnosis will be discussed in a later section.

Effect of Concurrent HIV Infection on Clinical Course of Syphilis

Early epidemiological studies of acquired immunodeficiency syndrome (AIDS) found a very high concordance of history of syphilis (or seropositivity to *T. pallidum*) with AIDS (defined clinically at that time), and this led some to speculate that syphilis might cause the immunodeficiency that defined AIDS. It is clear now that the correlation between AIDS and syphilis is related not only to shared behavioral risk factors but also to biological factors. Several studies have indicated that syphilis is a risk factor for both acquisition and transmission of HIV, with risk estimates ranging from 2.3 to 8.6 (Fleming and Wasserheit 1999). Syphilis continues to affect populations with high HIV prevalence. In Europe and the United States, this occurs particularly in men who have sex with men MSM. In 2009, among MSM presenting to syphilis surveillance network clinics with primary or secondary syphilis, a median of 44.4% were coinfected with HIV, with a range of 30% in Birmingham, AL to 74% in Baltimore, MD (CDC 2010a). The CDC has reported that the number of cases of infectious syphilis continues to rise, more than doubling since 2000, with the most dramatic increases in MSM (CDC 2010a). It has been speculated that the recent increasing incidence of syphilis may be due to disinhibition and resulting high-risk behaviors in the setting of effective antiretroviral treatment (ART). These behaviors include HIV serosorting and resulting unprotected sex, anonymous unprotected sex, sex with multiple partners, and sex under the influence of drugs, including methamphetamine use (CDC 2006; Spindler et al. 2007; Taylor et al. 2007).

It is also likely that higher rates of syphilis in HIV-infected individuals may be due to several immunological and bacteriological factors. Primary syphilis, with its chancre, can increase the acquisition and transmission of HIV by disrupting epithelial and mucosal barriers (Greenblatt et al. 1988). The infiltration of chancres by immune cells brings activated cellular targets to the open lesion where HIV acquisition may occur. Conversely, the infiltrating cells may be HIV infected, providing a ready means of transmission to the partner. *T. pallidum* lipoproteins induce the expression of CCR5 on macrophages and dendritic cells, which acts as a coreceptor for HIV entry into CD4+ T cells (Salazar et al. 2007a; Sheffield et al. 2007).

There is evidence that HIV may alter or worsen manifestations of syphilis in coinfected persons. Clinically, persons coinfected with HIV and syphilis are more likely to have multiple ulcers and simultaneous clinical manifestations of primary and secondary syphilis (Rompalo et al. 2001). Following treatment for syphilis, patients coinfected with HIV are more likely to fail to show expected reductions in syphilis serological titers (Rolfs et al. 1997; Ghanem et al. 2007); effective antiretroviral treatment (ART) that improves CD4+ T cell count reduces serological failure (Ghanem et al. 2008). For neurosyphilis, neurological manifestations are more common and more severe in the setting of HIV infection, and cerebrospinal fluid (CSF) abnormalities of neurosyphilis are more common in persons with a CD4+ T cell count of $\leq$350 cells/ml and/or a rapid plasma reagin (RPR) titer of $\geq$1:32 (Marra et al. 2004a; Libois et al. 2007; Ghanem et al. 2009). In the era of effective ART, persons with immune reconstitution due to ART are less likely to have CSF abnormalities suggestive of neurosyphilis.

Conversely, there is conflicting evidence about whether *T. pallidum* coinfection has a deletory effect on the immunologic and virologic status in HIV-infected persons. In several studies, syphilis was associated with increased HIV viral replication and a decrease in CD4+ T cell count, which improved following syphilis treatment (Buchacz et al. 2004; Kofoed et al. 2006; Palacios et al. 2007). However, another study reported no association of early syphilis infection on changes of CD4+ T cell count or HIV RNA concentrations present in the blood or semen (Sadiq et al. 2005). On the other hand, HIV-infected persons with neurosyphilis have higher CSF HIV RNA concentrations, suggesting a synergy between *T. pallidum* and HIV in the central nervous system (CNS) (de Almeida et al. 2010).

The long-term consequences of syphilis infection on the HIV-infected individual's prognosis are not known. In a prospective study, syphilis did not appear to affect HIV progression despite transient changes in CD4+ T cell counts and viral loads (Weintrob et al. 2010). This study design however did not consider stage of syphilis infection at the time of diagnosis and the response to syphilis treatment in the analysis.

Early CNS Invasion and Neurological Involvement

Most people consider CNS involvement by syphilis to be a manifestation of the tertiary stage of infection because the most dramatic manifestations of neurosyphilis (tabes dorsalis, general paresis) occur after many years of infection. However, it was recognized 100 years ago that neuroinvasion by *T. pallidum* occurs in the very early stages of infection, concurrent with dissemination of *T. pallidum* to other parts of the body. The precise mechanisms of neuroinvasion and the breaching of the blood–brain barrier are unknown but may be similar to the mechanisms used by *T. pallidum* to enter and exit the bloodstream. Invasion of the CNS, evidenced by the presence of *T. pallidum* in CSF, has been demonstrated in ~30% of patients with primary and secondary syphilis (Lukehart et al. 1988; Rolfs et al. 1997) and occurs independently of HIV infection. A distinction has been made between CNS "invasion," which occurs first, and "involvement," in which signs of *T. pallidum*-induced inflammation (increased CSF mononuclear white blood cell {WBC} and protein concentrations) are present (Moore and Hopkins 1930). Neurosyphilis is diagnosed when these CSF abnormalities reach threshold levels, by development of a reactive CSF-Venereal Disease Research Laboratory (CSF-VDRL) test or by clinical manifestations consistent with neurosyphilis. Some affected patients may have asymptomatic neurosyphilis, that is, they have no neurologic symptoms but have abnormal CSF measures only. Up to 40% of early syphilis patients and 25% of latent syphilis patients meet at least one diagnostic criterion for neurosyphilis (Lukehart et al. 1988).

A majority of immunocompetent patients with CNS infection appear to control or clear CNS *T. pallidum*, with only a minority developing neurological signs or symptoms. However, the factors that determine progression of CNS involvement to symptomatic neurosyphilis are unknown. Several studies indicate that a serum RPR titer $\geq$1:32 is significantly associated with neurosyphilis, irrespective of HIV coinfection or stage of syphilis infection (Marra et al. 2004a; Libois et al. 2007; Ghanem et al. 2009). While predictive, this finding provides no clue as to the factors controlling ultimate development of neurosyphilis.

Early neurosyphilis may be symptomatic and may appear during or following the primary or secondary stages of infection, especially in HIV-infected individuals (Musher et al. 1990). Symptoms include meningitis (headache, fever, and stiff neck), visual changes (blurred vision, photophobia, and other signs of ocular inflammation), hearing changes or hearing loss, and facial weakness. Rarely, patients may have mental confusion or changes in memory. In early neurosyphilis, the clinical manifestations are thought to be caused by local inflammatory responses to the presence of the organism in the meninges, eyes, and cranial nerves. Meningovascular neurosyphilis, manifesting as headaches and stroke, may appear 7–10 years following initial infection and is thought to be due to endarteritis and perivascular inflammation, often of the middle cerebral artery. HIV-infected individuals may be more likely to have symptomatic neurosyphilis (Marra et al. 2004a; Taylor et al. 2008), and these individuals tend to have high CSF WBC cell counts and CSF protein concentrations (Poliseli et al. 2008). ART treatment of HIV-infected patients decreases the odds of developing neurosyphilis by 65% (Ghanem et al. 2008), suggesting that immune reconstitution following ART may result in better control of the CNS infection due to an improved immune response against *T. pallidum*.

Diagnosis of asymptomatic neurosyphilis is complicated by the fact that none of the CSF measures currently used is very sensitive (CSF-VDRL) or specific (CSF WBC, CSF protein concentrations). In addition, HIV infection itself may cause an elevated CSF WBC count. CXCL13, a B cell chemokine that is elevated in the CSF of HIV-infected patients with neurosyphilis compared to HIV-infected patients with uncomplicated syphilis, may serve as a more specific and sensitive adjunct marker for neurosyphilis (Marra et al. 2010b). CXCL13 is also elevated in the CSF of persons with neurological involvement by *Borrelia burgdorferi*, a "spirochete cousin" of *T. pallidum* (Rupprecht et al. 2007).

Latent Syphilis: Immune Evasion, Surface-Exposed Antigens, and Recurrent Secondary Syphilis

Despite the effective local bacterial clearance that results in healing of primary and secondary lesions, treponemes remain in many tissues throughout the body without causing clinical signs or symptoms, called the latent stage. Latent syphilis is further divided into two stages: early (within the first year of infection) and late (infection of $\geq$1 year or unknown duration). During the latent stage, *T. pallidum* appears to seed the bloodstream intermittently, as evidenced by the fact that women with latent syphilis may deliver an infant with congenital syphilis, reflecting transplacental infection of the fetus during pregnancy. Because latent syphilis is, by definition, asymptomatic, sexual transmission is thought to be unlikely. In the retrospective Oslo study of untreated syphilis, however, approximately 25% of persons developed recurrent secondary symptoms, usually within the first year of infection (Gjestland 1955). Thus, persons with early latent syphilis ($<$1 year of infection) are considered to be potentially infectious by either obvious (e.g., recurrent rash) or occult (e.g., mucous membrane) lesions. There are no modern data on the frequency of recurrent secondary lesions, as patients are treated when they are first diagnosed, so recurrences are not noted. Given the reduced ability of HIV-infected persons to control residual *T. pallidum* organisms, it is not unreasonable to expect that recurrent lesions might be more common in untreated persons with HIV coinfection, although there is no direct evidence to support this idea. Regardless, it is recognized that untreated syphilis is a chronic infection, that *T. pallidum* can persist for decades, and that reactivation of treponemes and reappearance of clinical disease may occur, even after many years.

Given the fragility of *T. pallidum* in vitro, and the obvious active and effective local immune response during the early stages, it has been difficult to explain the ability of the bacterium

to persist during lifetime latency. Over the past several decades, a number of hypotheses have been proposed: (1) *T. pallidum* may reside in intracellular locations or immune privileged sites (Azar et al. 1970; Medici 1972; Sykes et al. 1974), thus avoiding interaction with antibodies and immune cells; (2) *T. pallidum* may hide in a "cloak" by coating its surface with host serum or extracellular matrix proteins or mucopolysaccharides, rendering it invisible to immune cells (Christiansen 1963; Fitzgerald and Johnson 1979; Alderete and Baseman 1980); or (3) specific or generalized immunosuppression of the host may occur during syphilis infection (Musher et al. 1975; Wicher and Wicher 1977a, b). There is some merit to the first two hypotheses, although the third hypothesis has been dismissed (Radolf and Lukehart 2006), as it is clear that there is a very effective humoral and cellular immune response that clears treponemes from early lesions and, in immunocompetent persons, controls infection during the decades of latency.

Twenty years ago, it was demonstrated that the rare treponemes that survive immune clearance in the primary lesion in vivo are able to resist phagocytosis by macrophages, even in the presence of immune serum (Lukehart et al. 1992). This was not due to inhibition of macrophage function but was hypothesized at the time to be due to some alteration of the surface-exposed antigens on the persistent bacteria, resulting in failure of immune serum to opsonize the bacteria. It has now been shown that *T. pallidum* can evade the acquired immune response by antigenic variation of surface proteins, providing a mechanistic explanation for the older observations. Antigenic variation is a well-recognized characteristic of pathogenic spirochetes, such as the relapsing fever Borrelia and the agent of Lyme disease, *Borrelia burgdorferi*. Like syphilis, each of these infections also demonstrates a multistage clinical course (Barbour et al. 1991; Zhang et al. 1997).

As mentioned above, freeze-fracture and cryoelectron tomography (cryo-ET) studies indicate that there are very few integral OMPs in *T. pallidum* compared to traditional Gram-negative bacteria (Radolf et al. 1989b; Walker et al. 1989; Izard et al. 2009; Liu et al. 2010). While *E. coli* has 6,000–10,000 demonstrable integral OMPs per μm^2, the surface of *T. pallidum* revealed only 100/μm^2 or 1% of the number in *E. coli* (Walker et al. 1991). The cryo-ET studies show detailed structures of the *T. pallidum* cellular envelope, highlighting an additional difference from Gram-negative bacteria: the peptidoglycan layer is more closely associated with the cytoplasmic membrane than the outer membrane in *T. pallidum*, thus perhaps explaining the exquisite fragility of the *T. pallidum* OM. Despite the confirmed findings of limited numbers of OMPs in *T. pallidum*, experimental evidence suggests that specific ECM-binding proteins and targets of opsonic antibody must be surface exposed on the intact bacterium. Biochemical approaches to the identification of surface-exposed proteins have been hampered by the sensitivity of the *T. pallidum* OM to damage upon manipulation of the bacteria in vitro, by structural differences from typical Gram-negative bacteria that render detergent-based selective solubilization approaches inappropriate, and by the very low copy number of surface-associated proteins. Some attempts have been made to immunostain surface proteins of *T. pallidum*, but the results have been very disappointing, with only 1–2% of bacteria staining even when high-titer polyvalent antiserum is used (Cox et al. 2010). Nonetheless, bioinformatic approaches, possible after the sequencing of the *T. pallidum* genome, have resulted in significant progress in the identification of putative *T. pallidum* OMPs.

Computational prediction programs have been used to analyze the *T. pallidum* genome, yielding a number of candidate surface proteins including those previously discussed as binding the extracellular matrix components (Cameron 2003; Cameron et al. 2004, 2005; Castro et al. 2007; Brinkman et al. 2008). Structural algorithms have also been utilized recently to identify candidate outer membrane proteins (Cox et al. 2010). These lists include Tp92 (TP0326), which was recently shown to be a BamA homolog (Cameron et al. 2000; Desrosiers et al. 2011), and many members of the *T. pallidum* repeat (Tpr) family (Centurion-Lara et al. 1999), which have homology to the major sheath protein of *Treponema denticola* (Fenno et al. 1996; Mathers et al. 1996). The 12-membered Tpr family is divided into three subfamilies according to amino acid homology: subfamily I (TprC, D, F, and I), subfamily II (TprE, G, and J), and subfamily III (TprA, B, H, K, and L) (Centurion-Lara et al. 1999). Many of these proteins are predicted to be in the outer membrane (Centurion-Lara et al. 1999; Giacani et al. 2005; Cox et al. 2010), and both cellular and humoral immune responses are induced by expression of these proteins during infection (Leader et al. 2003; Giacani et al. 2007b). Unpublished opsonization data support the surface exposure of many of these antigens. Expression of the subfamily II genes is regulated by the length of a homopolymeric guanosine repeat sequence (poly-G) directly upstream of the transcriptional start site of these genes: a repeat sequence containing greater than 9 G's results in no transcription and provides a phase variation mechanism for these proteins (Giacani et al. 2007a). Phase variation is employed by a number of bacteria to "turn off" expression of immunogenic proteins when their expression is no longer needed during infection, resulting in effective immune evasion. This may occur with the subfamily II Tpr proteins during syphilis infection, although direct evidence for this mechanism has not yet been described in vivo.

TprK is the best studied of the Tpr proteins. Although there is controversy in the older literature about whether TprK is an OMP (Centurion-Lara et al. 1999; Hazlett et al. 2001), there is now compelling experimental evidence that portions of the TprK protein are exposed on the surface of *T. pallidum* and that functional antibodies can access this protein in vivo. Studies in the rabbit model indicate that, during syphilis infection, strong and early antibody and T cell responses are elicited against TprK (Morgan et al. 2002a, b; Leader et al. 2003; Giacani et al. 2007b) and immunization with recombinant TprK provides partial protection against infectious challenge (Centurion-Lara et al. 1999; Morgan et al. 2002a). Antibodies developed in response to immunization with full-length recombinant TprK

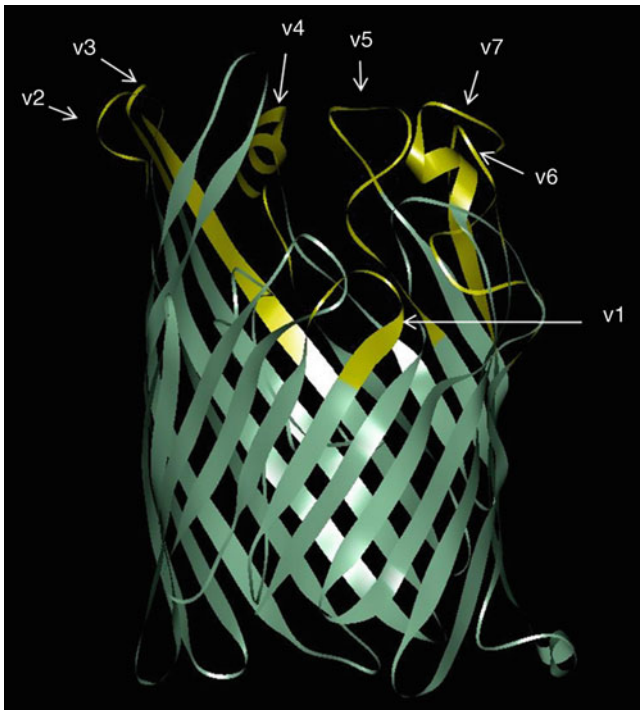

Fig. 20.5
Structural model of TprK, showing that V regions (yellow) are predicted to be surface exposed. Antigenic variation of the surface-exposed V regions of TprK produces new TprK variants that are positively selected by the immune response that effectively clears treponemes expressing earlier variants (Model courtesy of Arturo Centurion-Lara, Manish Mishra, and Ram Samudrala, University of Washington)

(Centurion-Lara et al. 1999) and peptides of TprK (unpublished results) can opsonize *T. pallidum* for phagocytosis by macrophages in vitro. Sequencing of *tprK* from many strains of *T. pallidum* revealed that impressive sequence diversity exists, even within an individual strain (Centurion-Lara et al. 2000; Stamm and Bergen 2000b), and that this sequence diversity is limited to seven discrete variable (V) regions within the gene and its encoded protein. Antibodies that develop during infection react predominantly with these V regions (Morgan et al. 2002b), and even minor changes in amino acids in these V regions abrogate binding of antibodies raised to other V regions (LaFond et al. 2006). Structural predictions for TprK indicate that the V regions, along with several conserved regions, form surface-exposed loops on the surface of the organisms (*Fig. 20.5*). These surface loops are hypothesized to be the targets of opsonic antibody.

In the rabbit model, TprK sequence diversity accumulates in *T. pallidum* strains during serial passage (LaFond et al. 2003, 2006) and during the course of infection (Centurion-Lara et al. 2004). A higher proportion of variants is found in vivo following development of acquired immunity, leading to the hypothesis that this is an antigenic variation system and that treponemes expressing variant TprK are positively selected by the immune response. A recent publication demonstrated the role of the immune response in selection of variants in two complementary ways, showing that (1) immunosuppression of infected rabbits reduces in vivo selection of variants and (2) prior immunization with a V6 peptide increases selection for V6 variants in vivo (Giacani et al. 2010). Molecular studies of *tprK* indicate that segmental gene conversion is the mechanism by which new variants arise: new variant sequences are derived from a large repertoire of unchanging "donor sites" located at a site on the chromosome distant from the expression site (*Fig. 20.6*) (Centurion-Lara et al. 2004). In our model of TprK antigenic variation, alterations in the V region sequences impair the binding of antibodies raised against the original infecting sequence, thus protecting the variant organisms from opsonophagocytosis. We hypothesize that it is these TprK-variant treponemes that survive bacterial clearance in the primary and secondary lesions to cause the later stages of disease, including recurrent secondary syphilis, as well as chronic latent and active tertiary infection.

Tertiary Syphilis

In some individuals, the smoldering chronic infection reactivates to cause tertiary syphilis, which occurs years to decades after initial infection and can affect multiple organs. In the retrospective study of Oslo patients from the pre-antibiotic era, approximately one-third of patients with untreated latent syphilis infection ultimately developed tertiary syphilis (Clark and Danbolt 1955; Gjestland 1955). Manifestations may include gumma, cardiovascular syphilis, and tertiary neurosyphilis. In the modern antibiotic era, tertiary syphilis is rarely seen, perhaps due to inadvertent syphilis treatment with antibiotics prescribed for other infections.

Gummas are granulomatous nodules with variable central necrosis. While they can appear as early as 2 years after initial infection, gummas are usually seen after 15–20 years of infection. Most commonly, the skin and bones are affected, but gummas are also seen in the liver, heart, brain, stomach, and respiratory tract. Based on observational clinical studies, the pathogenesis of gumma is thought to be due to an intense local cell-mediated response to a focus of replicating spirochetes (Magnuson et al. 1956). By the time a gumma is recognized clinically, however, treponemes are scarce (presumably due to the local immune response), and these lesions are generally not considered to be infectious. The rapid resolution of gummas following antibiotic treatment for syphilis supports the presence of *T. pallidum* as a trigger for the induction of the intense local response. This form of syphilis is also called "late benign" syphilis because gummas usually do not cause serious complications unless tissue destruction occurs in a vital organ.

Tertiary cardiovascular syphilis consists primarily of aortitis, usually of the ascending aorta. While most cases are asymptomatic, complications occur in 10% of untreated patients

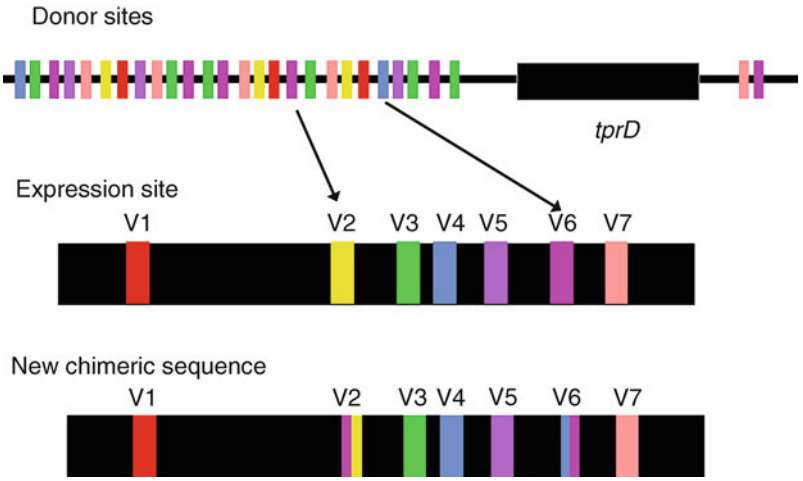

Fig. 20.6

Gene conversion model of TprK antigenic variation in which sequence segments from donor sites, located near the *tprD* locus and distal to the *tprK* expression site, replace portions of the V regions in the expression locus. This results in chimeric V regions that are expressed as antigenically variant TprK proteins. The sequences of the donor sites do not change during this process, indicating that gene conversion (rather that reciprocal recombination) is the mechanism (Centurion-Lara et al. 2004)

(Kampmeier 1964) and may include aortic regurgitation, coronary ostial stenosis, and saccular aneurysm (Jackman and Radolf 1989). *T. pallidum* invades the vasa vasorum, the blood vessels of the aortic adventitia, which leads to inflammation, obliterative endarteritis, adventitial scarring, medial necrosis, and destruction of the elastin fibers, resulting in an aneurysm. Confirming that infection is the pathogenetic mechanism underlying aneurysm formation, *T. pallidum* DNA has been detected using the polymerase chain reaction (PCR) in aortic aneurysm tissue, and the DNA was localized to the adventitia (O'Regan et al. 2002).

Early invasion of the CNS by *T. pallidum* may progress to meningovascular syphilis, resulting in stroke, usually occurring 7–10 years after infection. The presumed pathogenic mechanism is likely similar to aortitis. Stroke from syphilis infection may have prodromal symptoms of headache, vertigo, and personality changes, and in HIV-infected individuals, stroke may occur as early as a few months after initial infection (Johns et al. 1987).

Two to three decades after infection, general paresis (cognitive impairment, personality change, hyperreflexia) and tabes dorsalis (loss of vibratory sensation and joint position sense, paresthesia, sensory ataxia) may be observed. Both general paresis and tabes dorsalis can have concomitant pupillary abnormalities (the Argyll Robertson pupil) and optic atrophy, leading to blindness. These late forms of neurosyphilis are rarely seen today, and the mechanisms underlying neurologic symptoms of tertiary syphilis are not understood.

Congenital Syphilis

Congenital syphilis is a major public health concern, particularly in developing countries, where it accounts for up to 25% of stillbirth and neonatal morbidity (Schmid et al. 2007). Although readily preventable by early prenatal serological testing and antibiotic treatment of infected pregnant women, lack of access to care and lack of care-seeking behaviors have perpetuated this potentially devastating form of syphilis. The developing fetus can be infected with *T. pallidum* at any time during pregnancy, via seeding of *T. pallidum* into the bloodstream and consequent invasion of the placenta, and the highest rate of transmission occurs during the early stages of syphilis infection in the pregnant woman (Sheffield et al. 2002). The recent development and implementation of the use of treponemal point-of-care serological tests now provides the means to make prenatal testing available even in the most remote settings (Peeling et al. 2006). The great potential provided by these tests has recently led the World Health Organization and the US Centers for Disease Control (CDC) to identify prevention of congenital syphilis as a high priority "winnable battle" in public health. Antibiotic treatment of the woman during the first two trimesters of pregnancy will usually prevent fetal demise, congenital defects, low birth weight, and chronic infection in the infant. In untreated congenital syphilis, complications that may cause neonatal death include prematurity, pulmonary hemorrhage, hepatitis, and secondary infection (Fiumara 1975; Chapel 1988).

Similar to syphilis in human adults, congenital syphilis is divided into stages of infection. Early manifestations occur within the first 2 years of life and resemble adult secondary syphilis. Symptoms include rash, mucous patches, condylomata lata, anemia, hepatosplenomegaly, osteochondritis of the long bones, and renal disease. Approximately 50% of infants develop "snuffles" due to *T. pallidum* invasion of the nasal mucosa. Late manifestations occur after the age of 2, often in adolescence, and may persist despite ongoing treatment. These include interstitial keratitis, sensorineural hearing loss, neurosyphilis, arthropathy, joint effusions, gummatous periostitis of the palate and nasal

septum, Hutchinson's teeth (peg-shaped notched upper incisors), and other abnormalities of permanent teeth.

Diagnosis of Syphilis

Because *T. pallidum* cannot be cultured, measurement of antibodies is important for screening and diagnosis of syphilis infection. Two categories of antibodies, termed "nontreponemal" and "treponemal," have been used for this purpose. The "nontreponemal" antibodies, currently detected by the RPR and VDRL tests, were first identified in 1906 using the Wassermann reaction. In this first serological test for syphilis, patients' sera were reacted with an extract of liver from a fetus with congenital syphilis; later an extract of beef heart was used as the antigen. These "nontreponemal" antibodies react to phospholipids, such as cardiolipin, but the mechanism underlying the production of these antibodies continues to be debated. It had been thought that these antibodies arose due to an autoimmune reaction to tissue damage stimulated by *T. pallidum* infection. Examination of the *T. pallidum* genome, however, reveals that *T. pallidum*'s ability to synthesize lipids is extremely limited and it is highly likely that treponemal lipids are largely obtained from the host. The identification of several homologues of fatty acid transporters in the genome supports this hypothesis, although it is unknown whether *T. pallidum* modifies these host lipids. Interestingly, a monoclonal antibody with extraordinarily high killing activity for *T. pallidum* was derived following immunization with outer membrane vesicles of *T. pallidum* and was shown to have specificity for a surface-localized phosphorylcholine-containing component (Blanco et al. 2005). Persons with autoimmune conditions such as systemic lupus erythematosus may be falsely positive in the nontreponemal tests.

Treponemal antibodies are thought to be directed against *T. pallidum* polypeptides and are detected by the fluorescent treponemal antibody-absorbed (FTA-ABS) test or by agglutination in the *Treponema pallidum* hemagglutination (TPHA) or *Treponema pallidum* particle agglutination (TP-PA) tests. It is important to note that measurements of treponemal antibodies may be falsely reactive due to antibodies induced by cross-reactive antigens in commensal treponemes or treponemes associated with periodontal disease. Thus, sera used in the above tests are typically diluted in a "sorbent solution" in an attempt to remove these cross-reactive antibodies. During primary syphilis, IgM and IgG antibodies against treponemal antigens are detectable, appearing shortly after appearance of the chancre. As the patient develops secondary syphilis, IgM levels increase, and IgG1 and IgG3 constitute approximately 90% of the IgG antibodies in the serum (Baughn et al. 1988). After therapy, IgM levels decline slowly (Baker-Zander et al. 1985; Baughn et al. 1988), but IgG antibodies may persist for many years (Miller 1975; Hanff et al. 1982; Baker-Zander et al. 1985), leading some to speculate that *T. pallidum* may not be completely cleared from the host following antibiotic treatment. A similar humoral immune response has been observed in the rabbit experimental model (Lukehart et al. 1982, 1986).

Using a protein array of 882 polypeptides predicted to be in the *T. pallidum* proteome, 106 proteins were identified that were recognized by antisera from patients with syphilis (Brinkman et al. 2006). Two proteins that induce high titers of antibodies include TpN17 (TP0435) and TpN47 (TP0574). These well-known immunodominant lipoprotein antigens (Hanff et al. 1982; Lukehart et al. 1982; Baker-Zander et al. 1985; Lukehart et al. 1985; Akins et al. 1993; Hayden 2003) are currently utilized in the new point-of-care tests (Herring et al. 2006; Mabey et al. 2006), and in enzyme immunoassay (EIA) or chemiluminescence (CIA) tests for syphilis diagnostic testing.

Traditionally, *T. pallidum* infection has been diagnosed using a nontreponemal test for screening, with reactive results confirmed using treponemal serologic tests. The new EIA and CIA tests detect antitreponemal IgM and IgG antibodies, usually to recombinant TpN47 and TpN17 antigens, and are highly sensitive. The ability to automate these tests has led some large laboratories in the United States to use new syphilis screening algorithms that begin with a treponemal test. This shift has caused significant confusion among clinicians, as the appropriate interpretation of reactive tests is unclear.

The EIA and CIA tests have two major disadvantages: (1) they cannot distinguish between recent versus remote syphilis, nor between treated versus untreated infection, and (2) they are more sensitive than the FTA-ABS or agglutination tests and do not include the "sorbent" treatment of the serum. Consequently, when used for initial screening, reactive results are seen in many persons who are highly unlikely to have syphilis. Syphilis screening is conducted on a large number of persons from low-risk populations (e.g., prenatal screening or blood bank screening in developed countries).

Consequently, these reactive sera must be retested using the nontreponemal tests. A recent study reported that 58% of sera that are reactive in the EIA/CIA screening test are nonreactive in the nontreponemal tests (Park et al. 2011), leading to confusion for the clinicians, emotional trauma for the patient, and high costs for the public health departments who are charged with epidemiological follow-up on all syphilis cases. These results have led to concerns about the specificity of the antigens used in these tests for syphilis infection. Indeed, a 20-year-old published study reported that persons with periodontal disease, which is extremely common, carry oral treponemes that can be detected with monoclonal antibodies to the same TpN47 and TpN17 antigens used in many EIA/CIA tests. These patients have serum IgG that reacts with these antigens (Riviere et al. 1991), potentially causing false-positive EIA/CIA tests. Although screening with these tests is cost-effective for the laboratory because the tests are automated, the need for multiple follow-up tests to sort out inappropriately reactive results increases costs for the patient, public health departments, and insurance companies while making even more profit for the labs. A recent study has shown that, with the new algorithm necessary for interpreting the results from reactive EIA/CIA screening, this screening method requires significantly more confirmatory testing and may lead to unnecessary treatment (Owusu-Edusei et al. 2011).

Treatment

Penicillin has been used successfully to treat syphilis for over 60 years, with clinical resolution, prevention of disease progression, and interruption of sexual transmission. Unlike many other bacteria, *T. pallidum* remains penicillin sensitive. Thus, penicillin remains the treatment of choice for syphilis infection. *T. pallidum* has no known plasmids (an early report (Norgard and Miller 1981) has not been substantiated) and is not thought to be naturally competent for uptake of exogenous DNA, so mechanisms for acquisition of resistance genes are limited. Penicillin-binding proteins function in cell wall synthesis, and penicillin acts primarily by interfering with cross-linking of peptidoglycan during cell wall synthesis. Several *T. pallidum* proteins have been determined to bind penicillin (Cunningham et al. 1987; Radolf et al. 1989a; Weigel et al. 1994), including the immunogenic lipoprotein, TpN47, that is used in newer diagnostic tests for syphilis. In addition to binding penicillin, TpN47 also has been shown to have β-lactamase activity (Cha et al. 2004); however, this activity is inhibited by products of the reaction, and this has been proposed to explain, in part, why *T. pallidum* remains penicillin sensitive.

Because of the very long propagation time for *T. pallidum*, it is necessary to maintain appropriate drug levels for at least 10 days for effective treatment of early syphilis, either through repeated dosing of short-lived antibiotics or through use of a long-acting drug. Benzathine penicillin G (BPG) is a depot form of penicillin that provides treponemicidal levels for several weeks following a single dose. Although BPG must be administered by deep and painful intramuscular (I.M.) injections, the exquisite sensitivity of *T. pallidum* to penicillin and concerns about patient compliance with repeated dosing of alternative antibiotics have led to a very strong recommendation for the use of BPG for treatment of syphilis. The US Centers for Disease Control and Prevention (CDC) 2010 STD Treatment Guidelines recommend that treatment for uncomplicated early syphilis in adults be BPG administered intramuscularly as a single dose of 2.4 million international units (CDC 2010b). Patients with late latent or tertiary syphilis (without neurosyphilis) should be treated with 3 weekly doses of 2.4 million units of BPG. Treatment failure, which is usually defined serologically by failure of the RPR or VDRL titer to decline fourfold within 6–12 months after treatment, occurs approximately in 3–8% of individuals treated for early syphilis and 3–10% of individuals treated for late syphilis (Idsoe et al. 1972). Clinical failure to resolve syphilis lesions following BPG treatment is rare in the absence of reinfection.

One possible reason for serological failure is the failure of BPG to achieve therapeutic levels in the central nervous system, which is invaded by *T. pallidum* in ~30% of persons with early syphilis. Several studies have demonstrated the isolation or PCR identification of *T. pallidum* in CSF following BPG treatment (Lukehart et al. 1988; Rolfs et al. 1997), so it is clear that the organisms can survive BPG treatment in that setting. Consequently, a different form of penicillin, with better CNS penetration, is recommended for treatment of neurosyphilis to ensure treponemicidal levels of penicillin in the CSF. The recommended treatment for neurosyphilis is 18–24 million units of intravenous aqueous penicillin G daily for 10–14 days or, alternatively, 2.4 million units of I.M. procaine penicillin administered daily with 500 mg of oral probenecid taken four times daily for 10–14 days. After treatment with the neurosyphilis regimens, normalization of CSF WBC count, protein concentrations, and CSF-VDRL titer is used to measure adequacy of therapy. Normalization of serum RPR after treatment with neurosyphilis regimens predicts normalization of CSF abnormalities, suggesting that serum RPR is an indicator of successful neurosyphilis treatment (Marra et al. 2008). It is unclear, however, whether normalization of serum RPR after BPG treatment is indicative of normalization of unrecognized CSF abnormalities.

Syphilis in HIV-infected individuals has been associated with increased rates of BPG treatment failure (Rolfs et al. 1997; Ghanem et al. 2007; Horberg et al. 2010), and ART is associated with a reduction in the rate of serologic failure (Ghanem et al. 2008). In addition, compared to HIV-uninfected individuals, HIV-infected individuals with neurosyphilis are less likely to normalize CSF lab values and may take longer to resolve CSF abnormalities following treatment (Marra et al. 2004b; Ghanem et al. 2008). Despite these concerns, the CDC does not currently recommend lumbar puncture for identification of persons with neurosyphilis except in those with neurological symptoms and signs, and recommends that HIV-infected individuals undergo the same treatment for syphilis and neurosyphilis as HIV-uninfected individuals (CDC 2010b). These recommendations are controversial, particularly for HIV-infected persons who are not on ART. Given the higher rates of relapse in HIV-infected persons treated with BPG, however, these persons should be followed closely to monitor serologic and CSF response to therapy.

Alternative Antibiotic Treatments

Macrolides (e.g., erythromycin and azithromycin) and tetracyclines (e.g., tetracycline and doxycycline) have historically been used as alternatives to penicillin in penicillin-allergic patients (except for pregnant women). The tetracyclines and erythromycin require multiple daily doses for 2–4 weeks, and compliance with these regimens is not optimal. In contrast, azithromycin is a long-lasting antibiotic with demonstrated activity against syphilis infection in the rabbit model (Lukehart et al. 1990). Early syphilis in humans has been successfully treated with a single 1–2 g dose of azithromycin (Mashkilleyson et al. 1996; Hook et al. 1999; Gruber et al. 2000; Hook et al. 2002; Kiddugavu et al. 2005; Riedner et al. 2005; Hook et al. 2010) with efficacy equal to standard I.M. administration of BPG.

Azithromycin and other macrolide-like drugs are bacteriostatic antibiotics that inhibit bacterial protein synthesis by reversibly binding to the 23S rRNA of the 50S ribosomal subunit. Bacteria develop resistance to macrolides, in part, via single nucleotide mutations in the 23S rRNA gene (Vester and Douthwaite 2001), and an alarming rate of macrolide resistance is now seen in circulating *T. pallidum* strains. Until 2004, the

only recognized macrolide-resistant strain of *T. pallidum* was Street strain 14, isolated in 1977 from a patient who had failed long-term erythromycin therapy for secondary syphilis (Stapleton et al. 1985; Stamm et al. 1988). This particular strain is highly resistant to a number of macrolides as well as azithromycin (Stamm et al. 1988; Stamm and Parrish 1990) due to an adenine (A)-to-guanine (G) mutation at the position cognate to A2058 in the *E. coli* 23 S rRNA gene (Stamm and Bergen 2000a). In 2003, clinical failures following azithromycin treatment in San Francisco led to a broad examination of *T. pallidum* samples from multiple sites for similar mutations. Swab samples from lesions of syphilis patients from San Francisco, Baltimore, Seattle, and Dublin revealed the presence of the A2058G macrolide resistance mutation in 11–88% of sampled patients, and the association of this mutation with clinical failure of azithromycin treatment in San Francisco (Lukehart et al. 2004; Mitchell et al. 2006). A new mutation, A2059G, in the *T. pallidum* 23 S rRNA gene was identified in clinical specimens of persons failing spiramycin treatment in the Czech Republic (Matejkova et al. 2009). These mutations have been previously recognized in other bacteria to confer resistance to a number of macrolide-type antibiotics including erythromycin, azithromycin, spiramycin, and clarithromycin (Vester and Douthwaite 2001; Woznicova et al. 2010).

In San Francisco and Seattle, where surveillance for macrolide resistance is ongoing, the frequency of finding the A2058G mutation in samples from syphilis patients has increased steadily over time (Lukehart et al. 2004; Mitchell et al. 2006). Although there is no formalized global surveillance program for detection of macrolide-resistant *T. pallidum* strains, one clinical study indicated treatment failure in 132 syphilis patients who received azithromycin therapy in Shanghai from 2001 to 2008 (Zhou et al. 2010). During the past 8 years, molecular studies have revealed a frequency of these mutations of 100% in Shanghai (Martin et al. 2009), 36% in the Czech Republic (Matejkova et al. 2009), 44% in British Columbia (Morshed and Jones 2006), and 67% in London (Tipple et al. 2011b). These findings mandate a very strong caution against the use of azithromycin as first-line treatment for syphilis.

Three recently published successful trials of azithromycin treatment of syphilis in Africa (Kiddugavu et al. 2005; Riedner et al. 2005; Hook et al. 2010) have led some to conclude that macrolide-resistant strains are not present in Africa (Van Damme et al. 2009). It should be noted that patients were enrolled in these studies in 1994–1997, 2000–2003, and 2000–2007, either before, or very early in, the recognition of macrolide-resistant strains elsewhere in the world. There are no recent data on macrolide resistance from Africa or South America, and the absence of recent data does not indicate absence of resistant strains.

Jarisch-Herxheimer Reaction

Treatment for early syphilis frequently results in the Jarisch-Herxheimer (J-H) reaction, which occurs within hours of penicillin (or other antibiotic) administration and results in systemic symptoms such as fever, headache, arthralgias, and myalgias. In addition, syphilis lesions and other clinical symptoms may transiently increase in prominence. The J-H reaction is thought to be a systemic inflammatory response to *T. pallidum* products that arise from dead or dying spirochetes as antibiotic therapy commences. While *T. pallidum* lacks lipopolysaccharide, which can trigger systemic inflammatory changes in other infections, the many *T. pallidum* lipoproteins are highly expressed, can stimulate an inflammatory cytokine response, and may be responsible for this reaction.

Molecular Strain Typing of *T. pallidum*

Knowledge of the epidemiology of other infections has been significantly enhanced by the development of simple and highly discriminating molecular methods for strain typing. Until recently, no typing systems existed for *T. pallidum.* Particularly in light of the increasing incidence of infectious syphilis and the recognition of macrolide-resistant strains, the development and optimization of a sensitive and culture-independent method for differentiating one strain of *T. pallidum* from another is critical. Pillay and colleagues have described a typing method based on (1) determination of the number of 60-bp repeats in the acidic repeat protein (*arp*) gene and (2) sequence differences in the *T. pallidum* repeat (*tpr*) subfamily II genes (*tprE {tp0313}, tprG {tp0317}*, and *tprJ {tp0621}*) determined by restriction fragment length polymorphism (RFLP) analysis (Pillay et al. 1998). This method for subtype designation has been applied to bacterial DNA recovered from a variety of patient samples (including chancres, condylomata lata, mouth scrapings, earlobe scrapings, blood, and CSF) and to laboratory-passaged *T. pallidum* isolates from diverse geographic areas (Pillay et al. 1998, 2002; Sutton et al. 2001; Pope et al. 2005; Molepo et al. 2007; Florindo et al. 2008; Castro et al. 2009; Cole et al. 2009). Epidemiological studies of the strain types in San Francisco and Seattle in the last decade showed that the majority of cases were of strain subtype 14d (Katz et al. 2010; Marra et al. 2010a), which suggests a linked sexual network, while the studies listed above indicate variation in the distribution of predominant strain types by location in the USA and worldwide. A study sampling recent strain types present in Shanghai, China, showed that the majority of syphilis cases were 14f (Martin et al. 2009). One weakness of this typing scheme is its relatively low discrimination, and several laboratories have successfully evaluated additional gene targets to increase the utility of the typing scheme (Katz et al. 2010; Marra et al. 2010a). For example, the addition of the *tp0548* gene revealed the replacement of the formerly predominant 14d/f strain in Seattle by the 14d/g strain during the period from 1999 to 2008 (Marra et al. 2010a). Using the original typing method, these two strains would simply have been identified as 14d.

Molecular analysis has been used to determine whether macrolide-resistant isolates of *T. pallidum* belong to a single strain or whether resistance has arisen in multiple strains. The finding that the A2058G mutation was found in molecularly

distinct strains in Seattle suggests that the point mutation may be arising in real time in strains subjected to antibiotic selection. This hypothesis is supported by the report that resistant strains are more likely to be found in patients that had received macrolide antibiotics in the previous 6 months (Marra et al. 2006).

Strain typing may also be useful in identification of strains associated with particular clinical outcomes. The older syphilis literature speculates about "neurotropic strains" of *T. pallidum*, based upon the observation that sexual contacts of persons who developed neurosyphilis were also likely to develop neurosyphilis (Stokes et al. 1944). Studies using a rabbit infection model suggest that some strains are more likely to be neuroinvasive than others (Tantalo et al. 2005). This observation is supported by a recent strain typing study of patients infected with *T. pallidum,* which showed that patients infected with one specific *T. pallidum* strain type, 14d/f, had a higher rate of neurosyphilis compared to patients infected with other strain types (Marra et al. 2010a). This finding requires independent confirmation, and determining whether other strain types are associated with different clinical manifestations of syphilis requires careful future study.

Vaccine Development and Prevention

Despite the facts that inexpensive and highly effective treatment has been available for syphilis for decades and that the public health sector devotes substantial amounts of its budget to contact tracing and epidemiological follow-up of syphilis, syphilis has not been eradicated. The current epidemic of syphilis in China and the increasing syphilis incidence in many developed countries suggest that these long-used methods for disease prevention will not eradicate this infection. The best hope for syphilis control is development of a safe and effective vaccine that stops transmission and, optimally, provides sterile immunity for the immunized person.

Many attempts to produce a successful syphilis vaccine by immunizing rabbits with whole killed or attenuated *T. pallidum* (reviewed in (Lukehart 1985; Cullen and Cameron 2006)) have resulted in failure. Only one study, involving 60 intravenous inoculations of γ-irradiated *T. pallidum* over 37 weeks, demonstrated complete protection against homologous *T. pallidum* infection in the rabbit (Miller 1973). Because this protocol was so cumbersome and expensive, it was too impractical to test in humans. Nonetheless, it does provide proof of principle and yields information that may be important in the design of a practical and effective vaccine: (1) *T. pallidum* immobilizing immunity is not necessary for protection (although production of opsonic antibody was not tested) and (2) it was important to avoid significant physical manipulation of the treponemes used as immunogen to maintain the integrity of the fragile outer membrane of the organisms. Later studies have shown that immunization with individual recombinant *T. pallidum* antigens can stimulate production of an immune response, as evidenced by strongly reactive T cells and antibodies, including some antibodies with opsonic function (Centurion-Lara et al. 1999; Cameron et al. 2000). Infectious challenge of these rabbits, however, has resulted in only partial protection, with significantly attenuated lesion development, but not with sterile immunity (Cameron et al. 2000; Morgan et al. 2002a; Sun et al. 2004; Giacani et al. 2005). Studies using passive transfer of antibodies against *T. pallidum* (Turner 1939; Perine et al. 1973; Bishop and Miller 1976a) or against one of its lipid components (Blanco et al. 2005) also conferred only partial protection, emphasizing that cellular immunity plays an important role in protection against *T. pallidum* infection. The recent discovery of both phase and antigenic variation in several *T. pallidum* surface proteins makes the development of a protective vaccine even more formidable. However, studies testing the protective efficacy of immunization with conserved regions of multiple *T. pallidum* antigens are underway in the rabbit model.

Until a successful vaccine is developed, control of syphilis infection globally will depend upon diagnosis using simple rapid point-of-care tests for widespread screening of at-risk populations, prompt treatment of suspected cases and their partners, and education concerning the risk of unprotected sex. The long incubation period of syphilis and the availability of inexpensive and effective single-dose treatment make these approaches, if implemented broadly, very effective in reducing transmission.

Concluding Statement

Syphilis is one of the most interesting and perplexing diseases—with its multiple and variable clinical manifestations, and both diagnostic and management ambiguities. The organism that causes syphilis is one of the most challenging infectious agents to study because of its inability to be cultured or genetically manipulated, its physical fragility, and an animal model with limited immunological reagents. Despite these challenges, significant progress has been made over the past 10 years in the application of modern molecular techniques to understand the biological basis of the disease process, to develop new tools for diagnosis, to predict efficacy of treatment with alternative antibiotics, and to define the networks of transmission of infection through populations. Less progress has been made in understanding the mechanisms of pathogenesis of syphilis, which will remain quite problematic until a method for genetic manipulation of *T. pallidum* is developed. Much work remains to be done, and the future of syphilis research is dependent upon training the next generation of clinicians and researchers to appreciate, and become fascinated with, this mysterious infection.

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21 Chlamydial Diseases

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Abstract
Chlamydiae are unique among pathogenic bacteria in that they are completely dependent on their host species for survival. They are unable to multiply in any other environmental niche, and do not carry the minimum genetic code necessary to be self-sufficient. This absolute dependence on a specific host species is strongly reflected in the evolution of *Chlamydia* genomes. *Chlamydiae* have further evolved to occupy specific niches within their host species that manifest as tissue tropisms. These bacterial adaptations are counterbalanced by innate and adaptive host defenses. While *Chlamydia* research until recently has been hampered by an inability to genetically manipulate the bacteria, the wealth of pathogenic *Chlamydia* species, hosts, and tissue tropisms has provided a window into the complex relationship between these pathogens, their hosts, and the associated diseases. This chapter covers the spectrum of human illness caused by *Chlamydiae*, and incorporates experimental model data from closely related nonhuman *Chlamydia* species to better understand human disease. This approach is hopefully interesting and informative with the practical outcome of highlighting issues relevant to host defense and vaccine development.

Introduction

Chlamydiae are obligate intracellular bacterial pathogens that cause incidental and species-specific infections in humans. The genus name has been in flux. As the pendulum has swung back to the use of *Chlamydiae*, rather than subdividing the genus into *Chlamydiae* and *Chlamydophila*, the genus name *Chlamydiae* is used throughout this chapter. The major human pathogens within the genus are *Chlamydia psittaci*, *Chlamydia pneumoniae*, and *Chlamydia trachomatis*. These three *Chlamydia* species have different relationships with the human host that manifest as different types of infections. *C. psittaci* causes a disseminated infection, usually with a lung focus. *C. pneumoniae* is a common cause of pneumonia. *C. trachomatis* serovars A–C cause an eye infection known as trachoma, while serovars D–L cause sexually transmitted genital tract infections. The species-specific biology that distinguishes each *Chlamydia* species from the others appears to map to a region of the genome known as the plasticity zone. The second critical pathogenesis determinant for *Chlamydia* species appears to be whether a given species is capable of replicating in the monocyte/macrophage cell lineage. *Chlamydia* species capable of replicating in human monocyte/macrophages cause disseminated infections, while the "incapable others" cause limited local infections of epithelium. These two facets of the *Chlamydia*-host interaction appear to explain most aspects of human *Chlamydia* infections.

The plasticity zone contains genes related to species-specific evasion of host defenses mediated by the interferons, especially interferon gamma (IFN-γ). There are two basic gene evasion packages encoded within the plasticity zones of *Chlamydia* species (Fig. 21.1). Urogenital *C. trachomatis* serovars have a multistep enzymatic pathway for synthesizing tryptophan using environmental indole as a precursor. IFN-γ effects on human epithelial cells and macrophages include upregulation of indoleamine-2,3-dioxygenase (IDO), a host cell enzyme that depletes intracellular tryptophan, depriving intracellular replicating *Chlamydia* of an essential amino acid. To avoid this fate, human urogenital

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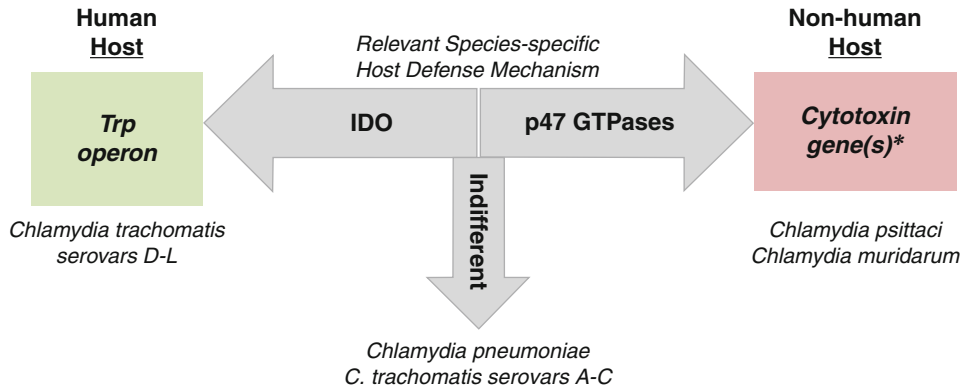

Fig. 21.1

Evasion of species-specific innate defense mechanisms induced by IFN-γ. * *Chlamydia muridarum* has three slightly degenerate copies of the large cytotoxin gene that are 95 %/35 % conserved/identical to the *YopT* peptidase domain; *C. psittaci* has one copy of the cytotoxin gene with 100 % identity to the *YopT* peptidase domain. *Chlamydia* cytotoxin homologies with the clostridial *TcdA/TcdB* domains are roughly 30 % conservation with low identity scores

C. trachomatis serovars in the presence of IFN-γ upregulate the *trp* operon and synthesize their own tryptophan (Wood et al. 2003; Belland et al. 2003). *C. pneumoniae* and *C. psittaci* do not have intact *trp* operons, suggesting that evasion of IDO is not critical for either pathogens life cycle when infecting the human respiratory tract. *C. trachomatis* trachoma serovars A–C lack critical components of the *trp* operon and cannot synthesize tryptophan from indole, suggesting that the indole-based salvage pathway is not critical during infections of the human eye. Conversely, all human *C. trachomatis* serovars infecting the genital tract (serovars D–L) have a functional *trp* operon. The rare trachoma (eye infection) serovar, serovar B, isolated from the genital tract had an intact *trp* operon (Caldwell et al. 2003), reinforcing the importance of the *trp* operon during human *Chlamydia* genital tract infections. IDO is expressed by human but not mouse reproductive tract epithelial cells (Roshick et al. 2006). *C. psittaci*, *C. pneumoniae*, and *C. muridarum* (a rodent version of *C. trachomatis serovar D*) cannot replicate in human epithelial cells in the presence of IFN-γ, even with exogenous indole, because they lack an intact *trp* operon. The human *Chlamydia* urogenital serovars *trp*-operon-IDO-evasion mechanism underscores evolutionary adaptations made by *Chlamydia* species to overcome innate defenses relevant to their specific mammalian host and environmental niche.

The second IFN-γ evasion package includes a large cytotoxin gene(s) with homologies to the large clostridial cytotoxin *TcdA/TcdB* helical and catalytic glycosyltransferase domains and the *Yersinia YopT* cytotoxin peptidase domain (Nelson et al. 2005). Based on these homologies, the C. *muridarum TC0437-0439* and *C. psittaci CPSIT_0606* large cytotoxins likely inactivate inducible p47 GTPases, a host defense mechanism present in rodents but not humans (Taylor et al. 2007; Bekpen et al. 2005). Clostridial TcdA/TcdB domains bind and glucosylate p47 GTPases (Voth and Ballard 2005). YopT peptidase cleaves modified p47 GTPases (Shao et al. 2002). Human *C. trachomatis* and *C. pneumoniae* strains do not have intact copies of the large cytotoxin gene. The large *Chlamydia* cytotoxin is truncated in *C. trachomatis serovar* D, preserving only the clostridial TcdA/TcdB-like domain; the large cytotoxin is completely absent in *C. trachomatis serovar L2* and in *C. pneumoniae* (Read et al. 2000; Thomson et al. 2008; Somboonna et al. 2011). Accordingly, human *C. trachomatis* strains infecting mouse epithelial cells cannot replicate in the presence of IFN-γ because the murine p47 GTPase defense system induced by IFN-γ starves the replicating bacteria of sphingomyelin or engulfs bacterial inclusions in autophagosomes trafficked to lysosomes for destruction, or both (Nelson et al. 2005; Al-Zeer et al. 2009; Coers et al. 2008). Mice have 23 different inducible p47 GTPases; humans have 0 inducible and 1 constitutively expressed p47 GTPase. Four different murine inducible p47 GTPases have been implicated in inhibition of human *C. trachomatis* serovar replication in mouse cells treated with IFN-γ; Irgm1, Irgm3, Irga6, and Irgb10 (Nelson et al. 2005; Al-Zeer et al. 2009; Coers et al. 2008; 2011). Ectopic expression of Irgb10 alone was sufficient to block human serovar replication in mouse cells (Coers et al. 2008). Unlike human *C. trachomatis* serovars, the *C. muridarum* rodent strain replicating in mouse epithelial cells is largely indifferent to effects of IFN-γ and p47 GTPases (Nelson et al. 2005; Al-Zeer et al. 2009; Coers et al. 2008; Jayarapu et al. 2010). *C. muridarum* (rodent pathogen) and *C. psittaci* (bird pathogen) have intact large cytotoxin genes consistent with evading an inducible p47 GTPase defense system in mice and likely in birds. Because human hosts lack an inducible p47 GTPase defense system (Bekpen et al. 2005), human *Chlamydia* strains were not under selective pressure to retain a 3000^{+} amino acid cytotoxin gene during their evolution.

Because *Chlamydia* species initially invade at mucosal epithelium, replication in epithelial cells is likely a critical first step for successful infections. *Chlamydia* species invading nonhuman hosts (rodents and birds) appear to have evolved to evade epithelial p47 GTPase-mediated innate defenses, while human *Chlamydia* species appear to have evolved to evade

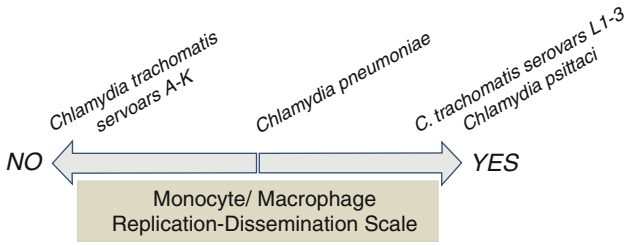

Fig. 21.2
Relative ability of *Chlamydia* species to replicate in macrophages and cause disseminated infections

epithelial innate defenses based on IDO. *Chlamydia* species lacking an intact *trp* operon, *C. psittaci* and *C. pneumoniae*, likely take advantage of a small window early in infection, preceding IFN-γ-triggered innate defenses, to infect monocyte/macrophages, or they replicate in an epithelial niche where IFN-γ-induced IDO may be less active or environmental indole unavailable (*the eye?*).

The ability of *Chlamydia* species to cause disseminated infections correlates well with their relative ability to replicate in human monocyte/macrophage lineages (Fig. 21.2). *C. psittaci* and *C. trachomatis lymphogranuloma venereum* (LGV) serovars $L_{1–3}$ are quite capable of infecting and replicating in human monocyte/macrophage lineages, while trachoma and urethritis serovars A–K of *C. trachomatis* are not (Yong et al. 1987). *C. pneumoniae* has an intermediate phenotype. It does not replicate well in human monocyte/macrophages but is able to persist within them by avoiding lysosomes, while non-LGV serovars of *C. trachomatis* are shunted into lysosomes and destroyed exponentially (Yong et al. 1987; Gaydos et al. 1996). The biology underlying successful replication in macrophages is not understood. It is widely believed that replication in macrophages is a critical step for *Chlamydia* species that cause disseminated infections. Genomic sequence comparisons of macrophage-tropic LGV serovars to genital and ocular *C. trachomatis* serovars that cannot replicate in macrophages did not reveal a clear macrophage-tropism gene or genes. Rather, the LGV sequencing showed that macrophage tropism is due to loss of function or subtle changes in gene function or expression levels, rather than convenient acquisition of a new virulence factor (Thomson et al. 2008).

Chlamydia species have a dual phase life cycle. The infectious form of the bacteria is the elementary body (EB), a dense minimally metabolically active spore-like form that attaches to and invades host cells to initiate infection. Once inside the host cell, the EB de-condense into large non-infectious reticulate bodies within an inclusion body bound by host cell membrane and begin replicating through binary fission (Fig. 21.3). *Chlamydiae* are highly evolved intracellular metabolic parasites. They have no ability to replicate or mature into infectious EB except within an intact eukaryotic cell. The window during which *Chlamydia* exists primarily in the non-infectious reticulate body form is analogous to a viral eclipse phase, a window in time when cell lysis effectively terminates infection.

Paradigms for *Chlamydia* pathogenesis are largely drawn from studies in small animal models, especially the mouse, e.g., rodent pathogen *C. muridarum* in Fig. 21.3. Animal models are essential because they allow experimental manipulation of the host to test hypotheses about protective immunity and immunopathology. Exempting the rodent p47 GTPase system, mouse immunity is very similar to that of humans. There are slight differences in TLR repertoires between mice (TLRs 1–9 and 11–13) and humans (TLRs 1–10). However, TLR2, critical in *Chlamydia* pathogenesis, is conserved in sequence (79% conserved; 71% identical) and function. There are subtle differences in IgG subsets and their relationship to Th1- and Th2-skewed immunity. Th2 immunobiology in mice favors IgG1 over IgG2a and IgG3 (Isakson et al. 1982; Bergstedt-Lindqvist et al. 1984); in humans, Th2 immunobiology favors IgG4 (Lundgren et al. 1989; Punnonen et al. 1993). All components of the T cell and B cell receptor complexes have homologs in mice and men. There are homologs/orthologs for the antigen presentation molecules. The interleukins are the same except humans additionally have IL-8 (chemokine), IL-26, IL-29, and IL-32. IL-26 is a cytokine of unknown function secreted by activated human Th17 cells (Donnelly et al. 2010). IL-29 is a 3rd human λ interferon; mice have two λ interferons (Donnelly and Kotenko 2010). IL-32 is associated with TNF-α and chemokine production (Kim et al. 2005); its receptor and significance are unknown. There are subtle differences in T cell memory cell surface phenotypes. However, based on high degrees of homology and conservation of function, the mouse immune response to *Chlamydia* infections likely mirrors the human response except for innate IFN-γ biology related to the murine p47 GTPase defense system and, perhaps, some subtle differences in Th2 and Th17 immunobiology.

Chlamydia Species: What They Do and How They Do It

Chlamydia psittaci

Clinical Disease

Psittacosis, also known as parrot fever, stands out as the only zoonotic *Chlamydia* infection of humans. *C. psittaci's* natural host species are birds, including parrots, parakeets, cockatiels, macaws, pigeons, and poultry. The infection is so common among birds that tetracycline antibiotics are commonly used as prophylaxis in commercial and hobby breeding populations and are required therapy during quarantine periods for imported exotic pet birds. In birds, *C. psittaci* causes a disseminated infection involving almost all organ systems including the respiratory and gastrointestinal tracts (Mohan 1984). Infected birds transmitting the disease to humans are typically ill but can appear asymptomatic, transiently shedding *C. psittaci* when stressed. The infection in humans is acquired by inhalation of dried airborne respiratory or gastrointestinal tract secretions from infected birds. There are also cases of bite transmission and

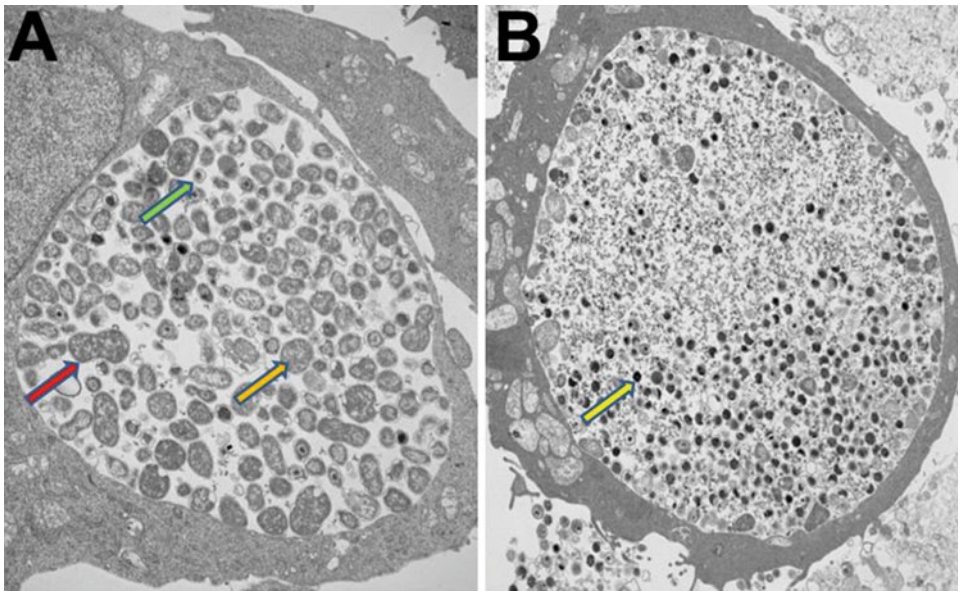

Fig. 21.3
Replication of *Chlamydia muridarum* (a rodent strain closely related to human *Chlamydia trachomatis* serovar D) in mouse epithelial cells. The large round structures in panels A and B are inclusion bodies within infected epithelial cells. (a) 16 h post infection: reticulate bodies predominate. *Orange arrow* – reticulate body; *red arrow* – reticulate body undergoing binary fission; *green arrow* – intermediate form condensing into an elementary body. (b) 32 h post infection: infectious elementary bodies predominate. *Yellow arrow* – elementary body. The "eclipse phase" for *C. muridarum* is roughly 2–15 h post infection

beak-to-mouth transmission in affectionate bird owners. *C. psittaci* is an incidental pathogen of human beings as infected humans have only rarely been implicated as the source of secondary human infections (Hughes et al. 1997). The incubation phase of the disease is 1–3 weeks, with affected individuals presenting with mild nonspecific upper respiratory tract symptoms and fever. Serologic studies in individuals with occupational exposures to birds (turkey farmers, veterinarians, zoo keepers, homing pigeon fanciers, pet store workers) support the theory that most infected individuals are able to resolve the infection without serious illness or medical intervention (Centers for Disease Control and Prevention 2000; Raso et al. 2010). Less fortunate individuals go on to develop a more severe disseminated disease that can have many manifestations. The most common presentation is an "atypical pneumonia" with a persistent relatively nonproductive cough. Other manifestations include hepatitis, pericarditis, endocarditis, meningoencephalitis, gastroenteritis, or fever of unknown origin with splenomegally. There is a post-infectious polyarthritis syndrome associated with *psittacosis* (Lanham and Doyle 1984). The diagnosis of *psittacosis* is usually made based on a significant exposure to birds, fevers, a chronic cough, and an abnormal chest x-ray showing focal consolidation with interstitial changes, frequently accompanied by enlargement of the draining lymph nodes in the chest known as hilar adenopathy. Pleural effusions are common, likely reflecting infection of the pleura. The pneumonia includes mucous plugging related to sloughing of respiratory epithelium. *Psittacosis* is predominantly diagnosed by the clinical presentation supported by serologic testing. Prior to an understanding of the disease and effective antibiotic therapy, *psittacosis* had a fatality rate of 20–40% (Macfarlane and Macrae 1983). Today, patients usually defervesce within 48 h of starting tetracycline or doxycycline and are typically treated for 2 weeks (Yung and Grayson 1988). While a fascinating and potentially fatal infection, currently less than 50 cases of *psittacosis* are reported to the US Center for Disease Control (CDC) each year.

Pathogenesis

C. psittaci infection is initiated by inhalation of infectious EB in dried avian respiratory or gastrointestinal secretions. *C. psittaci* is quite hardy compared to other *Chlamydia species* and can remain infectious for months at room temperature (Centers for Disease Control and Prevention 1997). In an experimental turkey model, the inhaled organisms initially infect respiratory tissues and then rapidly disseminate. Every tissue examined in infected birds including eyes, inner ear, sinuses, lungs, pericardium, liver, kidney, spleen, and gut had visible *Chlamydia* inclusions on immunohistopathology (Vanrompay et al. 1994). Investigations of avian host defense mechanisms against *C. psittaci* are limited, reflecting availability of immunologic reagents and potential risks associated with avian research. In the USA, *C. psittaci* is a risk group 2 pathogen; in some countries, *C. psittaci* carries the status of risk group 3.

Human host defense mechanisms against *C. psittaci* are not well understood. It is clear that humans treated with antibiotics for *psittacosis* can be reinfected (Gosbell et al. 1999). However,

serologic data suggest many individuals self-clear infections without coming to medical attention. *C. psittaci* readily infects and replicates in human monocyte-derived macrophages (Rothermel et al. 1983), consistent with its dissemination phenotype. In vitro *C. psittaci* is susceptible to interferon-mediated upregulation of IDO in human macrophages (Paguirigan et al. 1994) and likely cannot make its own tryptophan from indole as it lacks an intact *trp* operon.

Mice infected intraveneously with *C. psittaci* clear the infection in ≤2 weeks (Buendia et al. 1999) and may not need T cells to clear infection (McCafferty et al. 1994). In mice, *C. psittaci* is vulnerable to some alleles of murine p47 GTPases that dictate susceptibility differences between mouse strains (Miyairi et al. 2007). The most extensive *psittacosis* pathogenesis investigations have been done with the mammalian pathogen *C. abortus*. *C. abortus* is 91.3% homologous to *C. psittaci* by DNA sequence (Seth-Smith et al. 2011). The major difference between the *C. psittaci* and *C. abortus* is absence of the large cytotoxin gene in the plasticity zone of *C. abortus*. *C. abortus* like *C. psittaci* lacks a *trp* operon. There are rare human infections with *C. abortus*, most notably in pregnant women with animal husbandry related exposures (Meijer et al. 2004). *C. abortus* causes fetal loss in livestock (cattle, sheep, goats, and horses) and in the rare human infections. Zoonotic *C. abortus* infections of humans were likely more common when a larger portion of the population was involved in animal husbandry (Johnson et al. 1985).

C. abortus pathogenesis has been investigated in a mouse peritoneal infection model. In mice, resolution of a disseminated *C. abortus* infection requires CD8 T cells with little demonstrable role for CD4 T cells in the clearance mechanism. Adoptive transfer of immune CD8 T cells provided protection while adoptive transfer of immune CD4 T cells had little effect. Convincingly, in immune mice that had previously cleared a primary infection, CD8 depletion made mice susceptible to reinfection while CD4 depletion had no effect on established protective immunity (Buzoni-Gatel et al. 1992). Characterization of the cytokine pattern in infected mice during clearance showed a Th1 pattern (IFN-γ without IL-4) (Buendia et al. 1999). IFN-γ is critical for surviving an infection as mice treated with neutralizing antibody against IFN-γ did not survive (Del Rio et al. 2001). Surprisingly, IL-12 contributed to immunopathology rather than protection, in spite of its contributions to IFN-γ production (Del Rio et al. 2001).

Passive transfer of neutralizing monoclonal antibodies and immune serum protect fetuses in utero from IV challenges with *C. abortus* (Buzoni-Gatel et al. 1990). These data are difficult to interpret as it is not clear that *C. abortus* disseminates as an extracellular bacteria in the bloodstream during the course of natural infections. Interesting preliminary data suggest that terminal components of complement may exacerbate immunopathology during *C. psittaci* infections of mice (Ebeling et al. 2010). Based on the available data, mammalian host defenses against *C. psittaci*/*abortus* appear to be predominantly based on CD8 T lymphocytes, with the role of B cells and other humoral defense mechanisms still to be determined.

Chlamydia pneumoniae

Clinical Disease

Chlamydia pneumoniae is a human pathogen spread person-to-person by aerosolized respiratory secretions. There is only a single human serovar of *C. pneumoniae* known as TWAR (Grayston et al. 1990). Even before intercontinental travel was common, geographically distant clinical isolates of *C. pneumoniae* all identified as TWAR. For many years, it was thought that there were no animal reservoirs for *C. pneumoniae*; however, recent work has identified closely related *Chlamydia* animal pathogens including *C. pneumoniae* serovar LPCoLN, a common respiratory pathogen in koala bears (Bodetti et al. 2002; Myers et al. 2009). Identification of animal pathogens closely related to human *C. pneumoniae* TWAR suggests that TWAR evolved from a relatively recent zoonotic infection of humans that acquired the ability to be transmitted person-to-person.

C. pneumoniae is a common cause of inpatient and outpatient pneumonia, roughly 7–10% (Grayston et al. 1990; Lui et al. 2009; Lim et al. 2001). It affects the entire age spectrum from school age children (Kurz et al. 2009) to military recruits (Gray et al. 1994) and nursing home residents (Troy et al. 1997). Serologic studies suggest that *C. pneumoniae* infections are ubiquitous in most human populations, with seroprevalence rates of >50%. While low-grade endemic infections are likely the norm, there have also been documented outbreaks of *C. pneumoniae* infections in geographically localized groups including school children, military recruits, and nursing home residents (Troy et al. 1997; Lee et al. 2006; Ekman et al. 1993; Nakashima et al. 2006). The discrepancy between seropositivity rates and diagnosed cases of pneumonia supports the theory that most individuals clear or control the infection without seeking medical care or receiving antibiotic therapy. The incubation period preceding clinical disease is roughly 3 weeks. Many infected individuals likely have a mild upper respiratory tract infection that resolves without intervention. In more severely affected individuals, a mild upper respiratory tract infection evolves into a pronounced bronchitis or lower respiratory tract infection with a persistent nonproductive cough. Roughly half of patients with *C. pneumoniae* respiratory infections have wheezing as a significant component of their illness (Hahn et al. 1991). Affected individuals may or may not have fevers. Chest x-rays tend to show focal but non-dense infiltrates consistent with, but not diagnostic of, an "atypical" pneumonia. *C. pneumoniae* causes upper and lower respiratory tract infections, less often pharyngitis, otitis, or sinusitis. Current community-acquired pneumonia treatment guidelines either include use of antibiotics (macrolides, quinolones, doxycycline) that adequately treat *C. pneumoniae* as part of empiric therapy (e.g., IDSA/ATS (Mandell et al. 2007)) or recommend them for patients not responding to β-lactam therapy (penicillins or cephalosporins) or when *C. pneumoniae* is suspected (e.g., British Thoracic Society (Harris et al. 2011)). Unlike other *Chlamydia* infections, *C. pneumoniae* is not associated with post-infectious arthritis syndromes.

There are tantalizing associations of *C. pneumoniae* with chronic human diseases including atherosclerosis and adult-onset asthma. In the 1980s, an epidemiologic study in Finland showed a correlation between *C. pneumoniae* seropositivity and myocardial infarctions; the hypothesis that atherosclerosis was an inflammatory disease due to a chronic infection was born (Saikku et al. 1988). Similar studies have linked *C. pneumoniae* to strokes (Hasan 2011) and degenerative cardiac valve disease (Juvonen et al. 1997). Subsequent studies identified *C. pneumoniae* in atherosclerotic lesions by EM, PCR, and immunohistochemistry (reviewed in (Campbell and Kuo 2004)). Somewhat tempering the initial enthusiasm, similar findings were made for other intracellular human pathogens. A recent study utilizing PCR showed that *C. pneumoniae*, *Mycoplasma pneumoniae*, and hCMV were present in equal frequency in focal atherosclerotic lesions and histologically normal vascular tissue within individuals (Bayram et al. 2011). However, other studies focused on *C. pneumoniae* found *C. pneumoniae* only in atherosclerotic and not normal vascular tissue (Campbell and Kuo 2004). Multiple inflammatory diseases have been associated with myocardial infarctions including HIV (Friis-Moller et al. 2003) and periodontitis (Bahekar et al. 2007). These epidemiologic findings suggest that systemic inflammation may be the accelerant for progression of atherosclerotic lesions. The focal-chronic-vascular infection, chronic-inflammatory-systemic infection, and incidental-finding hypotheses have been tested in coronary artery disease clinical trials using antibiotic interventions. In multiple studies, antibiotic therapy with azithromycin or clarithromycin did not prevent secondary myocardial infarctions (O'Connor et al. 2003; Grayston et al. 2005; Anderson et al. 1999; Muhlestein et al. 2000; Jespersen et al. 2006). Other antibiotics including roxithromycin and gatifloxicin also showed no demonstrable benefit (Song et al. 2008). These studies argue against a role for chronic *C. pneumoniae* infection in progression of atherosclerotic disease and myocardial infarctions, at least once the atherosclerotic disease is advanced. However, azithromycin is not always bacteriocidal for *Chlamydia* species (Gieffers et al. 2001; Wyrick and Knight 2004). It is possible that bacteriostatic antibiotic therapies directed against persistent/chronic *C. pneumoniae* infections do not alter the natural history of disease because they do not eradicate the infection (Anderson 2005).

Results in clinical trials with asthmatic patients are slightly more promising. Minocycline therapy has a modest clinical benefit in chronic steroid-dependent asthma (Daoud et al. 2008). Macrolide antibiotics have limited benefits in adult asthma that do not correlate with evidence of concurrent *C. pneumoniae* infection or reinfection (Sutherland et al. 2010; Johnston et al. 2006; Black et al. 2001). Clarithromycin and azithromycin have anti-inflammatory effects at the level of neutrophils and epithelial cells (Simpson et al. 2008; Ribeiro et al. 2009). Minocycline inhibits metalloproteases that cause tissue damage during episodes of inflammation (Golub et al. 1985; Imtiaz et al. 2006). The link between *C. pneumoniae* and non-atopic asthma is uncertain as existing studies have shown limited benefits without a correlate for active *C. pneumoniae* disease. No study enrolled asthmatic patients based on elevated anti-*C. pneumoniae* IgM, which may be the best surrogate marker for active disease.

There are two cutaneous/mucocutaneous conditions that appear to be manifestations of localized *C. pneumoniae* infections. Acne rosacea, an inflammatory skin condition centered on and around the nose, has an association with *C. pneumoniae* by serology and immunohistochemistry of lesion biopsies (Fernandez-Obregon and Patton 2007). The condition in its severe form can involve the eye causing a mild trachoma-like infection (next section). Severe rosacea, granulomatous rosacea, is associated with significant disfigurement. The inflammatory infiltrate consists of lymphocytes, histiocytes, plasma cells, neutrophils, epitheloid cells, and giant cells similar to the lesions seen in *lymphogranuloma venereum* (upcoming *C. trachomatis* section). The condition is empirically treated with oral azithromycin, tetracycline/doxycycline, or topical clindamycin, all having activity against *C. pneumoniae*. The disease tends to relapse after discontinuing treatment. Giving some pause as to causality, the condition also responds to topical metronidazole, an antibiotic without known activity against *C. pneumoniae*. The second condition is gingival hyperplasia/overgrowth syndrome seen in transplant patients on cyclosporin A therapy. In one study, biopsies of this condition were almost uniformly positive (10 of 11 subjects) for *C. pneumoniae* by PCR, and affected individuals had elevated anti-*C. pneumoniae* IgG and IgM titers. The clinical disease and IgM antibody titers responded to azithromycin therapy, but the PCRs remained positive 1 year after treatment (Worm et al. 2004).

There are tantalizing associations of *C. pneumoniae* with late onset Alzheimer's disease including $^{+}$PCR, $^{+}$immunohistochemistry, and $^{+}$cultures (Dreses-Werringloer et al. 2009; Hammond et al. 2010). The narrative is logical and appealing. With advancing age and thymic involution, the host immune system loses control of a persistent *Chlamydia* infection that manifests in the CNS. Consistent with an infection, there is an inflammatory component to Alzheimer's disease (Tuppo and Arias 2005). The challenge will be to determine causality for a ubiquitous human pathogen like *C. pneumoniae*.

Pathogenesis

C. pneumoniae infections are initiated by inhalation of elementary bodies aerosolized by the coughing or sneezing of infected individuals. Inhaled EB infect respiratory epithelial cells and alveolar macrophages. On the monocyte/macrophage dissemination scale, *C. pneumoniae* falls in the gray zone. In vitro, viable *C. pneumoniae* persists in human alveolar macrophages without significant replication. However, in bronchoaveolar lavage (BAL) fluid from patients with *C. pneumoniae* pneumonia, there are large inclusions in alveolar macrophages suggesting in vivo replication (Rupp et al. 2009). Available clinical data argue that *C. pneumoniae* principally causes acute upper respiratory infections and pneumonia, while possibly contributing to some forms of chronic asthma, soft tissue, and CNS infections.

One longitudinal study based on serial chlamydial cultures of nine individuals with *C. pneumonia* respiratory infections sheds light on bacterial clearance. Two of the nine subjects presented for medical care with new onset wheezing as a major symptom. All patients were treated, and several retreated, with tetracycline or doxycycline courses of 5–21 days. Of the nine subjects, four cleared the infection with their initial course of antibiotic therapy; two subjects had delayed clearance at 2 and 3 months post antibiotic therapy. The three remaining patients did not clear the infection over 11 months in spite of repeated or prolonged courses of antibiotic therapy. The two patients with wheezing as part of their initial presentation received corticosteroids in addition to antibiotics and were among the three patients who did not clear. In the persistently culture positive patients, antibiotic therapy was associated with clinical resolution of the acute illness. Clearance of the infection, or resolution of the acute clinical symptoms in non-resolvers, was associated with decreases in elevated anti-*Chlamydia* IgM titers, suggesting that elevated IgM titers are a surrogate marker for clinically significant *C. pneumonia* infection (Hammerschlag et al. 1992). Lack of *C. pneumoniae* clearance with antibiotics for acute infection has also been seen in case studies with shorter follow-up (Yamazaki et al. 1990). *C. pneumoniae* reactivation or reinfection appears to be common in patients with compromised immunity such as advanced HIV/AIDS (Augenbraun et al. 1991; Gaydos et al. 1993) and recent lung transplant recipients (Glanville et al. 2005). Not surprisingly, there are no studies of the natural history of untreated symptomatic *C. pneumoniae* infections.

In human subjects hospitalized with pneumonia caused by *C. pneumoniae*, T cell subset analysis in the peripheral blood showed a CD8-predominant *C. pneumonia*-specific response in the early window after diagnosis, followed by a transition to a CD4-predominant *C. pneumoniae*-specific response 4 months post infection (Halme et al. 2000). There is no way to determine which T cell subset provided protection from reinfection or possibly contributed to future non-atopic asthma or atherosclerotic immunopathology.

Absence of a practicable species-matched animal model for *C. pneumoniae* infection has somewhat constrained investigations of its pathogenesis. In the species-mismatched *C. pneumoniae* mouse model, relatively large bacterial inoculums ($\sim 10^{6-7}$ infection-forming-units; IFU) are used to initiate a respiratory tract infection that is cleared over the course of ~4 weeks. In wild type mice, recovered *C. pneumoniae* IFU from the lungs do not exceed input IFU at any time point post infection. The *C. pneumoniae* genome lacks the large cytotoxin gene implicated as a species-specific virulence factor for evading rodent p47 GTPases. It is possible that mice are semi-permissive for *C. pneumoniae* infections because the murine IFN-γ-inducible p47 GTPase defense mechanism is sufficient to limit *C. pneumoniae* infection, as is the case in vitro for human *C. trachomatis* serovars infecting mouse cells in the presence of IFN-γ.

The relationship of p47 GTPases to the semi-permissive host status of mice for *C. pneumoniae* infections is unknown. A/J x C57BL/6 crosses mapped a rapid-clearance-but-greater-immunopathology phenotype seen in C57BL/6 mice to a region of chromosome 17 that includes the MHC loci (Min-Oo et al. 2008); the p47 GTPase loci are on chromosome 11. This study was not done to address p47 GTPases and does not necessarily address their role as there may not be any functional differences between the p47 GTPase alleles of A/J and C57BL/6 mice. The mouse combination used to map susceptibility to *C. trachomatis* serovar L2 intravenous infections was C3H/HeJ x C57BL/6 J, and the mechanism underlying susceptibility of C3H/HeJ mice is a lower level of *Irgb10* (p47 GTPase) expression, rather than a defect in function of C3H/HeJ *Irgb10* (Coers et al. 2008).

Caveats aside, the mouse model has provided important insights into *C. pneumonia*e lung pathogenesis. TLR2 and, to a lesser extent, TLR4 are the dominant pattern recognition receptors for host surveillance against *C. pneumoniae* infections (Prebeck et al. 2001). Infected MyD88 knockout mice lacking TLR2/4 signaling have significantly reduced neutrophil recruitment into the lungs and *better* outcomes than wild type mice including lower IFU lung titers. The improved lung immunopathology can be reproduced in wild type mice by depleting neutrophils with monoclonal antibody RB6-8 C5. Neutrophils recovered from lungs of infected wild type mice had small inclusions of uncertain significance; the major detrimental effect of neutrophils appeared to be enhancement of *C. pneumoniae* replication within respiratory epithelial cells based on associated neutrophil-epithelial cell interactions (Rodriguez et al. 2005). The small inclusions in neutrophils may serve as a Trojan horse strategy for infecting alveolar macrophages. In vitro studies showed that human neutrophils were effective vectors for infecting alveolar macrophages. Apoptotic-infected neutrophils release Ccl4 (MIP1β), attracting uninfected BAL (bronchoaveolar lavage) alveolar macrophages that ingested them to become productively infected (Rupp et al. 2009). In combination, the human and animal model data suggest that *C. pneumoniae* infects and replicates in both respiratory epithelial cells and alveolar macrophages and that recruitment of neutrophils facilitates replication in both cell types.

A seminal study defined the majority of immune parameters affecting clearance of *C. pneumoniae* from the lung. T cell immunity is necessary for clearance as *Scid* mice lacking T and B cells and CD4/CD8 dual knockout mice lacking T cells do not clear lung infections within 60 days. CD8 knockout and *Tap1*/β-2 microglobulin dual knockout mice (no MHC class I presentation) were compromised to the point that mice became productively infected (recovered IFU > input), while MHC class II knockout and CD4 knockout mice cleared the infection similar to wild type controls. In early stages, postinfection CD4 T cells are detrimental, as CD8 knockout mice (CD4 sufficient) had higher lung IFU titers than CD4/8 dual knockout mice, and *Scid* mice adoptively transferred with CD4 T cells had higher lung IFU titers than untreated *Scid* mice. However, CD8 knockout mice in the same study were able to resolve infections while CD4/8 dual knockout mice could not, demonstrating that CD4 T cells are also capable of mediating clearance (Rottenberg et al. 1999). Additional adoptive transfer

experiments into T cell-deficient mice demonstrated that either naïve CD4 or naïve CD8 T cells could contribute to clearing *C. pneumoniae* from the lung (Rothfuchs et al. 2004). However, depletion studies in wild type immune mice that cleared a previous infection showed that the CD8 T cell subset, not the CD4 T cell subset, was the critical subset for protecting the mice from reinfection (Penttila et al. 1999). In mice, the CD8 T cells mediating clearance include T cells that see N-terminal formylated bacterial peptides in the context of nonclassical MHC class 1b molecules (Tvinnereim and Wizel 2007). Clearance of *C. pneumoniae* from the lung can be mediated by either CD4 or CD8 T cells, but CD8 T cells predominate in "natural" immunity generated by infection and are more efficient than CD4 T cells in mediating clearance while minimizing immunopathology.

Natural killer T (NKT) cells have a significant role in molding the adaptive immune response to *C. pneumoniae* infections. CD1d and NKT knockout mice have increased susceptibility to *C. pneumoniae* infections with IFU recoveries that exceed input innocula and worsened immunopathology in the lungs that likely reflects a shift away from IFN-γ toward greater IL-4 and IL-5 production. Infected CD1d and NKT knockout mice had reduced frequencies of IFN-γ producing CD4 and CD8 T cells. Curiously, NKT knockout mice were more resistant to *C. muridarum* pulmonary infection with less immunopathology and reversed cytokine polarization; wild type mice with IL-4 dominant cytokine pattern and NKT knockout with an IFN-γ dominant cytokine pattern (Joyee et al. 2007). This may reflect the differing cell tropisms of the two *Chlamydia* species. The NKT cell skewing of T cell immunity toward Th1/Tc1 during *C. pneumoniae* infections was subsequently shown to occur at the level of dendritic cells (Joyee et al. 2008).

Based on the CD4/CD8 dual knockout result, B cells are not sufficient to clear primary infections (Rottenberg et al. 1999), but there is a role for anti-*Chlamydia* antibodies in wild type mice. Protection afforded by antibodies is associated with the IgA subclass and likely requires local production at the site of infection. Intranasal vaccination with MOMP reduced *C. pneumoniae* IFU titers in the lungs of wild type but not IgA knockout mice. The same MOMP vaccination delivered into the peritoneal space did not afford protection against nasal challenge (Rodriguez et al. 2006). Antibodies to *C. pneumoniae* are capable of neutralizing EB (Puolakkainen et al. 1995).

The mechanism use by CD8 T cells to clear *C. pneumoniae* infections is not known. Perforin knockout mice clear *C. pneumoniae* lung infections with the same kinetics as wild type mice (Rottenberg et al. 1999). Therefore, physical killing of infected cells when bacteria exist as non-infectious RB or non-infectious aberrant forms is not a critical effector mechanism. A second major defense mechanism mediated by T cells and IFN-γ is upregulation of inducible nitric oxide synthetase (*iNOS*). T cell and IFN-γ-mediated upregulation of *iNOS* and nitric oxide production are utilized for killing intracellular pathogens (Chakravortty and Hensel 2003). Consistent with susceptibility to IFN-γ-mediated host defenses, IFN-γ knockout mice (T cell sufficient) were more susceptible to *C. pneumoniae* than T cell-deficient mice with intact IFN-γ (Rothfuchs et al. 2004). In IFN-γ knockout and IFN-γ-receptor knockout mice, a *C. pneumoniae* challenge results in a productive infection with a >1 log expansion over input IFU (Rottenberg et al. 1999; Rothfuchs et al. 2004). Consistent with a protective role for T cells making IFN-γ, *Rag1*/IFN-γ dual knockout mice (no T cells or IFN-γ) succumb to *C. pneumoniae* infections within a month (Rothfuchs et al. 2004). Adoptive transfer of either naïve CD4 or CD8 T cells protected *Rag1*/γ_cR (Tcell/Bcell/NK cell-deficient) mice from lung immunopathology and reduced lung IFU titers, while adoptive transfer of IFN-γ-deficient naive CD4 or CD8 T cells led to severe immunopathology without an effect on lung IFU titers (Rothfuchs et al. 2004). Interestingly, *iNOS* knockout mice had only modestly increased *C. pneumoniae* bacterial loads in the lung that remained below the input inocula (Rottenberg et al. 1999). The modest decrease in bacterial clearance with *iNOS* deficiency suggests two things: (1) that nitric oxide production is not the only mechanism mediating clearance and (2) that IFN-γ is doing something more potent than upregulating *iNOS* and nitric oxide production. The possibilities include upregulating inducible p47 GTPases, upregulating IDO, upregulating MHC class I to augment presentation to CD8 T cells, facilitating lysis of infected cells when *Chlamydia* exist as non-infectious RB or aberrant bodies (persistence), upregulating MHC class II to augment presentation to CD4 T cells in order to "help" CD8 T cells, some other unknown direct antimicrobial effector mechanism, or combinations of the above. One should be cautious about overinterpreting the dramatic influence of IFN-γ in the *C. pneumoniae* mouse model, as this is a species-mismatched model, and the murine p47 GTPase defense system the wild card.

Chlamydia trachomatis

The major animal models for *C. trachomatis* infections are infection of nonhuman primates with human *C. trachomatis* serovar E and L2 and infection of mice with human *C. trachomatis* serovars, *C. muridarum*, and *C. caviae*. Limited by expense and existential issues, the Cynomolgus and Macaque models provide important insights into pathogenesis that most believe can be cleanly extrapolated to humans. The mouse models have been used extensively to investigate pathogenesis and have the advantage of a wealth of immunologic reagents and genetic tools including knockout mice. The *C. caviae* guinea pig model is somewhat constrained by availability of reagents and lack of knockout animals but is able to model both trachoma and infectious urethritis (Rank and Sanders 1992; Rank et al. 2003).

Clinical Disease

Trachoma

C. trachomatis serovars A through C cause trachoma, a devastating chronic infection/reinfection of the eyes that can progress to blindness. Trachoma is common in Africa and some

regions of the Middle East and Asia (Burton and Mabey 2009). It is a leading cause of blindness in areas where it is endemic. Unlike all other human *Chlamydia* infections, trachoma has an insect vector. Flies feeding on the exudates surrounding the eyes of sleeping infected individuals transmit the infection to new hosts while they sleep (Emerson et al. 2004). Close contact with infected family members under suboptimal housing conditions also likely transmits the infection. Short-lived immunity to one infecting serovar does not protect against other trachoma serovars. Infected individuals can be reexposed and infected multiple times with different serovars potentially exacerbating inflammatory damage to the eyes leading to blindness (Grayston et al. 1985). Trachoma treatment is family or community based. If there is a high prevalence of the disease, community-wide treatment with azithromycin or topical tetracycline is attempted. For isolated cases with a low incidence of disease in the community, only immediate family members are treated.

Infection/replication of *C. trachomatis* is limited to the conjunctival epithelium (el-Asrar et al. 1989a, b); there is no dissemination, consistent with its inability to replicate in monocyte/macrophages. Acute infection triggers a structured inflammatory response visible as follicles on the conjunctival surface. In conjunctival biopsies, the inflammatory infiltrate underlying the superficial epithelial infection includes T cells (no subset breakdown), plasma cells (IgA > other subtypes), neutrophils, and macrophages (el-Asrar et al. 1989a). Initial efforts toward developing *Chlamydia* trachoma vaccines based on chemically-inactivated whole organisms did not provide significant protection and were associated with exacerbated disease. Similar results were found in an experimental primate model for trachoma. These disappointing vaccine study results shifted the field toward development of a subunit vaccine (Schachter 1985). Demonstration that *Chlamydia* vaccination could exacerbate immunopathology without providing significant protection from infection raised the safety bar for development of all future *Chlamydia* vaccines.

Pathogenesis

There is epidemiologic evidence of protective immunity against trachoma. In endemic areas, the risk of infection, the apparent intensity of disease, and the duration of shedding all decrease with increasing age, consistent development of a protective immune response with prior infection(s) (Bailey et al. 1999). In subjects with scarring trachoma, peripheral blood T cell proliferative responses to cHSP60, MOMP, and EB were all lower than in non-scarred controls, while differences in T cell IFN-γ production between the two subject groups were minimal. Conjunctival swabs of subjects with scarring were more likely to have positive antigen and PCR testing for *C. trachomatis* suggesting persistent infection or a greater risk of reinfection, with either scenario reflecting a lack of protective immunity (Holland et al. 1993). Peripheral blood T cell proliferative responses to cHSP60, MOMP, and EB during active disease correlated with resolution during 6 months of follow-up; subjects unable to resolve the inflammation over 6 months had lesser T cell proliferative responses to chlamydial antigens. IFN-γ production did not correlate with disease resolution (Bailey et al. 1995). These two studies argue for a general protective role for T cell immunity against trachoma. Trachoma protection and immunopathology do not follow a convenient Th1 *versus* Th2 paradigm. The worst progressive scarring state, inflammatory trichiasis, appears to be driven by high levels of IL-6, IL-1β, IL-15, TNF-α, and chemokines including Ccl2 (MCP-1) and Cxcl9 (MIG) with deficiencies of IL-1Ra, IL-13, and IL-12p40 (Skwor et al. 2008). Severe trichiasis with active follicles appears to be driven by neutrophil-like inflammation rather than a T cell-mediated hypersensitivity reaction. Recently, active follicular trachoma was found to include elevated expression of IL-17a and Cxcl5, consistent with recruitment and activation of neutrophils, and there was evidence for non-chlamydial bacteria infections as a cofactor in disease (Burton et al. 2011). Perhaps the evolutionary loss of the *trp*-operon-IFN-γ-evasion mechanism from trachoma serovars supports the human study data showing no correlation of IFN-γ with protection from or resolution of active trachoma disease.

The role of antibody in trachoma is not clear. High titers of anti-*Chlamydia* IgG in tears were associated with a greater risk of acquiring disease, while presence of anti-*Chlamydia* IgA in tears trended toward protection from disease acquisition (Bailey et al. 1993). In one study, individuals with scarring trachoma were found to have lower systemic anti-*Chlamydia* IgA titers (Holland et al. 1993), perhaps suggestive of a protective function for anti-*Chlamydia* IgA. What is not knowable is whether a strong preexisting anti-*Chlamydia* IgA tear titer would protect against primary infection.

The pathogenesis of trachoma is studied in two animal models. There is a Cynomolgus monkey model that faithfully replicates the human disease including the initial infection inflammation with development of follicles and subsequent development of a pannus and conjunctival/corneal scarring with rechallenges (Taylor et al. 1981). Using this model, there is recent evidence for protective vaccine-mediated immunity generated using an attenuated plasmid-deficient *C. trachomatis* ocular strain (Kari et al. 2011).

The second animal model is the *C. cavaie* guinea pig model, guinea pig inclusion conjunctivitis (GPIC). *C. caviae* was originally isolated from a guinea pig with conjunctivitis, so this is a species-matched model. The *C. caviae* genome has been sequenced and there is good synteny (gene order alignment) with *C. trachomatis* urogenital serovars except in the plasticity zone (Read et al. 2003). *C. trachomatis* serovar D has 894 open reading frames (genes); 808 of them are shared with *C. cavaie*. *C. caviae* has an intact single copy of the cytotoxin gene and an intact *trp* operon. The guinea pig GPIC model is a reasonable reproduction of trachoma including the histopathology during the primary infection and recalls inflammatory T cell responses to *Chlamydia* antigen that cause significant ocular pathology.

Interestingly, absolute protection against rechallenge infection requires a previous ocular infection but rechallenge immunopathology does not. Guinea pigs that have cleared an ocular infection cannot be reinfected with GPIC and therefore have no immunopathology on rechallenge; no infection = limited antigen. However, ocular-immune animals rechallenged topically with *GPIC* protein extracts develop significant immunopathology absent an infection. Guinea pigs originally infected at non-ocular sites develop ocular infections on rechallenge that are cleared more quickly but associated with significant immunopathology (Watkins et al. 1986). If there were multiple serovars of GPIC, the human trachoma pathology related to recurrent infections with different serovars would likely be reproducible in the guinea pig model. The dominant antigen in the rechallenge hypersensitivity immunopathology is *Chlamydia* HSP60 (Morrison et al. 1989), a theme that will be revisited in the pathogenesis of serovars D–K. The second potential theme in *Chlamydia* pathogenesis is that benefits associated with anti-*Chlamydia* antibodies require local production at the site subject to infection. Perhaps the inability to reinfect ocular-immune guinea pigs relates to local IgA production that is absent if the primary infection occurred at a non-ocular site. A recently published study showed that neutrophil depletion did not affect time to clearance of *C. caviae* from the eye but dramatically improved gross inflammatory pathology and facilitated *Chlamydia*-specific IgA responses, suggesting that neutrophils play a detrimental role in shaping the adaptive immune response (Lacy et al. 2011). These data suggest that excessive neutrophil infiltration and activation diminishes the influence of local TGF-β on immunoglobulin class switching to IgA. The recent guinea pig data overlap the recent human trachoma data, both pointing toward neutrophils as the final masters of eye disasters.

Lymphogranuloma venereum

Clinical Disease

C. trachomatis serovars $L_{1–3}$ cause a sexually transmitted infection known as *lymphogranuloma venereum*. LGV infections are endemic in parts of Africa, Asia, and the Caribbean (Mabey and Peeling 2002). They are uncommon in the USA and Western Europe, except for a resurgence in MSM (men who have sex with men) communities (Spaargaren et al. 2005; Nieuwenhuis et al. 2004). Classic presentations of LGV infection are characterized by an initial papule(s), nodule(s), or shallow ulcer(s) on the internal or external genitalia, with subsequent dissemination to regional draining lymph nodes. Urethritis is uncommon. Acute primary infections related to anal sex are associated with rectal ulcers, proctitis, and proctocolitis. In classical presentations the initial skin lesion is painless and can be innocuous. In women, the lesion is commonly internal and not visualized. Patients who do not seek medical attention for the initial lesion may present 2 or more weeks later due to symptoms from dissemination to the regional draining lymph nodes. External skin lesions drain to the inguinal lymph nodes causing inguinal swelling, seen more often in men; internal cervical/vaginal lesions drain to deep pelvic lymph nodes causing pelvic pain, seen more often in women. The disseminated stage of the infection is commonly associated with fevers that can last for several weeks. The late stage of the infection is characterized by chronic inflammation with fibrosis and destruction of perineal tissue. Chronic complications of untreated LGV infection include elephantitis from obstructed lymphatics (scrotum or labia), inguinal, anorectal, and recto-vaginal fistulae due to suppuration of regional lymph nodes, perineal ulcers and fissures, and rectal strictures (Novy 1935). In the developed world, LGV's current foothold in the human population is predominantly as a sexually transmitted infection of the rectum related to anal sex. LGV proctitis appears to be facilitated by a positive HIV status and may be contributing to HIV transmission (Ronn and Ward 2011). In addition to dramatic clinical findings, LGV distinguishes itself by being unresponsive to short-term antibiotic therapies including single dose azithromycin therapy commonly prescribed for urogenital serovars D–K. LGV infections are treated with doxycycline or erythromycin for 3 weeks; azithromycin for 3 weeks is likely effective but unproven.

Pathogenesis

There is large void in data related to *lymphogranuloma venereum* pathogenesis. In skin lesions and infected lymph nodes, LGV histology includes lymphocytes, plasma cells, macrophages, neutrophils, epithelioid cells, and giant cells (stellate microabscesses). Within lesions, limited numbers of *Chlamydia* inclusions are seen in macrophages (Alacoque et al. 1984). Consistent with dissemination and replication in draining lymph nodes, LGV serovars are able to productively infect human monocyte/macrophages in vitro. Unlike their urogenital serovar D–K cousins, LGV serovars L_1–L_3 do not ascend to infect the Fallopian tubes. An informative 2007 HIV LGV proctocolitis case report included rectal immunohistochemistry pre-therapy, on therapy, and post therapy. In the setting of untreated advanced HIV, the predominant T cell subset infiltrating rectal lesions was $CD8^+$. With azithromycin and HIV antiretroviral therapy, the predominant T cell subset in lesions transitioned from $CD8^+$ to $CD4^+$. Final clearance of LGV was temporally associated with the CD4 dominant phase (van Nieuwkoop et al. 2007). While tempting, it is probably dangerous to draw conclusions about protective immunity and immunopathology from this clinical scenario. There is, or used to be, a nonhuman primate model for LGV proctitis. Rectal inoculation of Cynomolgus monkeys with serovar L2 causes a proctitis/proctocolitis similar to the human condition except that granulomata were not seen in rectal lesion histopathology. T cell infiltration into lesions was a relatively balanced mix of CD4 and CD8 T cells, slightly favoring the latter. The rectal lesions healed within 6 weeks even though the LGV cultures remained positive at that time point (Zeitz et al. 1988, 1989; James et al. 1987).

C. trachomatis serovar L2 is used in a species-mismatched mouse model via tail vein injection or trans-cervical injection into the uterus. In vitro replication of L2 in mouse cells is sensitive to inhibition by IFN-γ, likely due to absence of the large *Chlamydia* cytotoxin and existence of murine p47 GTPase defenses. It had been widely anticipated that knockout mice lacking p47 GTPase genes associated with *C. trachomatis* serovar L2 susceptibility to IFN-γ in mouse cells (Coers et al. 2008) would be permissive for human serovar infections of the mouse genital tract. Unfortunately, that was not the case. Dual p47 GTPase knockout mice had only a modest increase in levels of *Chlamydia* genomic DNA limited to early time points post infection compared to wild type mice (Coers et al. 2011). In this experimental model, vaginally infected mice do not develop local IgA responses and CD4 T cell depletion has minimal effects on bacterial clearance (Morrison et al. 2011). Pathogenesis data from infecting mice with serovar L2 has traditionally been interpreted as reflecting immunobiology of the urethritis serovars D–K. This may be problematic as natural infections with LGV serovars cause the clinical disease *lymphogranuloma venereum*, a disseminating infection from the genital tract epithelium to regional lymph nodes with formation of stellate microabscesses and localization of inclusions to macrophages within those lesions.

Urogenital Serovars D–K

Clinical Disease

C. trachomatis serovars D–K primarily cause genital tract infections but can also infect at other sites. Infants born to infected mothers are at risk for *C. trachomatis* neonatal pneumonia and conjunctivitis (Schachter et al. 1986). In STI clinic settings, there is an uncommon *C. trachomatis* D–K conjunctivitis seen in adults, typically associated with a coincident genital tract infection that suggests autoinoculation (Dawson and Schachter 1967). In ophthalmology clinic settings, roughly 20% of chronic conjunctivitis cases are due to *C. trachomatis* based on relatively insensitive culture techniques (Rapoza et al. 1990). An ophthamology outpatient clinic report, including longitudinal follow-up, showed that one quarter of patients with *C. trachomatis* conjunctivitis did not clear the infection as measured by conjunctival scrapings with DFA staining, even after three 2-week courses of oral roxithromycin/doxycline plus tetracycline eye washes given over 9 weeks. In that report, among those agreeable to screening, 39% of the patients had a coincident asymptomatic genital tract infection (Carta et al. 1994).

C. trachomatis serovars D–K cause the most common bacterial sexually transmitted infection in the USA and Western Europe. The Center for Disease Control (CDC) estimates that there are three million new *C. trachomatis* genital tract infections in the USA each year (CDC 2009), and there are similar numbers in Europe (Low 2004). In men, the infection causes a urethritis that can ascend to infect the prostate (prostatitis), *vas deferens*, and epididymis (epididymitis). In women, the infection begins as a cervicitis +/− urethritis and can ascend into the uterus (endometritis) and Fallopian tubes (salpingitis). Severe infections present as pelvic inflammatory disease (PID) and its variants, tubo-ovarian abscesses (TOA) and Fitz-Hugh-Curtis syndrome (pelvic-peritoneal infections with inflammation around the liver causing right upper quadrant pain). *C. trachomatis* D–K serovars are the most common etiology of PID, causing approximately 40% of cases (Paavonen and Lehtinen 1996). In men and women, smoldering relatively asymptomatic chronic infections and recurrent exposure and reinfection cause significant scarring within the reproductive tract. In women, infection-related scarring of the reproductive tract causes chronic pelvic pain and infertility and predisposes women to ectopic pregnancies that are potentially fatal. Whether *C. trachomatis* epididymitis causes infertility in men is uncertain (Ness et al. 1997), but in rare cases where infection-related scarring occludes both *vas deferens* at their junction with the prostate gland, the association with infertility is probable (Berger 1990). The significant gender difference in infectious sequelae likely relates to the complex structure and function of Fallopian tubes, readily compromised by scarring, versus the less complex pipe-like function of the upper male reproductive tract. In women, each episode of PID is associated with an additive 20% risk of infertility (Westrom 1994; Paavonen and Eggert-Kruse 1999). The risk of ectopic pregnancy after having PID is roughly 1–8%, and repeat episodes have additive risk (Westrom 1994; Hillis et al. 1997). A majority of women in infertility clinics with scarring-related infertility (tubal factor infertility; TFI) and positive *Chlamydia* serologies do not have a prior history of PID or prior diagnosis of *C. trachomatis* infections (Brunham et al. 1985); they are more likely to have a past history of pelvic pain (Wolner-Hanssen 1995).

Individuals exposed to *C. trachomatis* through sexual contact, oral-genital, penile-vaginal, or anal, are at high risk for acquiring the infection as the infectious inoculum for *C. trachomatis* is low. The dose delivered by an "average" infected male urethra may be less than 100 infection-forming units (IFU). IFU exposure in the female genital tract is typically <5,000 IFU but can be greater than 10,000 IFU (Geisler et al. 2001; Agrawal et al. 2009). In spite of an apparent IFU difference between genders, men and women are equal opportunity transmitters of the infection to their sexual partners, with an efficiency of roughly 70% (Quinn et al. 1996). The fact that average IFU exposures of <100 and >1,000 have the same rate of infection supports the proposition that only a few IFU are needed to transmit an infection. Condoms provide less robust protection against *C. trachomatis* infection (50–70%) than against HIV infection (80–90%), likely reflecting difficulties associated with using condoms and the low *Chlamydia* IFU exposure needed to transmit infections (Warner et al. 2006). *Chlamydia* infections facilitate HIV transmission (Laga et al. 1993).

C. trachomatis infected individuals experience one of two clinical outcomes. A majority of infected individuals, up to 75%, are asymptomatic or have mild symptoms that go unrecognized

and untreated (Zimmerman et al. 1990). *C. trachomatis* infections of the oropharynx and rectum in MSM are typically asymptomatic, such that limited conventional screening with urethral swabs or urine specimens may miss the majority of infections (Kent et al. 2005). This large pool of asymptomatic infected individuals of all sexual preferences poses a major challenge for public health measures aimed at decreasing the incidence and prevalence of *C. trachomatis* infections. The minority of individuals go on to develop symptomatic infections after an incubation period of 1–3 weeks. They present for medical care with urethral or vaginal discharges as their principal complaint. Associated symptoms can include dysuria, increased urinary frequency, and pelvic or scrotal pain. More severe infections in men present as epididymitis (scrotal pain). Severe infections in women present as pelvic pain due to pelvic inflammatory disease (PID), tubo-ovarian abscesses, and Fitz-Hugh-Curtis Syndrome. Asymptomatic infected women identified by screening do not necessarily progress to develop PID. 2–5% of untreated *asymptomatic* infected women identified by positive screening cultures develop PID with a median return interval of 2 weeks (Haggerty et al. 2010). Except for its most severe manifestations, *C. trachomatis* is a localized infection of reproductive tract epithelium without fevers or other signs or symptoms of systemic illness. Histopathology from human genital tract infections shows that *C. trachomatis* inclusions are limited to reproductive tract epithelial cells; no inclusions are seen in the stroma beneath the epithelium (Dean 1997). Symptomatic individuals and identified partners are typically treated with a single dose of azithromycin or 1 week of doxycycline therapy. Individuals with severe infections such as PID, TOA, and FHC initially receive parenteral (IM or IV) antibiotic therapy along with either doxycycline or azithromycin. In many health care settings, empiric treatment for *C. trachomatis* is initiated for urethral and cervical discharges prior to results of definitive testing (nucleic acid amplification or culture).

In a small subset of patients, *C. trachomatis* disseminates from the genital tract to cause persistent infections of the eye (uveitis/conjunctivitis) and synovium (arthritis) (Carter and Hudson 2010). The polyarthritis/uveitis syndrome is known as *Chlamydia*-induced reactive arthritis. It is reasonable to hypothesize that individuals with this uncommon manifestation of the infection have a defect in their immune defenses allowing *C. trachomatis* to persist or replicate in monocyte/macrophages, facilitating dissemination. Reactive arthritis appears to have been more common in the pre-antibiotic era. In the early 1970s, epidemiologic data suggested that roughly 1% of patients with genital "TRIC" infections would develop the reactive arthritis syndrome (Willcox 1975).

C. trachomatis infections in the post-antibiotic era would probably be considered a nuisance, "rhinovirus of the urethra," except for their infectious sequelae that include infertility and potentially fatal ectopic pregnancies. Those conditions are expensive to treat and take a significant physical and emotional toll on affected individuals. For that reason, and the expense and inconvenience of treating urethral and vaginal discharges, significant public health efforts have focused on reducing the incidence and prevalence of *C. trachomatis* infections. Unfortunately, aggressive test and treat programs appear to be somewhat counterproductive. That strategy is associated with early short-term reductions in new infections; however, over time, the incidence curve reverses and rates return to or exceed the preprogram level. This public health phenomenon is probably explained by the "arrested immunity hypothesis." Rebound rates of infection are thought to reflect compromised herd immunity due to early antibiotic interventions disrupting development of natural immunity (Brunham et al. 2005; Brunham and Rekart 2008; Rekart and Brunham 2008). The underlying premise of the arrested immunity hypothesis can be demonstrated in the *C. muridarum* genital tract mouse model. Administration of doxycycline sooner than 10 days after an experimental genital tract infection abrogates the recall protective cellular immune response, leaving mice susceptible to reinfection (Su et al. 1999).

What is the natural history of a *C. trachomatis* infection in symptomatic individuals who do not receive antibiotic therapy? Prior to the discovery of *C. trachomatis* and use of tetracycline to treat infections in the mid 1960s, individuals with non-gonococcal urethritis (NGU) were not dying in the streets of overwhelming urethral and vaginal discharges, suggesting some intrinsic ability to control the infection. Reliable details of the natural history of symptomatic *C. trachomatis* infections are scarce because the discovery that non-gonococcal urethritis (NGU) was a bacterial rather than viral infection and that tetracycline effectively treated NGU were nearly synchronous events (Holmes et al. 1975; Moulder 1966). However, an NGU review from 1965 suggests that perhaps 80% of symptomatic individuals resolved infections, leaving roughly 20% with low-grade discharges and chronic "urethrophobia," an introverted personality disorder with depression, along with unspecified rates of reactive arthritis, uveitis, and chronic prostatitis. In the pre-antibiotic era, the rate of NGU-associated urethral strictures was roughly 5% (Csonka 1965).

Pathogenesis

More recent epidemiologic studies have clearly shown that humans have the capability to clear *C. trachomatis* genital tract infections. A study of commercial sex workers in Kenya showed that the frequency of symptomatic *C. trachomatis* infections declined with increasing time in the profession, suggesting acquired resistance to reinfection and implying acquired protective immunity (Brunham et al. 1996). In the same vein, a prior history of STI is associated with lower levels of IFU shedding on reinfection (Barnes et al. 1990). Current epidemiologic studies show that 50% of asymptomatic infected women clear the infection within 6 months, while asymptomatic men take about twice that long to clear the infection (Morre et al. 2002; Molano et al. 2005; van den Brule et al. 2002; Geisler 2010). However, not all infected individuals can clear the infection as some have been documented to be chronically infected for years (Dean et al. 2000; Campbell et al. 1993; Patton et al. 1994). Self-clearance of genital tract infections and the

detrimental effects of antibiotic therapy on herd immunity provide examples in nature of protective immunity against *C. trachomatis* infection and offer hope that a protective vaccine can be developed.

By necessity, studies of human immunity to *C. trachomatis* genital tract infections generally been performed in STI clinic settings. The majority of subjects in these studies were seeking medical care for symptomatic *C. trachomatis* infections. Very crude extrapolation from pre-antibiotic NGU literature suggests that left untreated perhaps 10–20% of these individuals would not clear their infection, and some portion of the women would progress to PID and infertility. Individuals seen in STI clinics with symptomatic *C. trachomatis* infections have suboptimal immune responses. Optimal responses are likely those of completely asymptomatic individuals who clear infections without medical attention. Asymptomatic men may be suboptimal study subjects for investigating protective immunity because the male reproductive tract is resistant to tubal factor infertility, though one could consider a male-only vaccine focused on interrupting transmission. Theoretically, the optimal study subjects would be completely asymptomatic women who self-clear infections. These individuals are difficult to identify and enroll in clinical studies. Mindful of potential limitations, there are human data supporting roles for protective humoral- and T cell-mediated immunity against *C. trachomatis* genital tract infections.

Humans infected with *C. trachomatis* have a significant humoral immune response. In women with *C. trachomatis* cervicitis and positive cultures, the level of IFU shedding in cervical secretions was shown to inversely correlate with the anti-*Chlamydia* IgA titer in cervical secretions, suggesting a role for antibodies in controlling shedding (Brunham et al. 1983). However, in a subsequent study, levels of *Chlamydia*-specific IgA in cervical mucus did not show a relationship to rates of reinfection (Cohen et al. 2005), suggesting that antibody in genital tract secretions could not prevent infection even though it may play a role in reduced shedding (transmission). The latter results do not rule out the possibility that protective effects of antibody are masked by a coincident protective T cell response. Antibody-mediated reduction in shedding likely requires local production in the genital tract as serum anti-*Chlamydia* antibody titers have not correlated with evidence of functional immunity (Brunham et al. 1983; Cohen et al. 2005; Arno et al. 1994). A mechanism that would plausibly explain decreased shedding of viable EB would be neutralization. Antibodies to the *C. trachomatis* major outer membrane protein (MOMP) have been shown to neutralize elementary bodies (Su and Caldwell 1991).

Data from mouse models (upcoming section) support CD4 T cells making IFN-γ as being critical for clearing *Chlamydia* infections from the genital tract. The mouse model results correlate with human data for both CD4 T cells and IFN-γ. In addition to epidemiologic data, early human studies showed direct evidence for T cell immunity in controlling *Chlamydia* infections. In one STI clinic study, peripheral blood T cell proliferation to *Chlamydia* antigen was inversely related to intensity of IFU shedding, except in the subset of women (17%) with the most intense shedding, $>10^4$ IFU per endocervical swab (Brunham et al. 1983). In another STI clinic study, the likelihood of a positive *Chlamydia* culture was inversely correlated with age and peripheral blood T cell proliferation to *Chlamydia* antigen (Arno et al. 1994). Supporting a role specifically for CD4 T cells in human protective immunity, in a cohort of infertile Kenyan women seropositive and seronegative for *C. trachomatis*, the HLA class II allele DQA*0102 was shown to be negatively associated with infertility (Cohen et al. 2000). Inclusion of infertile women without serologic evidence of a previous *Chlamydia* infection controlled for genetic predispositions to infertility unrelated to *Chlamydia* infections. This HLA genomic association was subsequently mapped more finely to HLA allele DRB1*1503, an allele (gene) that is close (linked) to DQA*0102 (Cohen et al. 2003). CD4 T cells "see" immunogenic peptides bound to HLA class II molecules. Restated, the two previous studies provide reasonably strong evidence that a *Chlamydia*-specific CD4 T cell response restricted by an HLA class II allele protected Kenyan women infected by *C. trachomatis* from becoming infertile. Humans are an ethnically diverse/HLA diverse species. Because an individual's T cell receptor repertoire is determined both by the MHC/HLA alleles that are present and those that are absent (Huseby et al. 2003), the mapping of HLA-related phenomenon cannot necessarily be extrapolated between ethnic groups, potentially explaining differing results in a Finnish study that identified an IL-10 promoter polymorphism as a risk for infertility (Kinnunen et al. 2002a).

In women with active *C. trachomatis* infections, there are roughly equal numbers of CD4 and CD8 T cells with memory phenotypes present in the endocervix, ratio = 1:1. With treatment and resolution of the infection, the ratio of CD4 to CD8 T cells changes to 1.2:1 (Ficarra et al. 2008). The memory T cells in the cervix are enhanced for mucosal/skin homing receptors α4β7 and CLA1 (Kelly et al. 2009). By the criterion of "attendance," both T cell subsets appear to be playing some role in the response to infection. T cell clones specific for *C. trachomatis* have been isolated from individuals with a history of genital tract infections. Curiously, the majority of *Chlamydia*-specific CD8 T cell clones are not restricted by HLA-A,-B,-C, or CD1, the human class I presentation molecules (Matyszak and Gaston 2004; Gervassi et al. 2003). These "dissonant" CD8 T cells are not accounted for within any existing immunology paradigm. *Chlamydia*-specific CD4 T cell clones have also been derived from infected individuals, and among the mapped specificities are epitopes derived from *C. trachomatis* MOMP, enolase, *PmpD*, and CT579 (Ortiz et al. 1996; Goodall et al. 2001).

Mouse model data supporting a role for IFN-γ also correlate with human T cell response data. In a cohort of Kenyan female sex workers, peripheral blood T cell IFN-γ responses to *Chlamydia* HSP60 (cHSP60) correlated with reduced risk of subsequent *C. trachomatis* infections (Cohen et al. 2005). Similarly, women with a history of PID have decreased peripheral blood T cell IFN-γ responses to activation with cHSP60 antigen compared to women with single prior infections or multiple prior infections with the last episode >12 months earlier; the latter

two groups potentially having acquired immunity based on lack of recent reinfection. In that study, women with a history of PID had low IFN-γ responses that were similar to women without serologic evidence for a previous *C. trachomatis* infection (naïve controls), implying a predisposition to PID based on inferior T cell IFN-γ responses to *Chlamydia* infections (Debattista et al. 2002). cHSP60 and, to a lesser extent, cHSP10 are dominant target antigens for the primate and human T lymphocyte response against *C. trachomatis* infections (Lichtenwalner et al. 2004; Kinnunen et al. 2002b).

From human studies, there is also evidence of detrimental cellular immune responses to *C. trachomatis* infections. Two groups have shown that T cell responses to cHSP60 include exaggerated production of IL-10 in women with *Chlamydia*-tubal factor infertility (TFI) (Kinnunen et al. 2003; Srivastava et al. 2008); IL-10 is an anti-inflammatory cytokine that alters antigen presenting cell function and diminishes Th1 responses (Rutella et al. 2006). One of those studies also showed increased TNF-α production by T cells from women with *Chlamydia*-TFI (Srivastava et al. 2008); TNF-α is an inflammatory cytokine associated with scar formation. Additional studies showed that antibody and T cell responses to cHSP60 and cHSP10 correlated with PID and TFI (Toye et al. 1993; Claman et al. 1997; Spandorfer et al. 1999; LaVerda et al. 2000; Peeling et al. 1997; Eckert et al. 1997; Tiitinen et al. 2006). Independent research groups showed that antibody responses to cHSP60 associated with TFI and PID recognized a region of cHSP60 homologous to human HSP60, raising the possibility of infection-triggered autoimmunity (Arno et al. 1995; Domeika et al. 1998). A more recent study showed a correlation between seropositivity for cHSP60, TFI, and rate of recurrent infection (Dutta et al. 2008); implying that cHSP60 seropositivity identifies women at risk for infertility and also identifies a subset of women whose adaptive immune response to prior infections does not protect them from *C. trachomatis* reinfection.

Animal Model Pathogenesis Data for *C. trachomatis* Genital Tract Infections

While there is apprehension about the extent that animal model data can be extrapolated to humans, the animal models for *C. trachomatis* infection are reasonably good representations of the human disease. Female mice vaginally infected with *C. muridarum* and female guinea pigs vaginally infected with *C. caviae* develop infections that ascend to the upper reproductive tract resulting in salpingitis, hydrosalpinx, and infertility (Rank and Sanders 1992; Rank et al. 2003; de la Maza et al. 1994). Human *C. trachomatis* serovars D, E, and L2 inoculated vaginally into mice do not cause ascending infections and therefore do not cause hydrosalpinx or infertility. The only exception is a single *C. trachomatis* serovar D strain selected by passage in C3H/HeJ mice. That lab-selected strain has a frame shift mutation in CT135, a null phenotype. How this mutation allows the human serovar D strain to cause an ascending infection in mice is unclear (Sturdevant et al. 2010).

Infecting mice with human *C. trachomatis serovars* D–L has the advantage of using the "relevant pathogens," but extrapolation from those models to human pathogenesis should be done cautiously due to species-mismatched immunobiology. Human strains infecting mice must contend with an interferon-inducible p47 GTPase base defense system that does not exist in humans (Taylor et al. 2007; Bekpen et al. 2005). A consequence of this species-specific difference is that human *Chlamydia* strains infecting mice are likely overly sensitive to innate effects of IFN-γ and thereby lowering the bar for defining protective adaptive immunity.

Species-matched *Chlamydia* animal models carry the disadvantage of not using "the relevant pathogen." However, *C. muridarum* and *C. caviae* are closely related to the human *C. trachomatis* pathogens that they model. Their major genomic differences relate to evading innate p47 GTPase defenses, which is their relative advantage for studying adaptive immunity. The relative value of *Chlamydia* animal models is likely reflected by the degree to which they reproduce the human disease; if it looks like a duck, walks like a duck, and quacks like a duck, it is probably a (good representation of a) duck.

C. muridarum is closely related to *C. trachomatis* serovar D (Read et al. 2000). There are gene-for-gene homologs in identical gene order with the only major differences occurring in the plasticity zone. 810 open reading frames are shared between *C. muridarum* and *C. trachomatis* serovar D. In the plasticity zone, *C. muridarum* has three copies of the large cytotoxin gene (p47 GTPase evasion) and lacks the *trp* operon (IDO evasion). Vaginal infections with *C. muridarum* cause ascending infections with infectious sequelae that include hydrosalpinx and infertility (de la Maza et al. 1994; Barron et al. 1981). There are two isolates of *C. muridarum* in experimental use, Nigg and Weiss, which differ slightly in virulence (Ramsey et al. 2009).

The Lung

C. trachomatis pulmonary infections have been modeled using *C. muridarum* infections of the lung. The content below is an abridged version of pneumonia studies presented to highlight differences between the lung and genital tract immune compartments.

Innate Immunity

In the upcoming genital tract infection section, one major conclusion will be that absence of TLR2 or infections with plasmid-deficient *C. muridarum* that does not activate TLR2 are protective against immunopathology in the upper genital tract. In the genital tract, TLR2 wears a black hat. It is exactly the opposite in the lungs where TLR2 wears a white hat. TLR2 knockout mice have significantly worsened immunopathology in the lungs including greater neutrophil infiltrates and elevated levels of TNF-α, IFN-γ, and IFN-γ. The same worsening of

immunopathology was seen when wild type mice were infected with the plasmid-deficient *C. muridarum* that does not activate TLR2 compared to wild type *C. muridarum*. The only commonality between the lungs and the genital tract is that severity of immunopathology is independent of bacterial clearance, as there were no differences in lung clearance between wild type and TLR2 knockout mice, or plasmid-deficient and wild type *C. muridarum* (He et al. 2011).

Adaptive Immunity: T Cells

MHC class II knockout mice are compromised in their ability to clear primary *C. muridarum* pulmonary infections but are able clear them (Williams et al. 1997); MHC class II knockout mice are unable to clear *C. muridarum* genital tract infections (Morrison et al. 1995). Similar to the genital tract, IL-12 and IFN-γ are critical cytokines for protective immunity in the lung. One-day-old knockouts of IL-12, IFN-γ, or IFN-γ receptor do not survive *C. muridarum* pulmonary infections while 1-day-old wild type mice do (Jupelli et al. 2010). A major difference in cytokines between the lungs and the genital tract relates to the biology of IL-6 and IL-17. In genital tract infections, IL-6 and IL-17 knockout mice had no discernable differences in clearance or immunopathology (Perry et al. 1998; Scurlock et al. 2011). In the lungs, IL-6 knockout mice have increased *C. muridarum* replication (Williams et al. 1998), while in wild type mice, IL-17 depletion with antibody results in increased *C. muridarum* replication and worsening of immunopathology (Bai et al. 2009).

The Genital Tract

Innate Immunity

TLR2 is a dominant pattern recognition receptor for detection of early *C. muridarum* infection and the acute inflammatory cytokine response in the genital tract, while TLR4 does not play a significant role. On day 3–4 post infection, TLR2 knockout mice have reduced levels of Cxcl2 (MIP-2; neutrophil recruiting chemokine made by macrophages) and TNF-α in genital tract secretions with negligible differences in IL-6 and IFN-γ. No apparent changes in early acute phase cytokines were seen in TLR4 knockout mice. TLR2 knockout mice also had significantly decreased acute inflammatory cell infiltration early post infection. Neither TLR2 nor TLR4 knockout mice had changes in *C. muridarum* clearance kinetics compared to wild type mice. The major finding of the study was that TLR2 knockout mice were significantly protected from scarring of the oviducts, while inflammation and scarring in TLR4 knockout mice was comparable to wild type mice (Darville et al. 2003). In vitro data with oviduct epithelial cells complemented the knockout mouse data as TLR2 was shown to be the pattern recognition receptor associated with production of acute phase cytokines by infected oviduct epithelial cell lines (Derbigny et al. 2005). The TLR2 data maybe be explained in part by *Chlamydia* LPS being a stronger activator of TLR2 than TLR4 (Erridge et al. 2004).

The critical role of TLR2 in upper genital tract immune pathology is highlighted by studies with a *C. muridarum* strain cured of its plasmid. This attenuated strain of *C. muridarum* was able to infect and replicate in cells and mice but did so without activating TLR2. Wild type mice infected with the plasmid-deficient *C. muridarum* did not develop upper tract scarring. Interestingly, wild type mice that cleared a plasmid-deficient *C. muridarum* infection were protected from upper tract scarring when subsequently infected with wild type *C. muridarum*. The mechanism of protection was unrelated to preventing the secondary infection or accelerating bacterial clearance as plasmid-deficient-immune mice had similar or slower rates of clearance on rechallenge than *C. muridarum*-immune mice (O'Connell et al. 2007). A follow-up study showed that plasmid-deficient-*C. muridarum* infections were associated with reduced neutrophil recruitment to the oviducts compared to wild type *C. muridarum* infections. To prove causality, an attempt was made to deplete neutrophils with an anti-Ly6G antibody, 1A8, but the authors found that the antibody depleted mature but not immature neutrophils. Depletion of mature neutrophils had no effect on intensity of shedding or time to clearance of *C. muridarum* infections (Frazer et al. 2011).

The principal effector cell types in innate immunity against extracellular bacterial infections are neutrophils and macrophages. In Balb/c mice, neutrophil depletion using monoclonal antibody RB6-8 C5 caused increased IFU shedding in the genital tract during the first week of the infection (Barteneva et al. 1996). Another lab, using Balb/c mice and monoclonal RB6-8 C5 antibody showed that antibody treatment depleted neutrophils from the blood and genital tract but had no effect on intensity of *C. muridarum* shedding or time to clearance. However, they did find a significant protective effect of neutrophil depletion on development of immunopathology in the oviducts (Lee et al. 2010). Additional mouse model data supporting a detrimental role for neutrophils in infection-associated oviduct immunopathology include pharmacologic inhibition of neutrophil metalloproteases (Imtiaz et al. 2006) and genetic disarming of neutrophils' ability to produce reactive oxygen species (ROS; NADPH oxidase knockout mice) (Ramsey et al. 2001a); both interventions reduced scarring and dilatation of the oviducts without affecting the intensity of infection or the rate of clearance. In the species-mismatched human serovar L2 trans-cervical uterine infection model, antibody depletion of neutrophils in dual p47 GTPase knockout mice (Irgm1 and Irgm3) delayed clearance of bacterial DNA to the same extent as CD4 depletion and dual neutrophil and CD4 depletions. These data were interpreted as potentially showing a role for IFN-γ and neutrophils in clearance (Coers et al. 2011). With the exception of the serovar L2 study, the bulk of the data suggest that neutrophils have little to no beneficial effect on clearance of *Chlamydia* from the genital tract. Instead, they appear to contribute to tissue injury as a prerequisite for hydrosalpinx formation.

Definitive data on the role of macrophages during *Chlamydia* genital tract infections are not available; however, a recent study suggested that an increased influx of TNF-α^+ macrophages compensated for a decreased Th1 response in IL-17 knockout mice allowing them to clear infections with wild type kinetics (Scurlock et al. 2011).

Epithelial cells likely play a role in innate defenses. In male subjects with *C. trachomatis* infections, there are elevated levels of the epithelial defensin HD5 present in urethral secretions. The majority of HD5 was present in an inactive pro-defensin form that required proteolytic cleavage to be bacteriocidal against *C. trachomatis*. In subjects with evidence of significant neutrophil-mediated inflammation, HD5 was detectable in its active form, suggesting an anti-chlamydial partnership between neutrophils and epithelial cells in the reproductive tract (Porter et al. 2005). Conversely, infected epithelial cells release inflammatory cytokines that may contribute to immunopathogenic scarring in the upper reproductive tract (Rasmussen et al. 1997; Stephens 2003; Johnson 2004).

The innate immune response provides a bridge to the adaptive immune response though recognition of microbial evasion and early cytokine/APC events that influence the character of adaptive immunity. The innate response to *C. muridarum* in the genital tract includes an intense early interferon response that includes type 1 IFN-α and IFN-β and type 2 IFN-γ. IFN-γ levels in genital secretions peak on day 4 and drop to undetectable levels by day 14 (Scurlock et al. 2011); IFN-β levels in genital secretions peak on day 4–5 post infection and drop rapidly down to low levels by day 6 post infection (Derbigny et al. 2010). This early innate blast of interferons (and TNF-α) likely accounts for poor replication capacity of human urogenital serovars in the mouse (Morrison et al. 2011; Perry et al. 1997), and the early two log decrease in *C. muridarum* shedding in wild type mice. IFN-γ and TNF-α synergize to induce *iNOS* transcription and nitric oxide production (Ding et al. 1988; MacMicking et al. 1997; Vila-del Sol et al. 2007; Paludan et al. 2001). Both human and mouse *Chlamydia* strains are susceptible to high levels of nitric oxide (Igietseme et al. 1996, 1997). The principle source of early IFN-γ in genital secretions is natural killer cells. Depletion of NK cells with anti-asialo-GM1 antiserum decreases early IFN-γ mRNA transcripts in the genital tract and day 4 IFN-γ producing cells from draining iliac lymph nodes. The outcome of NK depletion was prolonged shedding of *C. muridarum* and a shift toward Th2-like immunobiology as evidenced by increased anti-*Chlamydia* IgG1 levels in serum (Tseng and Rank 1998). The source of IFN-β detectable in genital secretions is likely infected epithelial cells. Infected oviduct epithelial cell production of IFN-β is largely but not entirely dependent on TLR3, and TLR3 knockout mice have negligible IFN-β in genital tract secretions (Derbigny et al. 2010). Conversely, macrophages infected with *C. muridarum* secrete IFN-β via a signaling pathway(s) dependent on MyD88, suggesting TLRs7-9 (Nagarajan et al. 2005). Loss of the majority of IFN-β from genital tract secretions in TLR3 knockout mice suggests that epithelial cells were the source of IFN-β in genital secretions, though not necessarily IFN-β present in genital tract tissues.

In experiments done three decades ago, type 1 interferon treatment of *mouse* L cells had negative effects on replication of a *C. trachomatis* serovar L2 but not *C. psittaci* (Byrne and Rothermel 1983). In hindsight, this may have been the first demonstration of the murine inducible p47 GTPase defense mechanism as type 1 interferons alone are poor inducers of iNOS (Ding et al. 1988). To the surprise of most investigators, type 1 interferon receptor (IFNR1) knockout mice and wild type mice treated with neutralizing anti-IFN-β antibody had reduced susceptibility to *C. muridarum* infection with faster clearance and less oviduct immunopathology (Nagarajan et al. 2008; Prantner et al. 2011). The enhanced immune protective immune response in the absence of IFNR1 signaling included a more vigorous CD4 T cell and IFN-γ response to the infection. In vitro studies with *C. muridarum*-specific CD4 T cells and oviduct epithelial cell lines suggested that the negative effect of type 1 interferons on bacterial clearance was due to IFN-β suppression of IFN-γ-mediated upregulation of epithelial MHC class II. The reduced MHC class II induction was associated with reduced IFN-γ production by CD4 T cells activated by infected epithelial cells and reduced CD4 T cell recognition of infected epithelial cells (Jayarapu et al. 2009). Mice deficient in MyD88, the signaling molecule for TLR2, 4, 5, and 7–9, have delayed clearance of *C. muridarum* from the genital tract (Nagarajan et al. 2011). The MyD88 knockout mice were deficient in the early innate blast of IFN-γ provided by natural killer (NK) cells and had delayed recruitment of CD4 T cells to the reproductive tract, likely explaining the delay in clearance and highlighting the bridge between innate and adaptive immunity (Nagarajan et al. 2011). These studies raise the interesting possibility that an individual's susceptibility to *C. trachomatis* infection and downstream immunopathology could be influenced by the magnitude of their initial natural killer cell response or the relative balance between type 1 and type 2 interferon levels in the genital tract during the transition from innate to adaptive immunity.

Adaptive Immunity: B Cells and Ig

B cell- and antibody-mediated immunity is one of the interesting and unresolved components of the adaptive immune response to *Chlamydia* in mice and men. In the *C. caviae* guinea pig GPIC model, passive transfer of immune serum reduced the intensity of genital tract shedding without decreasing the time to clearance (Rank and Batteiger 1989). A humoral immune response is not critical for clearing primary *C. muridarum* infections of the mouse genital tract as B cell-deficient mice cleared *C. muridarum* primary infections with normal kinetics (Su et al. 1997). Human studies in *C. trachomatis* infections suggest that anti-*Chlamydia* IgA in cervical secretions reduced the intensity of shedding. Testing that hypothesis in the mouse model showed no difference in clearance of *C. muridarum* from the genital tracts of IgA knockout and wild type mice (Morrison and Morrison 2005a). That study was done in mice that were T cell sufficient, leaving open the possibility that protective humoral immunity was masked by a potent protective T cell response.

Testing protective humoral immunity in mice lacking CD4 T cells is equally problematic as those mice do not mount meaningful antibody responses (e.g. (Williams et al. 1997; Morrison et al. 1995)). Asking an un-"helped" population of B cells to mount an immunoglobulin response to an invading pathogen likely does not test their mettle. In the setting of a protective T cell response, the only way to "see" a role for B cells would be to deplete the T cells after they have had a chance to "help" the B cells generate antibody. When that was done in *Chlamydia* pathogenesis experiments, a role for antibodies in protective immunity was discovered.

B cells and antibody have a role in clearing secondary infections, i.e., rechallenge of mice that previously cleared a primary infection. Immune wild type mice depleted of CD4 T cells are able to clear a secondary challenge, while immune B cell-deficient mice depleted of CD4 T cells cannot. Passive transfer of anti-chlamydial antibodies into immune B cell-deficient/CD4 T cell-depleted mice reconstitutes the ability to clear a secondary infection. However, passive transfer of immune serum or antibody to naïve mice does not protect them from primary infection, and importantly, the specificity of the antibody during secondary infections seems to be of limited importance. Anti-MOMP, anti-LPS, and serum from immune mice were all capable of mediating the T cell-independent protection secondary clearance mechanism, while anti-cHSP60 did not reconstitute protection (Morrison and Morrison 2005b). This is informative as anti-LPS antibody has no potential to neutralize EBs and argues against an antibody neutralizing effect, directly or via complement. Failure of anti-cHSP60 antibody suggests that the antibodies must recognize an EB surface exposed antigen. Failure of passive antibody transfer to protect against primary infections does not necessarily argue against other innate mechanisms, e.g., ADCC. Failure to protect naïve mice may be related to the relevant cells for mediating ADCC, such as activated macrophages, not being prepositioned in the genital tract to address a primary infection. An alternative antibody contribution to protection against secondary infections could be enhanced antigen presentation. Professional APC bearing antigen-specific surface Ig are 1,000 times more efficient at presenting antigen (Rock et al. 1984) (i.e., can present antigens present at very low concentrations, potentially accelerating an adaptive immune response). A role for Fc receptors in anti-*Chlamydia* secondary responses has been previously demonstrated. Fc receptor knockout and wild type mice clear infection at the same rate and shed with the same intensity; however, on rechallenge, the protective immune response is dramatically compromised in the Fc receptor knockout mice (Moore et al. 2002). In that study, activated peritoneal macrophages were able to mediate enhanced killing of *C. muridarum*-infected epithelial cells in the presence of anti-*Chlamydia* immune mouse serum, which also enhanced antigen presentation and T cell activation. One thorny issue with an ADCC mechanism is the nature and identity of the *Chlamydia* antigen on infected cell surfaces that could be seen by anti-*Chlamydia* antibody. One possibility would be *Chlamydia* LPS that is detectable on infected cell membranes and to a lesser extent on adjacent non-infected cells (Karimi et al. 1989). Antibody depletion of CD4 or CD8 or both CD4 and CD8, after clearance of the primary infection, had no effect on the antibody-dependent secondary clearance mechanism (Morrison and Morrison 2001). Based on those results, the candidate effect cell lineage could be macrophages, mast cells, or NK cells. B cells are not likely as immune serum can reconstitute protection in immune B cell-deficient mice. The antibody-dependent/αβ TCR T cell-independent secondary clearance mechanism is one of the major remaining mysteries of protective immunity in the genital tract.

Lack of a critical role for antibodies in clearing primary infections should not be interpreted to mean that anti-*Chlamydia* antibodies are unimportant. An early mouse vaccine study based on a MOMP neutralizing epitope delivered subcutaneously showed no efficacy against *C. trachomatis* serovar D genital tract infections, even with evidence of a neutralizing IgG in genital secretions. Those experiments suggested that neutralization alone was not a potent protection mechanism (Su et al. 1995). However, subsequent vaccination studies with refolded native MOMP protein showed protection against *C. muridarum* infections of the genital tract (Pal et al. 2005). The importance of native protein conformation to vaccination efficacy implies a role for antibody as αβ TCR T cells "see" only short denatured peptides. Two recent studies, one combining inactivated EB with an intracellular nonelementary body *Chlamydia* protein (CPAF) and a second study vaccinating B cell sufficient and deficient mice with native MOMP, have shown improved protection with vaccine strategies that generate anti-*Chlamydia* antibodies directed against EB (Farris et al. 2010; Li et al. 2010). The precise mechanism of antibody-dependent enhanced vaccine-protection has not been determined.

Adaptive Immunity: T Cells

Doxycycline interruption studies defined the adaptive phase of the anti-*Chlamydia* cellular immune response in the genital tract to be significant and durable by day 10 post infection in C57BL/6 mice (Su et al. 1999). Investigation of the cellular infiltrate during the adaptive clearance phase showed a predominance of CD4 T cells with lesser numbers of CD8 T cells and B cells. Curiously, when infection and acute inflammation resolved, left behind in the uterus were perivascular clusters of CD4 T cells, with fewer clusters of CD8 T cells, both associated with macrophages and occasional B cells. These immune structures (microfollicles) remain assembled out to day 70 post infection and likely beyond. These immunologic clusters were less obvious in the vagina and oviduct. The significance of these structures is unknown, but they may represent a rapid response mechanism based on prepositioned effector cells (Morrison and Morrison 2000).

Early seminal work with T cell depletions in guinea pigs (Rank and Barron 1983) and studies utilizing immunocompromised mice (Rank and Barron 1983) showed that T cells were critical for clearance of primary chlamydial genital tract infections. Athymic nude mice lacking T cells challenged with

C. muridarum became chronically infected, entirely unable to clear the genital tract infections (Rank et al. 1985). Knockout mouse studies showed that αβTCR$^+$ T cells are critical to clearance while γδTCR$^+$ T cells play no apparent role (Perry et al. 1997). Because *Chlamydiae* are intracellular pathogens, early host defense hypotheses focused on CD8 T cells. *Chlamydia*-specific CD8 T cell lines and clones could be derived from immune mice, and they facilitated bacterial clearance in adoptive transfer experiments (Ramsey and Rank 1991; Igietseme et al. 1994a; Starnbach et al. 1994). However, a critical role for CD8 T cells in clearance of *Chlamydia* from the genital tract was set aside by experiments utilizing β-2 microglobulin knockout mice. β-2 microglobulin is a critical component of MHC class Ia and Ib heterodimers (human equivalents HLA-A,-B,-C, and CD1) that present foreign peptides and glycolipids to CD8 T cells and natural killer T cells (NKT cells), respectively. Despite a paucity of CD8 T cells and NKT cells, β-2 microglobulin-deficient mice cleared *C. muridarum* genital tract infections with kinetics similar to that of wild type mice (Morrison et al. 1995). Current studies support a greater role for the *Chlamydia*-specific CD8 T cell response in immunopathology rather than clearance and protection. CD4 knockout mice (sufficient in CD8 T cells and B cells) have higher infertility rates than CD8 knockout mice (sufficient in CD4 T cells and B cells) after clearing primary and secondary *C. muridarum* genital tract infections (Igietseme et al. 2009). The CD8 T cell-mediated damage to the upper reproductive tract requires TNF-α production by CD8 T cells but also from non-T cell sources (Murthy et al. 2011).

Data from mouse models strongly support a dominant role for CD4 T cells in protective immunity; however, the original and most precise statement of protective immunity in the mouse model is that it is critically dependent on MHC class II (Morrison et al. 1995; Morrison and Caldwell 2002). MHC class II heterodimers present foreign peptides to CD4 T cells. MHC class II knockout mice are unable to clear primary *C. muridarum* genital tract infections. That data, plus normal clearance in β2-microglobulin knockout mice and supporting CD4 T cell depletion studies, are broadly interpreted as showing a dominant role for CD4 T cells and no significant role for CD8 T cells in clearance of infection. This generalization risks being overly broad and may limit consideration of important CD8 immunobiology. The relevant caveats are that CD8 T cells responses are frequently dependent on CD4 T cell responses during primary infections (Shedlock and Shen 2003), i.e., no MHC II may also mean a weak or absent CD8 response, and CD4-deficient mice are able to clear *C. muridarum* genital tract infections with a relatively modest 10-day delay compared to wild type control mice (Morrison et al. 1995). In addition, *Chlamydia*-specific CD8 T cells in humans are predominantly HLA-A,-B,-C, and CD1 unrestricted (Matyszak and Gaston 2004; Gervassi et al. 2003), i.e., unusual. In mice, MHC class II-restricted CD8 T cells comprise roughly 10% of "normal" cellular immune responses (Golding and Singer 1985). The fact that CD4 knockout mice clear primary infections demonstrates that CD8 T cells can mediate clearance; the only question is the relevance of that clearance mechanism in the setting of a "normal" immune response. Our current understanding of *Chlamydia*-specific CD8 T cell immunobiology is incomplete and likely important for understanding genital tract pathogenesis.

CD4 T cells play the dominant role in protective immunity in the mouse model. CD4 T cell depletion of immune B cell-deficient mice renders them susceptible to reinfections with *C. muridarum* that they cannot clear (Morrison and Morrison 2005b). Mice depleted of CD4 T cells do not clear primary infections (Morrison et al. 2011). Those data plus the inability of MHC class II knockout mice to resolve primary infections strongly support the proposition that CD4 T cells are the dominant effector cell type mediating protective immunity in the genital tract. While informative, this conclusion is incomplete as it is clear that not all *Chlamydia*-specific CD4 T cells mediate protective immunity in adoptive transfer studies (Igietseme et al. 1993, 1994b), and not all vaccinations that generate *Chlamydia*-specific CD4 T cell responses are protective (Yu et al. 2010, 2011). Identification of the critical effector T cell subset also does not reveal the mechanism that terminates *Chlamydia* replication in genital tract epithelium.

The protective contributions of cytokines to genital tract immunity have been analyzed using knockout mice. Cytokines shown to contribute to protective immunity in the genital tract include IL-1β, TNF-α, IFN-γ, and IL-12. IL-1β knockout mice have delayed clearance compared with wild type mice but also reduced oviduct pathology (Prantner et al. 2009). IL-1β has complex immunobiology encompassing innate and adaptive immune responses. In innate immunity, it participates in increasing IL-6 and TNF-α production, induces expression of defensins in non-hematopoetic cells, recruits and activates neutrophils and macrophages, and affects prostaglandin synthesis among its many activities (Dinarello 1991; Liu et al. 2002). In adaptive immunity, IL-1β contributes to γδ T cell production of IL-17 (Sutton et al. 2009) and Th17 differentiation (Chung et al. 2009). IL-17 receptor knockout mice have altered inflammatory infiltrates but no discernible differences in time to clearance or immunopathology compared to wild type mice (Scurlock et al. 2011). TNF-α receptor knockout mice have more intense genital tract shedding but not significantly prolonged time to clearance of *C. muridarum*. TNF-α receptor knockout mice are more permissive for *C. trachomatis* serovar D infections (Perry et al. 1999a). Biologic activities of TNF-α have some overlap with IL-1β including positive feedback for IL-1 and IL-6 production. IFN-γ knockout mice clear the first 99.9% of *C. muridarum* from the genital tract with near normal kinetics during the first 3 weeks of infection but allow *C. muridarum* to disseminate to extragenital sites and cannot clear the last two logs of *C. muridarum* from the genital tract (Perry et al. 1997, 1999a; Cotter et al. 1997). Antibody depletion of IL-12 delays genital tract clearance by roughly 4 weeks. With IL-12 depletion, there was a significant delay in IFN-γ and IL-6 (and presumably TNF-α) splenocyte responses. The detrimental effect of IL-12 depletion on genital tract clearance was much greater than was the absence of IFN-γ in IFN-γ knockout mice (Perry et al. 1997). It is possible that the higher intensity shedding in IL-12 depleted

mice reflects the combined relative deficiencies of both IFN-γ and TNF-α, the latter inferred based on decreased IL-6 splenic responses. IL-22 is an interesting cytokine because it regulates innate immunity at mucosal surfaces including induction of defensins in epithelial cells (Wolk et al. 2004). IL-22 knockout mice have been generated (Zheng et al. 2007), but there are no published data using them in *Chlamydia* models of infection. The above studies have provided important insights into *Chlamydia* pathogenesis; however, cytokine deficiencies with pleiotropic effects on host innate and adaptive immunity do not identify specific effector mechanisms responsible for terminating *Chlamydia* replication in reproductive tract epithelium.

A useful and widely used paradigm for understanding T cell-mediated immunity is based on cytokine profiles. The first and broadest cytokine profiles were Th1 (IFN-γ/IL-2 producers) versus Th2 (IL-4 producers) (Mosmann et al. 1986). Th1 cells are associated with resolution of infections caused by intracellular pathogens while Th2 responses are associated with resolution of infection by extracellular pathogens. Adoptive transfer experiments utilizing IFN-γ sufficient and deficient mice have shown that CD4 T cell protection against *C. muridarum* and *C. trachomatis* serovar L2 in the genital tract requires IFN-γ (Li et al. 2008; Gondek et al. 2009) and that *Chlamydia*-specific Th2 cells are ineffectual in protective immunity (Gondek et al. 2009; Shaw et al. 2002; Hawkins et al. 2002). Vaccination studies with single antigens have also shown that IFN-γ is important for protective immunity in the genital tract (Murthy et al. 2007), but importantly, recent studies have shown that IFN-γ by itself is not sufficient to mediate protection. One *C. muridarum* vaccine antigen protective in the genital tract is *PmpG-1*, containing the CD4 T cell epitope $PmpG_{303–311}$ (Yu et al. 2009, 2010). When *PmpG-1* was given with a CpG-ODN adjuvant, it generated a *Chlamydia*-specific CD4 T cell response that was almost exclusively a mono-IFN-γ profile that did not protect mice from *C. muridarum* genital tract infections. When the same *PmpG-1* antigen was given with a DDA/TDB adjuvant, the CD4 T cell response included an IFN-γ/TNF-α profile that provided significant protection in the genital tract (Yu et al. 2010). Th1 cells making IFN-γ/TNF-α/IL-2 were previously shown in *Leishmania* vaccine studies to be a protective T cell phenotype and were given the moniker "multifunctional Th1" (Darrah et al. 2007). Recent work in the *C. muridarum* model has shown that protective immunity against *C. muridarum* genital tract infections correlates with a CD4 T cell cytokine profile of IFN-γ/TNF-α with or without IL-2 (Yu et al. 2011).

A useful parameter for categorizing effector mechanisms of protective immunity against *Chlamydia* infections of the genital tract is the immunobiology of IFN-γ. IFN-γ has already come up in the context of its role in innate host defenses (IDO and p47 GTPases). IFN-γ is also an integral component of adaptive immunity. It has important effects on antigen presentation including transition to immunoproteosomes that process foreign proteins into antigenic peptides (T cell epitopes) and upregulation of MHC class I, MHC class II, and ICAM-1 on professional (dendritic cells, macrophages, B cells) and semiprofessional antigen presenting cells (epithelial cells). Epithelial cells express very little MHC class II unless exposed to IFN-γ. Upregulation of MHC class II is likely critical in host defense against *C. trachomatis* replicating in epithelial cells lining the reproductive tract because CD4 T cells utilize MHC class II to "see" infected epithelial targets. MHC class II knockout mice cannot clear *Chlamydia*. Correlation between IFN-γ-induced MHC class II and CD4 T cell recognition of *C. muridarum*-infected epithelial cells has been demonstrated in vitro (Jayarapu et al. 2009). IFN-γ also upregulates expression of epithelial inducible nitric oxide synthetase (*iNOS; nos2*) that generates nitric oxide, the effector molecule for one of the two known CD4 T cell-mediated mechanisms for controlling *Chlamydia* replication in epithelial cells.

IFN-γ Dependent Cellular Immunity (*iNOS*)

Because replication *of C. trachomatis* urogenital serovars D–K is largely limited to reproductive tract epithelium, it is reasonable to presume that protective CD4 T cell responses must terminate *C. trachomatis* replication in epithelial cells. A universal feature of adaptive T cell immunity is the ability of T cells to "see" infected cells presenting microbial peptides bound to antigen presentation molecules on their cell surface; therefore, CD4 T cells likely interact physically with infected epithelial cells to mediate protective immunity. Consistent with general features of adaptive cellular immunity, the first CD4 T cell-mediated mechanism for terminating *Chlamydia* replication in epithelial cells requires physical interaction between T cells and infected epithelial cells.

In vitro CD4 T cells can terminate *Chlamydia* replication by upregulating expression of epithelial *iNOS* to generate *Chlamydiacidal* levels of nitric oxide, a chemical antiseptic analogous to hydrogen peroxide. This mechanism requires IFN-γ and physical contact between CD4 T cells and infected epithelial cells via LFA-1 on the T cell interacting with ICAM-1 on the epithelial cell. IFN-γ alone induces *iNOS* transcription in epithelial cells, but the physical interaction is required to boost nitric oxide levels to sterilizing levels (Igietseme et al. 1996).

A CD4 protective mechanism mediated by IFN-γ/*iNOS*/nitric oxide was a satisfying mechanism for controlling an intracellular pathogen such as *C. trachomatis*. However, the reality of protective immunity was more complex than the initial glimpse at its mechanisms. Mice deficient in *iNOS* were not compromised in clearance of *C. muridarum* genital tract infections (Igietseme et al. 1998; Ramsey et al. 1998), and IFN-γ-knockout mice cleared 99.9% of *C. muridarum* from the genital tract with near normal kinetics (Perry et al. 1997, 1999a; Cotter et al. 1997). This was disconcerting, as were subsequent experiments showing that mechanisms for killing/lysing infected epithelial cells via perforin and FasL were also dispensable for clearing primary *C. muridarum* infections from the genital tract (Perry et al. 1999b). If induction of nitric oxide production and physical killing of infected epithelial cells were not critical and antibodies were not critical, then there was no known host

defense mechanism for understanding resolution of *C. muridarum* genital tract infections. However, critical experiments showed that *iNOS* was important for sterilizing immunity (Ramsey et al. 2001b). Viable *C. muridarum* could be recovered from the genital tracts of *iNOS*-deficient but not wild type mice that previously self-cleared genital tract infections when they were treated with cyclophosphamide, a lymphocyte, neutrophil, and monocyte/macrophage toxin.

The IFN-γ and *iNOS* knockout mouse data showed that sterilizing immunity was dependent on IFN-γ and *iNOS* but also that there were IFN-γ- and *iNOS*-independent mechanisms for clearing *C. muridarum* from the genital tract. The existing data could be readily explained if there were two or more redundant CD4 T cell mechanisms for terminating *Chlamydia* replication in epithelial cells. It was perplexing that neither *iNOS* nor T cell-mediated lysis of infected epithelial cells were critical for controlling genital tract infections as knockout mice singly deficient in either function could still clear infection. However, recent data suggest that the majority of *Chlamydia*-specific CD4 T cells recognize infected epithelial cells at or after 15 h post infection and do not physically lyse them for another 12^{+} h after recognition, ~30 h post infection. Physical killing of *C. muridarum*-infected epithelial cells by CD4 T cells occurs well after the "eclipse phase" when non-infectious reticulate bodies predominate. That study suggested that *Chlamydia*-specific T cells terminate *Chlamydia* replication by directly killing EB within intact epithelial cells (Jayarapu et al. 2010).

IFN-γ Independent Cellular Immunity (*Plac8*)

Evidence for an *iNOS* and IFN-γ-independent CD4 T cell mechanism came from in vitro studies utilizing *Chlamydia*-specific CD4 T cell clones. In a panel of *Chlamydia*-specific CD4 T cell clones derived from mice that self-cleared *C. muridarum* genital tract infections, it was noted that some clones were better than others at controlling *C. muridarum* replication in epithelial cells. The best clones were able to terminate *C. muridarum* replication in the absence of exogenous IFN-γ and in the presence of inhibitors of *iNOS*. Blocking the ability of this potent subset of CD4 T cell clones required both inhibition of *iNOS* and inhibition of T cell degranulation. This was evidence for a second CD4 T cell mechanism that was not dependent on nitric oxide production. Because these same T cell clones recognized infected epithelial cells well after the *Chlamydia* eclipse phase, it was unlikely that physical destruction of the epithelial "*Chlamydia* incubator" was the relevant mechanism for terminating infection. The existing data suggested that the *iNOS*-independent pathway (degranulation-dependent) involved injection of a preformed T cell *Chlamydia* microbicide into the epithelial cytosol that directly attacked elementary bodies (Jayarapu et al. 2010).

The major T cell granule microbicide in humans is granulysin. However, mice do not have a granulysin homolog, implying existence of an additional T cell microbicide active against *Chlamydia*. Microarray experiments comparing expression of the most potent CD4 T cell clones to less effective CD4 T cell clones identified a small cysteine-rich protein known as *Plac8* (also called onzin) as a candidate T cell granule *Chlamydia* microbicide (Johnson et al. 2012). *Plac8* knockout mice were previously shown to be more susceptible to *Klebsiella* peritonitis (Ledford et al. 2007), implying at least an indirect antibacterial function for *Plac8*. The *Chlamydia*-specific CD4 microarray data was followed by infectious challenge of *Plac8*-deficient mice that revealed a compromised ability to clear *C. muridarum* genital tract infections. *Plac8* knockout mice have a ~20 day delay in clearance of *C. muridarum* compared to wild type controls. Continuous treatment of *Plac8* knockout mice with the *iNOS* inhibitor N-monomethyl-L-arginine (MLA) rendered *Plac8* knockout, but not wild type mice, nearly incapable of clearing a genital tract infection over 8 weeks. That experiment demonstrated that there were redundant mechanisms for clearing *C. muridarum*: one dependent on *iNOS* and the other dependent on *Plac8*. Mice deficient in either *Plac8* or *iNOS* could clear a *C. muridarum* genital tract infection, but dual-deficient mice, genetically deficient in *Plac8* and pharmacologically deficient in nitric oxide production, were severely compromised in their ability to clear a genital tract infection (Johnson et al. 2012).

Plac8 is a 12.4 kD protein that localizes to neutrophil granules and is expressed by a subset of T cells. Confusingly, it does not have a conventional signal peptide to put it into vesicular trafficking pathways necessary for delivery to granules. However, its co-purification with granules and absence from cytosolic cell fractions argues that it is in the granule and likely has an atypical signal peptide. Details about *Plac8*'s role as either the facilitator or effector molecule for the *iNOS*-independent mechanism for terminating *C. muridarum* replication in epithelial cells remains to be determined. Until that *Plac8* biology is clarified, it is unclear whether *Plac8* directly kills EBs or facilitates delivery or activity of an unknown effector molecule that does.

In summary, there are three major adaptive immunity mechanisms for clearing *Chlamydia* genital tract infections in the mouse model: two CD4 T cell mechanisms and one antibody-dependent mechanism (Fig. 21.4). The originally described CD4 T cell mechanism is *iNOS* and IFN-γ dependent, while the second CD4 T cell mechanism is *Plac8* dependent. The CD4 T cell mechanisms are potent and singly sufficient (i.e., redundant) for clearing primary genital tract infections; however, sterilizing immunity requires both *iNOS* and *Plac8*. Interestingly, neither *iNOS*-dependent nor *Plac8*-dependent T cell mechanisms require cytolysis. Instead, both T cell mechanisms neutralize *Chlamydia* trapped within an intact epithelial cell, a "toxic bag" model of sterilizing immunity. In fact, premature cytolysis would likely be detrimental to both T cell mechanisms. *iNOS* expression has been demonstrated in epithelial cells lining the human reproductive tract (Tschugguel et al. 1998; Al-Azemi et al. 2010; Ekerhovd et al. 1999), and murine *Plac8* has a highly conserved human homolog (Ledford et al. 2007). There are no reasons a priori to dismiss *iNOS* and *Plac8* as being relevant protective effector mechanisms in humans. The third adaptive immunity mechanism is B cell and antibody dependent with an

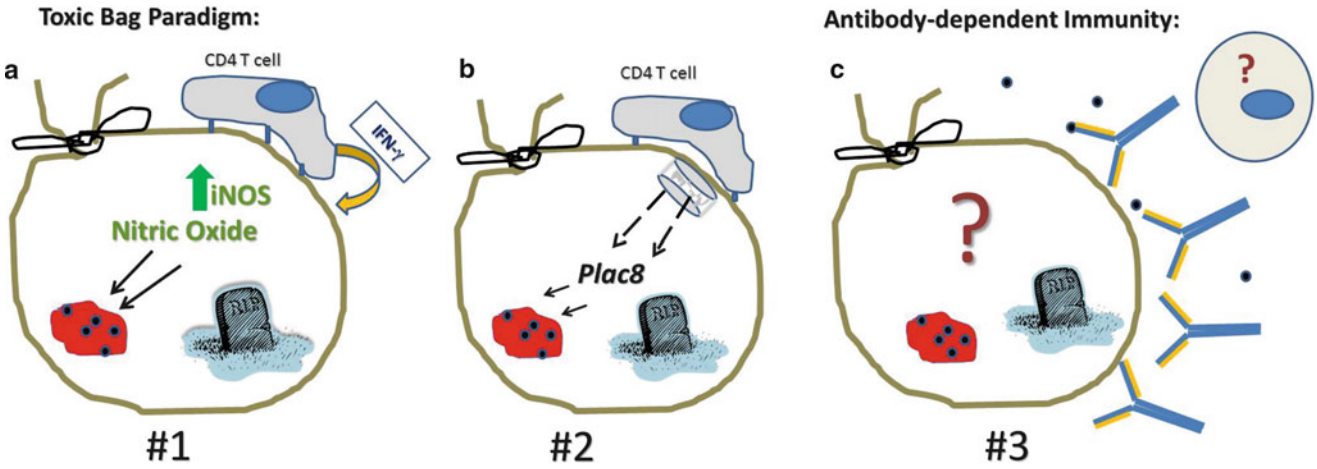

Fig. 21.4

Summary of adaptive immunity mechanisms for clearing *C. muridarum* genital tract infections. (a) CD4 T cell and IFN-γ/TNF-α upregulation of epithelial iNOS and nitric oxide production to terminate replication. (b) CD4 T cell degranulation and *Plac8*-dependent termination of replication. (c) The mysterious B cell and antibody-dependent clearance mechanism

unknown effector mechanism and unknown effector cell type. That mechanism is not dependent on neutralizing antibody but may require antibody recognition of intact EB. Whether the B cell and antibody-dependent effector mechanism acts on intracellular or extracellular *Chlamydia*, and how, is currently unknown.

Conclusions

(A) *Comparisons between Chlamydia species.* There is an unproven but appealing association between the ability to replicate in macrophages, the ability to disseminate, and the relative roles of CD8 *versus* CD4 T cells in mediating clearance. In mouse models, pathogens capable of replicating in macrophages, e.g., *C. psittaci* and *C. pneumoniae*, require protective immunity based on CD8 T cells, while *Chlamydia* species that are largely limited to replicating in epithelium, e.g., *C. trachomatis* urogenital serovars and *C. muridarum*, require CD4-mediated clearance mechanisms. It is tempting to speculate that the cell tropism of each *Chlamydia* species determines the relevant protective cellular immune response(s).

(B) *The role of innate immunity.* TLR2 is the principal sentry for detecting invasion by *Chlamydia* species, possibly because *Chlamydia* LPS binds and activates TLR2 more potently than TLR4. Neutrophils, in spite of their best intentions, appear to play detrimental roles during *Chlamydia* infections. They are associated with lung and genital tract damage during infections with *C. pneumoniae* and *C. trachomatis*, and in the case of *C. pneumoniae*, neutrophils serve as Trojan horses for infecting unsuspecting alveolar macrophages and facilitating replication in epithelial cells. During natural and experimental trachoma infections, neutrophils are intimately involved in detrimental inflammation and may antagonize local immunity by interfering with IgA class switching. In experimental models and natural infections, early innate cytokine responses likely dampen *Chlamydia* replication, e.g., IFN-γ and TNF-α induction of *iNOS*, until the adaptive immunity becomes engaged in bacterial clearance.

(C) *The role of B cells and antibodies. Chlamydia*-specific antibodies are likely to be beneficial but play a secondary role in host defense against natural *Chlamydia* infections. Humoral immunity is an important consideration in vaccine-generated immunity. It is unclear whether neutralization is an important protective mechanism or whether anti-*Chlamydia* IgA has a unique role in protective immunity. Critical details of the antibody-dependent secondary clearance mechanism including the effector cell type remain to be determined.

(D) *Other aspects of adaptive immunity.*

1. Local immunity is important for epithelial pathogens. Secondary immunity in the eye and genital tract appears to include pre-positioning of effector cells after resolution of the initial infection. Antibody, to the extent that it is protective, likely needs to be locally produced.
2. IFN-γ is important but potentially overrated due to experimentation in species-mismatched models complicated by the existence of the rodent inducible p47 GTPase innate defense system.
3. There are two CD4 T cell effector mechanisms for terminating replication in epithelial cells: one based on IFN-γ/TNF-α and iNOS and the other on Plac8. These mechanisms are potent and redundant for clearing the genital tract, but both are required for sterilizing immunity. Both mechanisms appear to kill EB within intact epithelial cells. It is unclear to what extent CD8 T cells contribute to terminating replication in epithelial cells or what mechanism(s) CD8 T cells use to

terminate replication in non-epithelial lineages including monocyte/macrophages.

4. Bacterial clearance and severity of immunopathology are generally inversely related but are not inextricably linked.
5. Successful resolution of a Chlamydia infection without immunopathology may depend on the relative balance of multiple factors including IFN-β, IFN-γ, IL-1β, TNF-α, IL-10, and IL-12 during the transition from innate to adaptive immunity. TNF-α appears to be a Jekyll and Hyde cytokine with one foot in protection and the other in immunopathology.

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22 Bacterial Toxins

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Introduction

Toxins were the first bacterial virulence factors to be identified and were also the first link between bacteria and cell biology. Cellular microbiology was, in fact, naturally born a long time ago with the study of toxins, and only recently, thanks to the sophisticated new technologies, has it expanded to include the study of many other aspects of the interactions between bacteria and host cells. This chapter covers mostly the molecules that have been classically known as toxins; however, the last section also mentions some recently identified molecules that cause cell intoxication and have many but not all of the properties of classical toxins. ❯ *Tables 22.1* and ❯ *22.2* show the known properties of all bacterial toxins described in this chapter, while ❯ *Fig. 22.1* shows the subunit composition and the spatial organization of toxins whose structures have been solved either by X-ray crystallography or by quick-freeze deep-etch electron microscopy.

Abbreviations: SEA-SEI, staphylococcal enterotoxin A through I; TSST-1, toxic shock syndrome toxin 1; SPEA, B, and C, streptococcal pyrogenic enterotoxins A, B, and C; ETA and B, exfoliative toxins A and B; MHC, major histocompatibility complex; Vβ or Vγ, T-cell-receptor variable domains; LukF, leucocidin F; PA, protective antigen; RTX, repeats-in-toxin; CryIA, CytB, G_i, G_s, G_o, G_t, G_{olf}, GTP-binding proteins; MAPKK1 and 2, mitogen-activated protein kinases 1 and 2; EF2, elongation factor 2; Rho, Rac, and Cdc42, GTP-binding proteins that control assembly of actin stress fibers; IL2, 4, and 5, interleukins 2, 4, and 5; TeNT, tetanus neurotoxin; VAMP, vesicle-associated membrane protein; BoNT, botulism neurotoxin; SNAP, synaptosome-associated protein; YOP, *Yersinia* outer-membrane proteins; AvrRxv, plant pathogen virulence protein; Ipa, invasion plasmid antigen; ICE, interleukin-converting enzyme; Sop, *Salmonella* outer-membrane protein;

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Table 22.1
Classes of toxins described in the text, their features and activity

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
Toxins acting on the cell surface	Immune system (superantigens)	SEA-SEI, TSST-1, SPEA, SPEC, SPEL, SPEM, SSA, and SMEZ	*Staphylococcus aureus* and *Streptococcus pyogenes*	Binding to MHC class II molecules and to Vβ or Vγ of T-cell receptor	T-cell activation and cytokines secretion	SEB SEC2, SEC3, SED, SEH TSST1, SPEA SPEC
		MAM	*Mycoplasma arthritidis*	Binding to MHC class II molecules and to Vβ or Vγ of T-cell receptor	Chronic inflammation	+
		YPMa	*Yersinia pseudotuberculosis*	Binding to MHC class II molecules and to Vβ or Vγ of T-cell receptor	Chronic inflammation	–
		SPEB	*S. pyogenes*	Cysteine protease	Alteration in immunoglobulin-binding properties	+
		ETA, ETB, and ETD	*S. aureus*	Trypsin-like serine proteases	T-cell proliferation, intraepidermal layer separation	ETA, ETB
	Surface molecules	BFT enterotoxin	*Bacteroides fragilis*	Metalloprotease, cleavage of E-cadherin	Alteration of epithelial permeability	–
		AhyB	*Aeromonas hydrophila*	Elastase, metalloprotease	Hydrolyzation of casein and elastine	–
		Aminopeptidase	*Pseudomonas aeruginosa*	Elastase, metalloprotease	Corneal infection, inflammation and ulceration	–
		ColH	*Clostridium histolyticum*	Collagenase, metalloprotease	Collagenolytic activity	–
		Nhe	*Bacillus cereus*	Metalloprotease and collagenase	Collagenolytic activity	–
	Cell membrane			Cell membrane permeabilization	Cell death	
	Large pore-forming toxins	PFO	*C. perfringens*	Thiol-activated cytolysin, cholesterol binding	Gas gangrene	+
		SLO	*S. pyogenes*	Thiol-activated cytolysin, cholesterol binding	Transfer of other toxins, cell death	–
		LLO	*Listeria monocytogenes*	Induction of lymphocyte apoptosis	Membrane damage	–
		Pneumolysin	*S. pneumoniae*	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–
	Small pore-forming toxins	Alveolysin	*B. alveis*	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–
		ALO	*B. anthracis*	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–
		α-Toxin	*S. aureus*	Binding of erythrocytes	Release of cytokines, cell lysis, apoptosis	+
		PVL leukocidin (LukS-LukF)	*S. aureus*	Cell membrane permeabilization	Necrotic enteritis, rapid shock-like syndrome	LukF

		γ-Hemolysins (HlgA-HlgB and HlgC- HlgB)	*S. aureus*	Cell membrane permeabilization	Necrotic enteritis, rapid shock-like syndrome	HlgB
		β-Toxin	*C. perfringens*	Cell membrane permeabilization	Necrotic enteritis, neurologic effects	–
	RTX toxins	Hemolysin II	*B. cereus*	Cell membrane permeabilization	Hemolytic activity	–
		CytK	*B. cereus*	Cell membrane permeabilization	Necrotic enteritis	–
		HlyA	*E. coli*	Calcium-dependent formation of transmembrane pores	Cell permeabilization and lysis	–
	Membrane-perturbing toxins	ApxI, ApxII, and ApxIII	*A. pleuropneumoniae*	Calcium-dependent formation of transmembrane pores	Lysis of erythrocytes and other nucleated cells	–
		LtxA	*A. actinomycetem comitans*	Calcium-dependent formation of transmembrane pores	Apoptosis	–
		LktA	*P. haemolytica*	Calcium-dependent formation of transmembrane pores	Activity specific versus ruminant leukocytes	–
	Other pore-forming toxins	δ-Hemolysin	*S. aureus*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	–
		Aerolysin	*A. hydrophila*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+
		AT	*C. septicum*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+ (model)
	Insecticidal toxins	PA	*B. anthracis*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+
		HlyE	*E. coli*	Perturbation of the lipid bilayer	Osmotic lysis of cells lining the midgut	+
		CryIA, CryIIA, CryIIIA, etc.	*Bacillus thuringiensis*	Destruction of the transmembrane potential	Osmotic lysis of cells lining the midgut	CryIA, CryIIIA
		CytA, CytB	*B. thuringiensis*	Destruction of the transmembrane potential	Osmotic lysis of cells lining the midgut	CytB
		BT toxin	*B. thuringiensis*	Destruction of the transmembrane potential	Cytocidal activity on human cells	–
Toxins acting on intracellular targets	Protein synthesis	DT	*Corynebacterium diphtheriae*	ADP-ribosylation of EF-2	Cell death	+
		PAETA	*P. aeruginosa*	ADP-ribosylation of EF-2	Cell death	+
		SHT	*S. dysenteriae*	N-glycosidase activity on 28S RNA	Cell death, apoptosis	+
	Signal transduction	PT	*Bordetella pertussis*	ADP-ribosylation of Gi	cAMP increase	+
		CT	*Vibrio cholerae*	ADP-ribosylation of Gs	cAMP increase	+
		LT	*E. coli*	ADP-ribosylation of Gs	cAMP increase	+
		α-Toxin (PLC)	*C. perfringens*	Zinc-phospholipase C, hydrolase	Gas gangrene	+
		Toxins A and B (TcdA and TcdB)	*C. difficile*	Monoglucosylation of Rho, Rac, Cdc42	Breakdown of cellular actin stress fibers	–

Table 22.1 (continued)

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
		Adenylate cyclase (CyaA)	*B. pertussis*	Binding to calmodulin ATP → cAMP conversion	cAMP increase	–
		Anthrax edema factor (EF)	*B. anthracis*	Binding to calmodulin ATP → cAMP conversion	cAMP increase	+
		Anthrax lethal factor (LF)	*B. anthracis*	Cleavage of MAPKK1 and MAPKK2	Cell death, apoptosis	+
		Cytotoxin necrotizing factors 1 and 2 (CNF1, 2)	*E. coli*	Deamidation of Rho, Rac and Cdc42	Ruffling, stress fiber formation.	CNF1 (catalytic domain)
		DNT	*Bordetella species*	Transglutaminase, deamidation or polyamination of Rho GTPase	Ruffling, stress fiber formation	–
		CDT	*Several species*	DNA damage, formation of actin stress fibers via activation of RhoA	Cell-cycle arrest, cytotoxicity, apoptosis	–
	Cytoskeleton structure	Toxin C2 and related proteins	*C. botulinum*	ADP-ribosylation of monomeric G-actin	Failure in actin polymerization	–
		Lymphostatin	*E. coli*	Block of interleukin production	Chronic diarrhea	–
		Iota toxin and related proteins	*C. perfringens*	Block of interleukin production	Chronic diarrhea	+ (C2I)
	Intracellular trafficking	TeNT	*C. tetanii*	Cleavage of VAMP/synaptobrevin	Spastic paralysis	+ (Hc domain)
		BoNT/B, D, G, and F neurotoxins	*C. botulinum*	Cleavage of VAMP/synaptobrevin	Flaccid paralysis	BoNT/B
		BoNT/A, E neurotoxins	*C. botulinum*	Cleavage of SNAP-25	Flaccid paralysis	BoNT/A
		BoNT/C neurotoxin	*C. botulinum*	Cleavage of syntaxin, SNAP-25	Flaccid paralysis	–
		Vacuolating cytotoxin VacA	*H. pylori*	Alteration in the endocytic pathway	Vacuole formation, apoptosis	–
		NAD glycohydrolase	*S. pyogenes*	Keratinocyte apoptosis	Enhancement of GAS proliferation	–
Toxins injected into eukaryotic cells	Mediators of apoptosis	IpaB	*Shigella*	Binding to ICE	Apoptosis	–
		SipB	*Salmonella*	Cysteine proteases	Apoptosis	–
		YopP/YopJ	*Yersinia species*	Cysteine protease, blocks MAPK and NFkappaB pathways	Apoptosis	–
	Inositol phosphate metabolism	SopB	*Salmonella species*	Inositol phosphate phosphatase, cytoskeleton rearrangements	Increased chloride secretion (diarrhea)	–
		IpgD	*S. flexneri*	Inositol phosphate phosphatase, cytoskeleton rearrangements	Increased chloride secretion (diarrhea)	–
	Cytoskeleton	ExoS	*P. aeruginosa*	ADP-ribosylation of Ras, Rho GTPase	Collapse of cytoskeleton	+ (GAP domain)

		C3 exotoxin	*C. botulinum*	ADP-ribosylation of Rho	Breakdown of cellular actin stress fibers	+
		EDIN-A, B and C	*S. aureus*	ADP-ribosylation of Rho	Modification of actin cytoskeleton	EDIN-B
		SopE	*S. typhimurium*	Rac and Cdc42 activation	Membrane ruffling, cytoskeletal reorganization, proinflammatory cytokine production	+
		SipA	*S. typhimurium*	Rac and Cdc42 activation	Membrane ruffling, cytoskeletal reorganization, proinflammatory cytokine production	+
		IpaA	*Shigella species*	Vinculin binding	Depolymerization of actin filaments	–
		YopE	*Yersinia species*	GAP activity toward RhoA, Rac1 or Cdc42	Cytotoxicity, actin depolymerization	+
		YopT	*Yersinia species*	Cysteine protease, cleaves RhoA, Rac, and Cdc42 releasing them from the membrane	Disruption of actin cytoskeleton	–
		VirA	*Shigella flexneri*	Inhibition of tubulin polymerization	Microtubule destabilization and membrane ruffling	–
	Signal transduction	YpkA	*Yersinia species*	Protein serine/threonine kinase	Inhibition of phagocytosis	–
		YopH	*Yersinia species*	Tyrosine phosphatase	Inhibition of phagocytosis	+
		Tir	*E. coli EPEC*	Receptor for intimin	Actin nucleation and pedestal formation	–
		CagA	*H. pylori*	Tyrosine phosphorylated	Cortactin dephosphorylation	–
		YopM	*Yersinia species*	Interaction with PRK2 and RSK1 kinases	Cytotoxicity	+
		SptP	*S. typhimurium*	Inhibition of the MAP kinase pathway	Enhancement of *Salmonella* capacity to induce TNF-alpha secretion	+
		ExoU	*P. aeruginosa*	Lysophospholipase A activity	Lung injury	–
Toxins with unknown mechanism of action		Zot	*V. cholerae*	?	Modification of intestinal tight junction permeability	–
		Hemolysin BL (HBL)	*B. cereus*	Hemolytic, dermonecrotic and vascular permeability activities	Food poisoning, fluid accumulation and diarrhea	–
		BSH	*L. monocytogenes*	?	Increased bacterial survival and intestinal colonization	–

Abbreviations: *SEA-SEI* staphylococcal enterotoxins, *TSST* toxic shock syndrome toxin, *SPE* streptococcal exotoxin, *SSA* streptococcal superantigen, *SMEZ* streptococcal mitogenic exotoxin z, *MAM*, Mycoplasma arthritidis mitogen, *YPMa Y. pseudotuberculosis*-derived mitogen, *ETA* and ETB, exfoliative toxins, *ColH* collagenase, *Nhe* nonhemolytic enterotoxin, *PFO* perfringolysin O, *SLO* streptolysin O, *LLO* listeriolysin O, *ALO* anthrolisin O, *AT* α-toxin, *PA* protective antigen, *DT* diphtheria toxin, *PAETA Pseudomonas aeruginosa* exotoxin A, *SHT* Shiga toxin, *PT* pertussis toxin, *CT* cholera toxin, *LT* heat-labile enterotoxin, *DNT* dermonecrotic toxin, *CDT* cytolethal distending toxin, *TeNT* tetanus neurotoxin, *RTX* repeats in the structural toxin, *Hly* hemolysin, *Cry* crystal, *BoNT* botulinum neurotoxin, *Ipa* invasion plasmid antigen, *Sip Salmonella* invasion protein, *EDIN* epidermal cell differentiation inhibitor, *Sop Salmonella* outer protein, *Ipg* invasion plasmid gene, *Yop* Yersinia outer protein, *GAP* GTPase-activating protein, *GAS* group A *Streptococcus*, *Vir* virulence protein, *YpkA* Yersinia protein kinase A, *Tir* translocated intimin receptor, *EPEC* enteropathogenic *E. coli*, *CagA* cytotoxin-associated gene A, *SptP Salmonella* protein tyrosine phosphatase, *VAMP* vesicle-associated membrane protein, *ICE* interleukin-1β-converting enzyme, *SNAP* synaptosome-associated protein, *MAPKK* mitogen-activated protein kinase kinase, *Zot* zonula. occludens toxin, and *BSH* bile salt hydrolase

Table 22.2
Toxins classified according to their enzymatic activities

Toxin	Substrate	Effect
Glucosyltransferases		
Clostridium difficile toxins A and B	Rho/Ras GTPases	Breakdown of cytoskeletal structure
Deamidases		
E. coli CNF1	Rho, Rac, and CdC42	Stress fiber formation
Bordetella DNT	Rho	Stress fiber formation
ADP-ribosyltransferases		
DT	Elongation factor EF-2	Cell death
PAETA	Elongation factor EF-2	Cell death
PT	G_i, G_o, and transducin	cAMP increase
CT	G_s, G_t, and G_{olf}	cAMP increase
E. coli LT	G_s, G_t, and G_{olf}	cAMP increase
Clostridium botulinum C2	Actin	Failure in actin polymerization
P. aeruginosa ExoS	Ras	Collapse of cytoskeleton
Clostridium botulinum C3	Rho	Breakdown of cellular actin stress fibers
N-Glycosidases		
Shiga toxin	Ribosomal RNA	Stop of protein synthesis
Metalloproteases		
Bacillus anthracis LF	Macrophages	Disruption of normal homoeostatic functions
Clostridium tetanii TeNT	VAMP/synaptobrevin	Spastic paralysis
C. botulinum BoNTs	VAMP/synaptobrevin, SNAP-25	Flaccid paralysis

Abbreviations: *CNF1* cytotoxin necrotizing factor 1, *DNT* dermonecrotic factor, *DT* diphtheria toxin, *PAETA Pseudomonas aeruginosa* exotoxin A, *PT* pertussis toxin, *CT* cholera toxin, *LT* heat-labile enterotoxin, *ExoS* exoenzyme S, *LF* lethal factor, *TeNT* tetanus neurotoxin, *BoNT* botulinum neurotoxin, *VAMP* vesicle -associated membrane protein, and *SNAP-25* synaptosome-associated protein of 25 kDa

Tir, translational initiation region; CagA, cytotoxin-associated gene A; YpkA, *Yersinia* protein kinase A.

For abbreviations, refer to the footnote in ❯ *Table 22.1*.

Toxins have a target in most compartments of eukaryotic cells. For simplicity, the toxins are divided into three main categories (❯ *Fig. 22.2*): (1) those that exert their powerful toxicity by acting on the surface of eukaryotic cells simply by touching important receptors, by cleaving surface-exposed molecules, or by punching holes in the cell membrane, thus breaking the cell permeability barrier (panel 1); (2) those that have an intracellular target and hence need to cross the cell membrane (these toxins need at least two active domains, one to cross the eukaryotic cell membrane and the other to modify the toxin target) (panel 2); and (3) those that have an intracellular target and are directly delivered by the bacteria into eukaryotic cells (panel 3).

Toxins Acting on the Cell Surface

See ❯ *Tables 22.1* and ❯ *22.2* for a summary of the principal features of toxins described in this section.

Toxins Acting on the Immune System (Superantigens)

Superantigens (❯ *Fig. 22.2*, panel 1) are bacterial and viral proteins that share the ability to activate a large fraction of T lymphocytes. They are bivalent molecules that have been shown to simultaneously bind two distinct molecules, the major histocompatibility complex (MHC) and the T-cell receptor variable domains (Vβ or Vγ; Kotzin et al. 1993; ❯ *Fig. 22.3*). Binding of these molecules to MHC class II requires no prior processing and occurs outside the antigen-binding groove. This results in the activation of between 2 % and 15 % of all T cells, ultimately leading to T-cell proliferation, production of a variety of cytokines, and expression of cytotoxic activity.

Bacterial superantigens, also known as pyrogenic toxins, comprise a class of secreted proteins mostly produced by *Staphylococcus aureus* and *Streptococcus pyogenes* (Bohach et al. 1990; Alouf and Muller-Alouf 2003). So far, they include the group of staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and SEI), exfoliative toxins (ETA and ETB), toxic shock syndrome toxin-1 (TSST-1; Dinges et al. 2000),

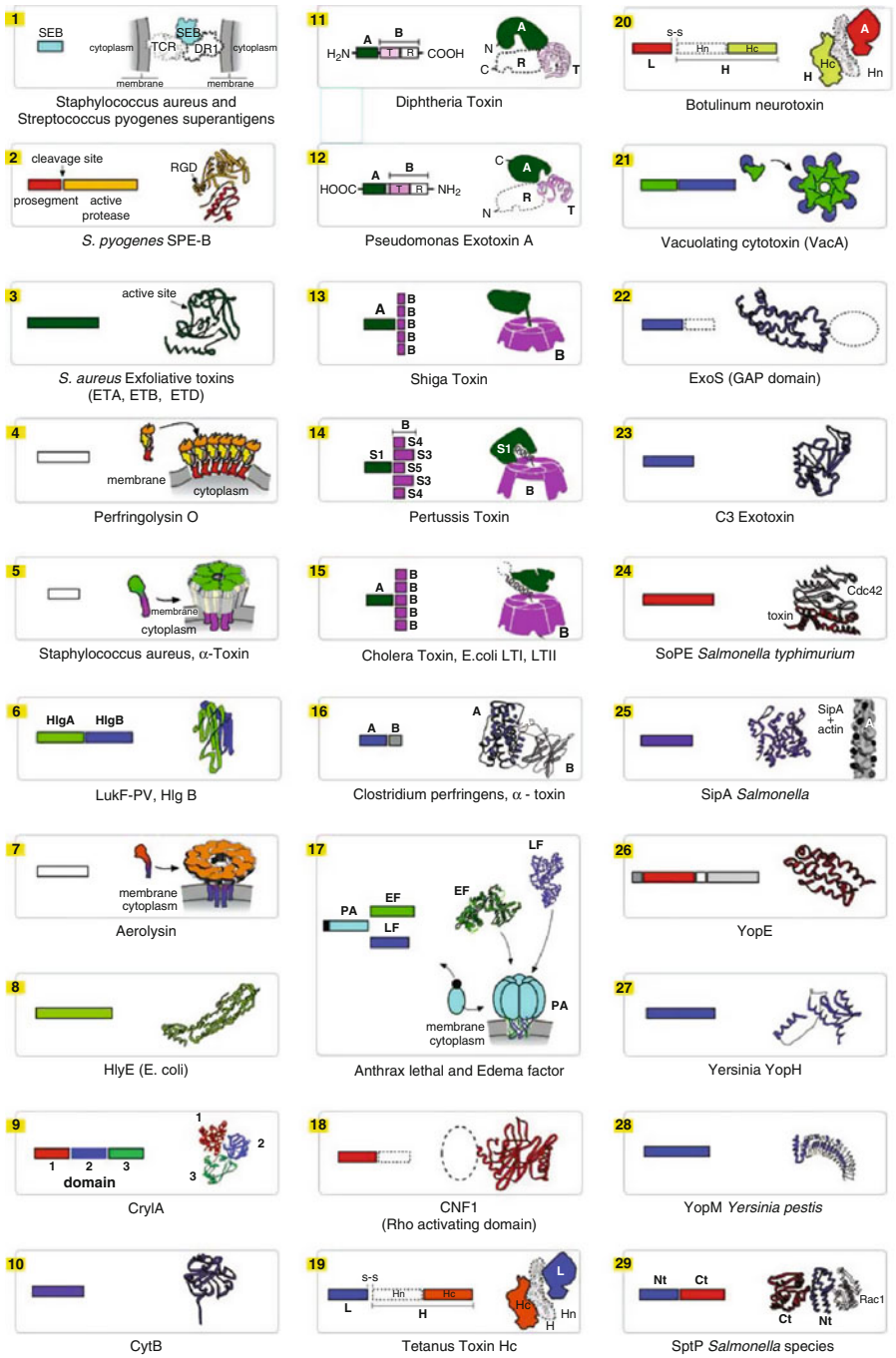

Fig. 22.1

Structural features of bacterial toxins. (*Left*) Scheme of the primary structure of each toxin. For the A/B toxins, the domain composition is also shown. The A (or S1 in PT) represents the catalytic domain, whereas the B represents the receptor-binding domain. The A subunit is divided into the enzymatically active A1 domain and the A2 linker domain in Shiga toxin, CT, *Escherichia coli* LTI and LTII, and PT. The B domain has either five subunits, which are identical in Shiga toxin, CT, and *E. coli* LTI and LTII and different in size and sequences in PT, or two subunits (the translocation [T] and the receptor-binding [R] subunits) in DT, *Pseudomonas* exotoxin A, botulinum toxin, and tetanus toxin. (*Right*) Schematic representation of the three-dimensional (3D) organization of each toxin. For *Staphylococcus* enterotoxin B, the protein is shown in the ternary complex with the human class II histocompatibility complex molecule (DR1) and the T-cell antigen receptor (TCR). For Salmonella SptP, the structure is shown in the transition state complex with the small GTP -binding protein Rac1. Similarly, toxin SopE is represented in complex with its substrate Cdc42. In the case of *E. coli* CNF1 and *Pseudomonas* ExoS, only one domain has been crystallized. In the case of SipA, a 3D reconstruction of SipA bound to F-actin filaments is also reported. For all toxins, the schematic representation is based on the X-ray structure, except that for VacA, whose structure has been solved by quick-freeze, deep-etch electron microscopy

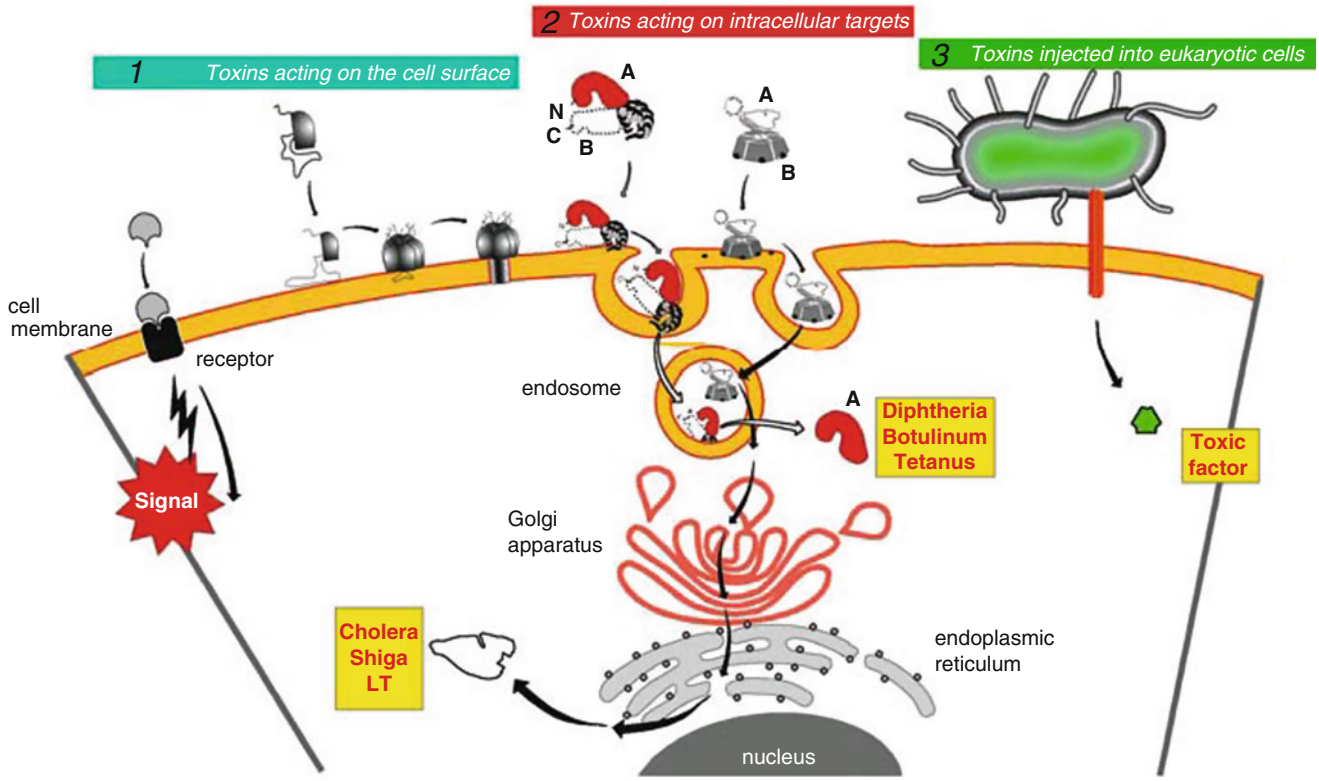

Fig. 22.2

Schematic representation of the three groups of bacterial toxins. Group 1 toxins act either by binding receptors on the cell membrane and sending a signal to the cell or by forming pores in the cell membrane, perturbing the cell permeability barrier. Group 2 toxins are A/B toxins, composed of a binding domain (B subunit) and an enzymatically active effector domain (A subunit). Following receptor binding, the toxins are internalized and located in endosomes, from which the A subunit can be transferred directly to the cytoplasm by using a pH-dependent conformational change or can be transported to the Golgi and the endoplasmic reticulum (ER), from which the A subunit is finally transferred to the cytoplasm. Group 3 toxins are injected directly from the bacterium into the cell by a specialized secretion apparatus (type III or type IV secretion system)

streptococcal pyrogenic enterotoxins (SPEA and SPEC; Papageorgiou et al. 1999), and streptococcal superantigen SSA (Sundberg and Jardetzky 1999).

These toxins play an important role in diseases such as the staphylococcal toxic shock syndrome induced by TSST-1 (Schlievert et al. 1981), vomiting and diarrhea caused by staphylococcal enterotoxins, and the exanthemas caused by the pyrogenic streptococcal exotoxins. Furthermore, these toxins have been linked to the pathogenesis of several acute or chronic human disease states such as the Kawasaki syndrome (Leung et al. 1993), which is the leading cause of acquired heart disease among children in the United States, and to the pathogenesis of other life-threatening events such as food poisoning (Blackman and Woodland 1995).

In addition to their functional similarities, the staphylococcal enterotoxins share a number of genetic and biochemical characteristics, as well as similar primary (Schlievert et al. 1995) and 3D structures (Swaminathan et al. 1992; Prasad et al. 1993; Papageorgiou et al. 1995; Schad et al. 1995). The genes for these toxins are generally carried on plasmids, bacteriophage chromosomes, or other heterologous genetic elements (Lindsay et al. 1998; Zhang et al. 1998), and all of them are translated into a precursor protein containing an amino-terminal signal sequence that is cleaved during export from the cell. The mature products are small non-glycosylated polypeptide molecules with molecular weights ranging from 20 to 30 kDa and are moderately stable to chemical inactivation, proteolysis, and denaturation by boiling.

Staphylococcal and streptococcal superantigens share 20–80 % sequence similarity (*Fig. 22.4*); in particular, staphylococcal SEA is more related to SEE and SED, whereas SEB has greater homology with SEC, TSST-1, and streptococcal superantigens SPEA and SSA. The overall homology found in the staphylococcal enterotoxins has been suggested to stem from duplication of a gene encoding a common "ancestral" toxin (Iandolo 1989).

Computer analysis of the *S. pyogenes* genome has revealed the presence of novel superantigen genes, and among them, the one coding for the mitogenic exotoxin Z (SMEZ). This toxin is particularly similar to the SPEC group of superantigens and, although present in all group A streptococci (GAS) strains, it shows extensive allelic variation. Further genetic characterization has shown that SMEZ is the most potent bacterial superantigen so far discovered and that it strongly contributes to the immunological effects of GAS both in vitro and in vivo by eliciting a robust cytokine production (Unnikrishnan et al. 2002).

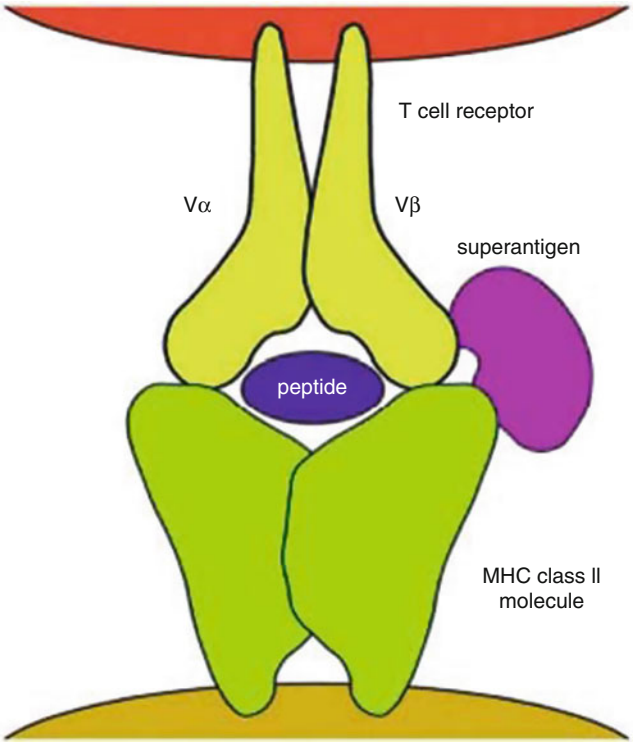

Fig. 22.3
Schematic representation of the interaction of a superantigen with a major histocompatibility complex (MHC) class II molecule and T-cell receptor

Three novel streptococcal genes (*spe-g*, *spe-h*, and *spe-j*) have been identified from the *Streptococcus pyogenes* M1 genomic sequence, while a fourth novel gene (*smez-2*) was isolated from the strain 2035. Of these, SMEZ-2, SPE-G, and SPE-J are most closely related to streptococcal pyrogenic exotoxin SPEC, whereas SPE-H is more similar to the staphylococcal toxins than to any other streptococcal toxin (Proft et al. 1999).

Finally, other pyrogenic toxin superantigens recently discovered by genome mining include proteins SPEL and SPEM produced by several isolates of *S. pyogenes* of the M18 serotype. The corresponding genes are contiguous and coded within a bacteriophage. Both toxins were shown to be lethal in different animal models and to directly participate in the host-pathogen interaction in some acute rheumatic fever (ARF) patients (Proft et al. 2003).

Crystallographic structures are currently available for most of the described staphylococcal and streptococcal superantigens, such as SEA (Schad et al. 1995), SEB (Swaminathan et al. 1992), SEC2 (Papageorgiou et al. 1995), SEC3 (Fields et al. 1996), SED (Sundstrom et al. 1996), TSST-1 (Prasad et al. 1993, 1997), SPEA (Papageorgiou et al. 1999), SPEB (Kagawa et al. 2000), SPEC (Roussel et al. 1997), and SSA (Sundberg et al. 1999). However, the primary sequence homology among superantigens does not assure homology in their secondary and tertiary structures, and vice versa; in fact, SEA, SEB, SEC, and TSST-1, despite their low level of sequence similarity, all fold into very similar 3D structures. Below are the X-ray structures of SEA, SEB, and TSST-1 that share a very similar fold despite low levels of sequence similarity that range from less than 20 % identity in the case of SEA and TSST-1 to 33 % in the case of SEA and SEB.

All of these toxins have a characteristic two-domain fold composed of a β-barrel at the *N*-terminus and a β-grasp at the C-terminus connected by a long α-helix that diagonally spans the center of the molecule (◉ *Fig. 22.5*). Moreover, all of these toxins are characterized by a central disulfide bond (with the exception of TSST-1, which has no cysteines) and by a Zn^{+2} coordination site which is believed to be involved in MHC class II binding (Abrahmsen et al. 1995).

The presence of two zinc-binding sites in SPEC indicates different modes in the assembly of the MHC-superantigen-T-cell receptor (TcR) trimolecular complex (Petersson et al. 2001).

The crystal structures of SEB and TSST-1 in complex with an MHC class II molecule and those of SEC2/SEC3 in complex with a TcR Vβ chain have been solved (Li et al. 1998; Fields et al. 1996). As an example, the complex between SEB and the Vβ domain of a TcR is reported (◉ *Fig. 22.6*).

Superantigen molecules have also been identified in other pathogens, where they represent important virulence determinants.

MaM is a T-cell mitogen produced by *Mycoplasma arthritidis*, which contributes to the acute and chronic inflammatory disease mediated by this organism (Cole and Atkin 1991). The recently determined X-ray structure of MaM in complex with HLA-DR1 has revealed that this protein has a fold and a mode of binding, which are entirely different from those of the known pyrogenic superantigens (Zhao et al. 2004; ◉ *Fig. 22.7*).

Another superantigenic toxin is the YPMa produced by a subset of *Yersinia pseudotuberculosis* strains. This 14.5-kDa protein was originally purified from bacterial lysates and found to exert a mitogenic activity on human peripheral blood mononuclear cells. Although the precise role of this protein is currently unknown, the data show that YPMa contributes to the virulence of *Y. pseudotuberculosis* in systemic infection in mice (Carnoy et al. 2000; Donadini et al. 2004).

Other toxins that have long been known as superantigens are the streptococcal pyrogenic exotoxin B (SPEB), a virulence factor with cysteine protease activity produced by all isolates of group A streptococci, and the exfoliative toxins A and B produced by *S. aureus* (◉ *Fig. 22.2*, panels 2 and 3) (Burns et al. 1996).

Although these proteins strongly contribute to the virulence of the corresponding microorganism, their role as mitogenic factors has been disproved when it was shown that all the nonrecombinant forms were in fact contaminated with trace amounts of the SMEZ superantigen (Unnikrishnan et al. 2002).

SPEB appears to contribute to *S. pyogenes* pathogenesis in several ways, including proteolytic cleavage of human fibronectin and vitronectin, two abundant extracellular matrix proteins involved in maintaining host tissue integrity. SPEB causes a cytopathic effect on human endothelial cells and represents a critical virulence factor in human infection and in mouse models of invasive disease. Despite low levels of sequence similarity, this toxin can be considered as a structural homologue of the papain superfamily that also includes the mammalian

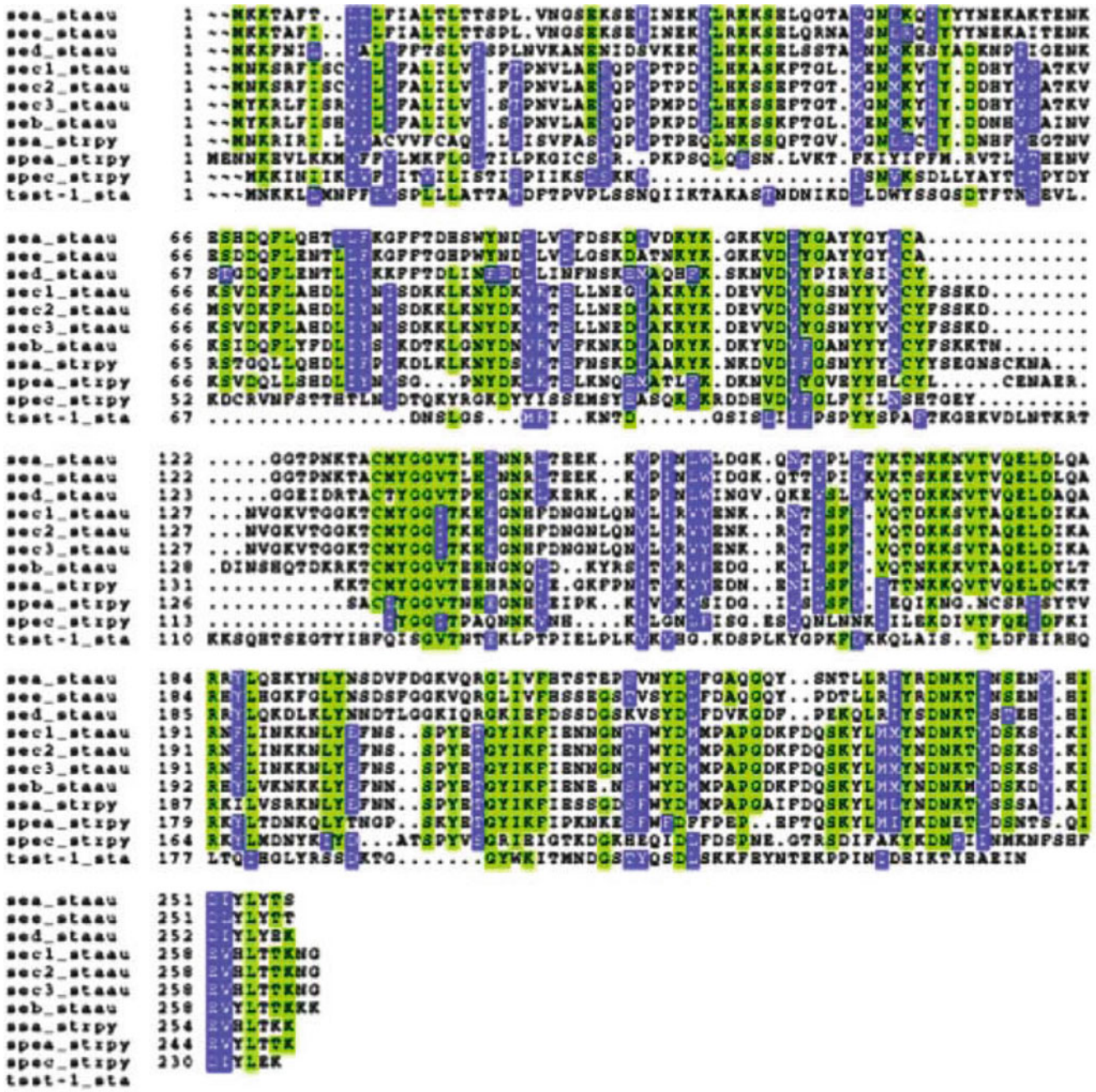

Fig. 22.4

Multiple sequence alignment of staphylococcal and streptococcal superantigens. *Green* indicates identity, whereas *blue* stands for amino acid similarity

cathepsins B, K, and L (Kagawa et al. 2000). Like other proteases, the enzyme SpeB is produced as an inactive precursor (zymogen) of 40 kDa which, following autolytic cleavage of the N-terminal 118 residues, is converted to the mature, active 27.6-kDa protease. The catalytic site lacks the Asn residue generally present in the catalytic Cys-His-Asn triad, which is in this case substituted by a Trp. The structure also reveals the presence of a surface-exposed integrin-binding Arg-Gly-Asp (RGD) motif that is a feature unique to SpeB among cysteine proteases and is linked to the pathogenesis of the most invasive strains of *S. pyogenes* (Stockbauer et al. 1999). Sequence analysis performed on more than 200 streptococcal isolates has revealed an overall limited structural variation in SPEB, with the entire active site being completely conserved. Interestingly, the prominent finger loop that extends from the N-terminal domain (*Fig. 22.8*) is also invariant, suggesting that antibodies directed against this region could be effective therapeutic agents.

The exfoliative toxins ETA and ETB of *Staphylococcus aureus* are produced during the exponential phase of growth and excreted from colonizing staphylococci before being absorbed

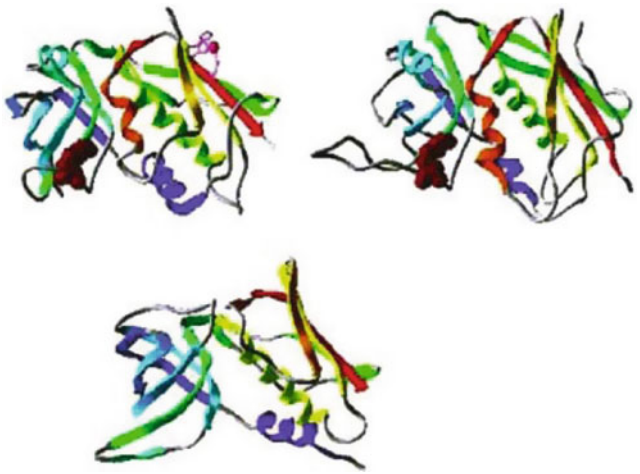

Fig. 22.5
Comparison of the X-ray structures of SEA (*left*), SEB (*right*), and TSST-1 (*below*). The colors follow the secondary structure succession where the N-terminus is *blue*, the C-terminus is *red*, and the long central helix is *pale yellow*. The zinc atom and the coordination site are colored *pink*, and the cysteines involved in the disulfide bond are *dark-red*

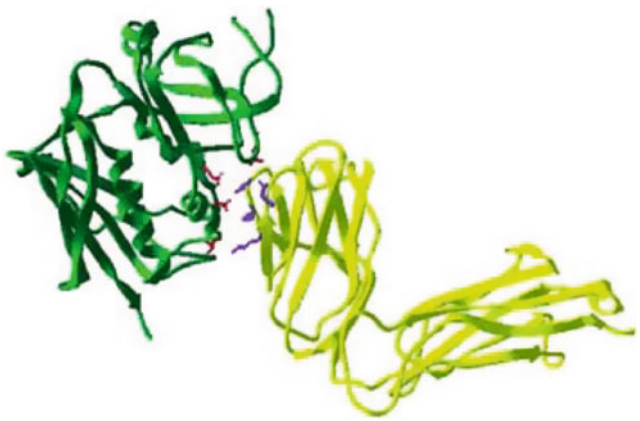

Fig. 22.6
Crystal structure of the complex between SEB (*green*) and TcR (*yellow*). The residues involved in hydrogen bonds between the two molecules have side chains colored in *red* and *blue*, respectively

into the systemic circulation. They have been recognized as the causative agents in staphylococcal scalded skin syndrome, an illness characterized by specific intraepidermal separation of the layers of skin between the stratum spinosum and the stratum granulosum (Ladhani et al. 1999). The two ETs are about 40 % identical, with no apparent sequence homology to other bacterial toxins. Both superantigens have been proved to act as serine proteases, and this enzymatic activity could be one of the mechanisms hypothesized as the cause of epidermal separation (Monday et al. 1999). In fact, at least in the case of ETA (*Fig. 22.8*), substitution of the active site serine residue with

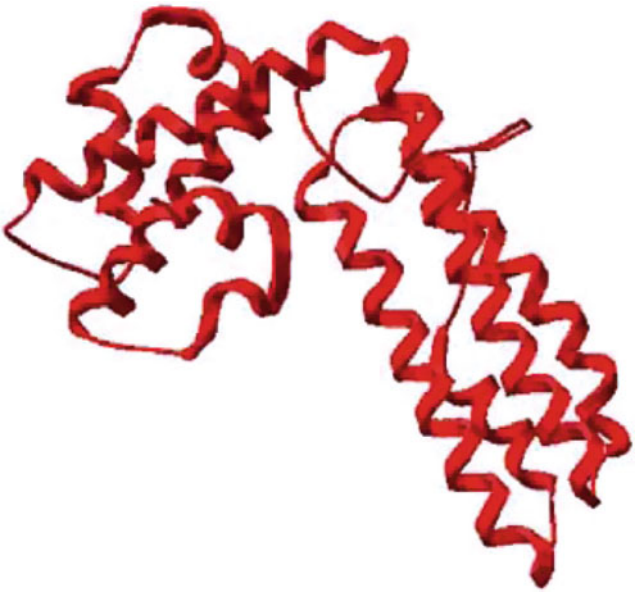

Fig. 22.7
Three-dimensional structure of MaM superantigen

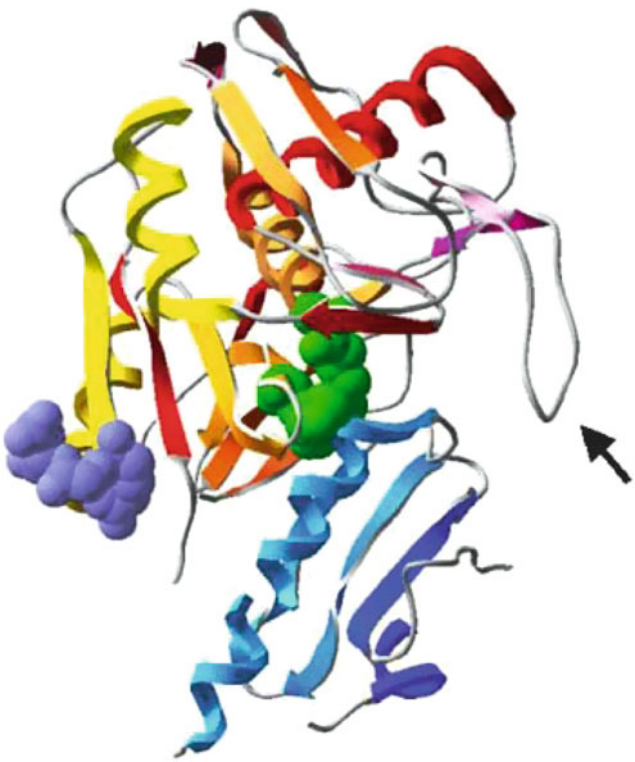

Fig. 22.8
The three-dimensional structure of the precursor form of streptococcal cysteine protease SpeB. The prosegment (*blue*) and active protease (*yellow-orange*) are indicated with different color scales. The solvent-exposed Arg-Gly-Asp (RGD) motif is *violet*, and the active site (Cys-47-His-195-Trp-212) is buried by the prosegment and is colored in *green*. The highly conserved finger loop is also indicated (*arrow*)

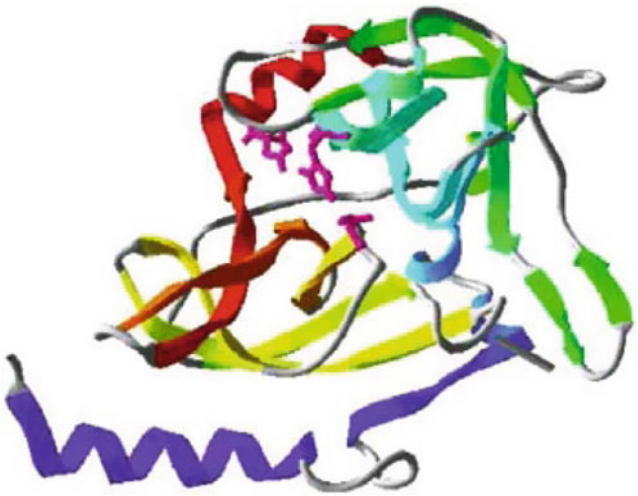

Fig. 22.9
Crystal structure of exfoliative toxin A (ETA) of *Staphylococcus aureus*. The three residues of the catalytic triad responsible for the serine protease activity are colored in *magenta*

cysteine abolishes its ability to produce the characteristic separation of epidermal layers but not its ability to induce T-cell proliferation (Redpath et al. 1991). The two ETs are about 40 % identical, with no apparent sequence homology to other bacterial toxins. The overall structures of ETA and ETB are similar to that of the chymotrypsin-like serine protease family of enzymes, with the catalytic triad being composed of His-57, Asp-102, and Ser-195 (Vath et al. 1997, 1999).

Recently, a novel member of the exfoliative group of toxins has been discovered in *S. aureus*. This protein, termed "ETD," is encoded within a pathogenicity island, which also contains the genes for a serine protease and the edin-B gene. When injected in neonatal mice as recombinant protein, ETD has been shown to induce exfoliation of the skin with loss of cell-to-cell adhesion in the upper part of the epidermis (Yamaguchi et al. 2002) (*Fig. 22.9*).

Toxins Acting on Surface Molecules

Bacteroides fragilis enterotoxin (BFT) is a protein of 186 residues that is secreted into the culture medium. The toxin has a zinc-binding consensus motif (HEXXH), characteristic of metalloproteases and other toxins such as tetanus and botulinum toxins. In vitro, the purified enterotoxin undergoes autodigestion and can cleave a number of substrates, including gelatin, actin, tropomyosin, and fibrinogen. When added to cells in tissue culture, the toxin cleaves the 33-kDa extracellular portion of E-cadherin, a 120-kDa transmembrane glycoprotein (responsible for calcium-dependent cell-cell adhesion in epithelial cells) that also serves as a receptor for *Listeria monocytogenes*. In vitro, BFT does not cleave E-cadherin, suggesting that the membrane-embedded form of E-cadherin is necessary for cleavage.

BFT causes diarrhea and fluid accumulation in ligated ileal loops. In vitro, it is nonlethal but causes morphological changes such as cell rounding and dissolution of tight clusters of cells. The morphological changes are associated with F-actin redistribution. In polarized cells, BFT is more active from the basolateral side than from the apical side, decreases the monolayer resistance, and causes dissolution of some tight junctions and rounding of some of the epithelial cells, which can separate from the epithelium. In monolayers of enterocytes, BFT increases the internalization of many enteric bacteria such as *Salmonella*, *Proteus*, *E. coli*, and *Enterococcus* but decreases the internalization of *L. monocytogenes* (Sears 2001).

BFT belongs to a large family of bacterial metalloproteases that usually cleave proteins of the extracellular matrix. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* elastases (aminopeptidase and AhyB) and *Clostridium histolyticum* collagenase (ColH) are the best-known examples (Yoshihara et al. 1994; Cascon et al. 2000; Cahan et al. 2001).

Lately, a novel member of this family of protein toxins has been identified in *Bacillus cereus*. The protein, termed "Nhe" (nonhemolytic enterotoxin), is a 105-kDa metalloprotease, which shares homologies to the above-mentioned elastases and collagenases. Biochemical characterization has shown that Nhe possesses both gelatinolytic and collagenolytic activities (Lund and Granum 1999).

Toxins Acting on the Cell Membrane

Protein toxins forming pores in biological membranes occur frequently in gram-positive and gram-negative bacteria (Braun and Focareta 1991). Pore-forming toxins, also known as "lytic factors," work by punching holes in the plasma membrane of eukaryotic cells, thus breaking the permeability barrier that keeps macromolecules and small solutes selectively within the cells (Sugawara et al. 1997; Gilbert 2002; *Fig. 22.2*, panel 1). Because erythrocytes have often been used to test the activity of these toxins, some of them are also called "hemolysins"; however, whereas erythrocytes appear to be very good targets in vitro, they are never the main physiological targets of this class of proteins in vivo (Tomita et al. 1997).

The pathogenicity of the toxin-producing organisms in eukaryotes is clearly related to the toxins they produce. Furthermore, pore-forming toxins represent the most potent and versatile tool with which invading microbes damage the host cell (Bhakdi et al. 1994). Cell permeabilization exerted by the toxic activity of these proteins generally results in release of cytokines, activation of intracellular proteases, induction of apoptosis, and finally, death of the eukaryotic cell.

To generate channels and holes in the cell membrane, this class of toxins must be able to fold in a characteristic amphipathic structure typical of porins (Weiss et al. 1991; Cowan et al. 1992), with one side facing the internal hydrophilic cavity and the other side interacting with the lipid chains or the nonpolar segments of integral membrane proteins.

Most of the toxins are produced or stored in a protoxin inactive form. The activation step varies from the cleavage of an N_{term} acidic peptide, as in the case of melittin, to a C_{term}

proteolytic cleavage as in aerolysin (van der Goot et al. 1992); in the particular case of the gram-negative hemolysins (cytolysins), these toxins are usually synthesized as precursor proteins, then covalently modified to an acylated, active form and finally secreted via specific export systems, which differ for various types of hemolysins (Issartel et al. 1991; Stanley et al. 1994). All such steps increase the affinity for the membrane, which appears to be essential for activity.

A large proportion of these proteins are produced by gram-positive bacteria and can be divided into large pore-forming and small pore-forming toxins on the basis of the dimension of the holes produced on the plasma membrane and also of the kind of interaction that they establish with the eukaryotic receptor. In addition, the pore-forming, repeats-in-toxin (RTX) family of toxins includes a large group of Ca^{+2}-dependent hemolysins (secreted by both gram-positive and gram-negative bacteria), which are characterized by a conserved glycine- and aspartate-rich motif of nine amino acids (Welch 1991; Coote 1992). Given their predominant role on cellular membranes, we have included in this section also the so-called membrane perturbing toxins and the insecticidal toxins produced by *Bacillus thuringiensis*.

Large Pore-Forming Toxins

This class of cytolysins (◉ *Fig. 22.2*, panel 2) comprises more than 20 family members, which are generally secreted by taxonomically diverse species of gram-positive bacteria and which have the common property of binding selectively to cholesterol on the eukaryotic cell membrane. Each toxin consists of a single 50- to 80-kDa polypeptide chain, and they are characterized by a pretty remarkable sequence similarity, also suggesting possible similar 3D structures. These proteins are produced by *Streptococcus pyogenes*, *S. pneumoniae*, *Bacillus*, a variety of *Clostridia*, including *Clostridium tetani* and *C. perfringens*, and *Listeria*.

To date, the best characterized are perfringolysin O (PFO), a virulence factor of *Clostridium perfringens*, which causes gas gangrene (Rossjohn et al. 1997), streptolysin O, secreted by *Streptococcus pyogenes* (Kehoe et al. 1987), alveolysin, produced by Bacillus alvei (Geoffroy et al. 1990), and pneumolysin, the major causative agent of streptococcal pneumonia and meningitis (Rossjohn et al. 1998).

In addition to its role as a cytolysin, listeriolysin O (LLO), which is an essential virulence factor of *Listeria monocytogenes* (Gedde et al. 2000), has also been shown to induce lymphocyte apoptosis with rapid kinetics (Carrero et al. 2004).

These toxins share a similar mechanism of action, which consists of an interaction of monomeric toxin with target cells via cholesterol (their receptor), followed by oligomerization and insertion into the host cell membrane (Heuck et al. 2003); this process ultimately results in serious membrane damage with formation of large pores with diameters exceeding 150 Å. All these toxins contain a common motif (boxed in ◉ *Fig. 22.10*), which is located approximately 40 amino acids from the carboxy-terminus; this motif includes a Cys residue, which if oxidized abolishes the toxin's lytic activities. Lytic activity can be restored only upon addition of reducing agents such as thiols. However, despite their designation as "thiol-activated cytolysins," thiol activation is clearly not an important property of this group of toxins (Billington et al. 2000). Interestingly, the membrane-bound receptor, cholesterol, plays an important role in the oligomerization step as well as in membrane insertion and pore formation.

Crystallographic data are available only for the thiol-activated cytolysin (perfringolysin O; PFO; ◉ *Fig. 22.1*, panel 4) of *Clostridium perfringens* (Rossjohn et al. 1997). Nevertheless, given the high degree of sequence conservation (◉ *Fig. 22.10*) detected within this class of protein toxins (ranging from the 43 % identity of PFO and listeriolysin, to the 72 % identity of PFO and alveolysin), this structure can be considered the prototype of the entire family (◉ *Fig. 22.11*).

PFO is an unusually elongated rod-shaped molecule mainly composed of β-sheets; the monomer is made of four discontinuous domains, indicated with different colors in the picture. Domain 1 (green) has an α/β structure, containing a seven-stranded antiparallel β-sheet. Domain 2 (blue) consists mainly of four β-strands, while domain 3 (yellow) is comprised of an α/β/a structure. Finally, domain 4 (red) is folded into a compact β-sandwich consisting of multiple-stranded sheets.

The mechanism of membrane insertion is not clear; in fact, no canonical transmembrane domains can be identified along the primary structure and no significant patches of hydrophobic residues can be mapped on the surface of the molecule. Nevertheless, a model of the membrane-bound state, which takes into account the interaction with the cholesterol receptor as the first step for penetration of the hydrophobic bilayer core, has been proposed on the basis of electron microscopy and other experimental data. Several chemical modifications and mutagenesis studies have suggested the cholesterol-binding site to be located at the tip of domain 4 (◉ *Fig. 22.12*), and in particular, it has been mapped within the highly conserved, Trp-rich segment (Michel et al. 1990; Hill et al. 1994). Proteolysis studies have further demonstrated that domain 4 is also the membrane-spanning domain, although the distribution of charged and hydrophobic residues on the β-sheet of this region is not compatible with an insertion into the lipid bilayer. From these studies, it has emerged that only the tip of the β-barrel domain D4 is responsible for membrane insertion and that a major conformational rearrangement takes place during pore formation (Shepard et al. 1998; Shatursky et al. 1999).

Taken together, these observations suggest a model of oligomer insertion. After the toxin binds to the cholesterol molecule, the aliphatic side chains neutralize the charged residues present on the β-sheet (blue) of domain 4 and then trigger membrane penetration. Consistent with this model is the hypothesis that the highly hydrophobic Trp-rich loop could lead to and promote the final penetration step.

Furthermore, on the basis of recent data, a mechanism has been proposed whereby insertion into the bilayer occurs only

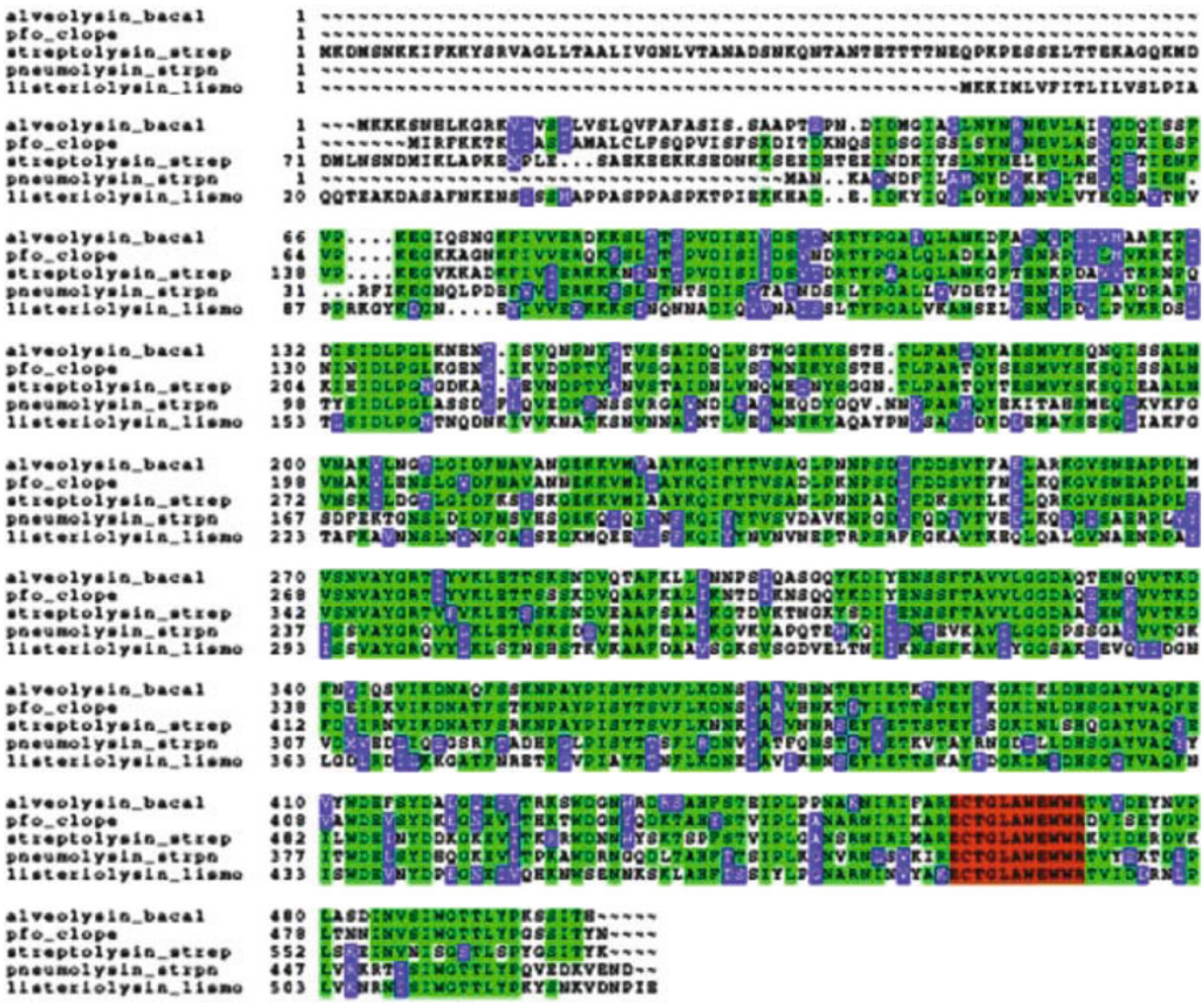

Fig. 22.10

Multiple sequence alignment of proteins belonging to the class of large pore-forming toxins. *Green* indicates identity, whereas *blue* stands for amino acid similarity

after PFO monomers have assembled into a pre-pore state. Monomer-monomer interactions therefore not only promote insertion, but cooperative interactions between PFO monomers appear to be required to drive transmembrane insertion and β-barrel formation (Hotze et al. 2002). Recently, a protein belonging to this class of cytolysins has been identified in *Bacillus anthracis* and named "anthrolysin O" (ALO). This putative toxin is able to bind erythrocytes and could have a role in the virulence of anthrax (Shannon et al. 2003).

Small Pore-Forming Toxins

The family of small pore-forming toxins acts by creating very small pores (1–1.5 nm of diameter) in the membrane of host cells, thus allowing their selective permeabilization to solutes with a molecular mass less than 2 kDa. Alpha-toxin (α-hemolysin) is the prototype of a group of pore-forming toxins produced by most pathogenic strains of *Staphylococcus aureus* (Gray and Kehoe 1984a; Song et al. 1996; Gouaux 1998; ❯ *Fig. 22.1*, panel 5); other members of this family include leukotoxins, such as leukocidin F (LukF), leukocidin S (LukS), Panton-Valentine leukocidin (PVL), and γ-hemolysin (Prèvost et al. 1995; Tomita and Kamio 1997; Olson et al. 1999; Pedelacq et al. 1999; Cooney et al. 1993), and the β-toxin of *Clostridium perfringens* (Steinthorsdottir et al. 2000; Tweten 2001; Magahama et al. 2003). These staphylococcal and streptococcal proteins are secreted as water-soluble monomers and assemble on the surface of susceptible cells to form heptameric transmembrane channels of approximately 1 nm in diameter (Finck-Barbancon et al. 1993; Sugawara et al. 1997; ❯ *Fig. 22.13*).

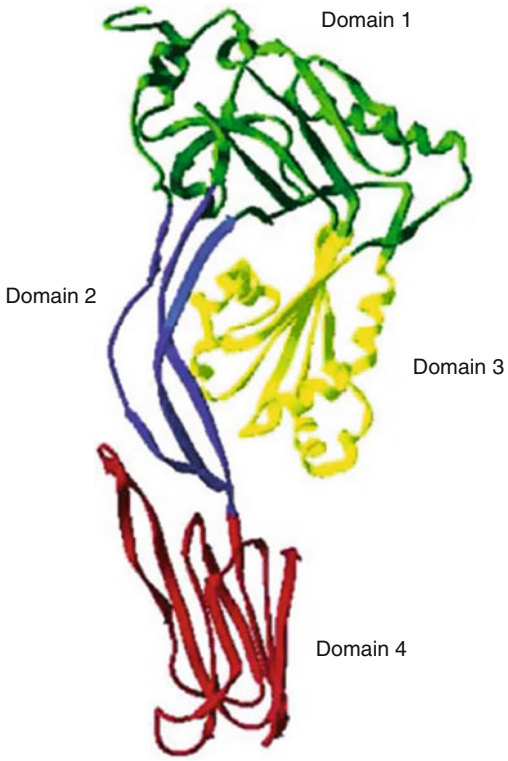

Fig. 22.11
Crystallographic structure and domain organization of perfringolysin O (PFO) produced by *Clostridium perfringens*

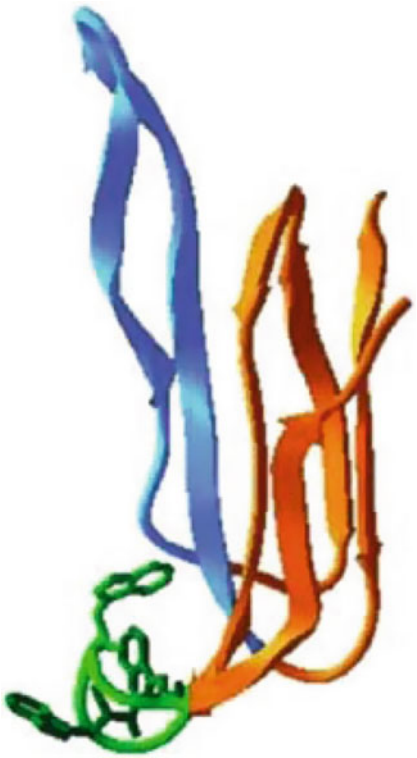

Fig. 22.12
Graphical representation of domain 4 of perfringolysin. The Trp-rich loop along with tryptophan side chains are colored in *green*. In blue is the β-sheet probably involved in membrane insertion

The monomers have molecular weights of 33 kDa and are related in sequence and function (Fig. 22.14).

These toxins bind to human erythrocytes, monocytes, platelets, lymphocytes, and endothelial cells, causing (at high concentrations) membrane rupture and cell lysis and death. Alpha-toxin has been recently shown to be the major mediator of caspase activation and apoptosis (Haslinger et al. 2003).

The structure of the transmembrane pore of staphylococcal α-toxin has been solved and has confirmed the heptameric structure of the oligomer (Song et al. 1996; Fig. 22.15). The complex is mushroom-shaped and measures 100 Å in height and up to 100 Å in diameter; the aqueous channel forms the transmembrane pore and spans the length of the entire complex, ranging from 14 to 46 Å in diameter.

Each protomer (Fig. 22.16) is mainly composed of β-strand elements; two of these in particular constitute the stem domain, which contributes to the formation of the transmembrane pore in the heptameric form of the complex; a glycine-rich segment that is probably involved in solvent interaction characterizes this domain.

Leukotoxins and γ-hemolysin (HγII) should be grouped together, inasmuch as they form two types of bicomponent complexes (LukF + LukS and LukF + HγII) that exhibit leukotoxic and hemolytic activity, respectively (Tomita and Kamio 1997). Panton-Valentine leukocidin (PVL) is a closely related toxin carried by 2 % of clinically isolated *S. aureus* strains and is also composed of type F and S components (Prèvost et al. 1995).

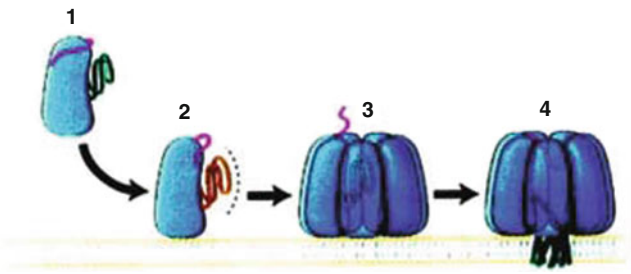

Fig. 22.13
General mechanism of assembly for small pore-forming toxins: the stem region is initially folded against in the body of the water-soluble monomer; upon binding to the membranes and oligomerization, it subsequently undergoes conformational rearrangement and promotes insertion into the lipid bilayer

The components of each protein class are produced as nonassociated, water-soluble proteins that undergo conformational changes and form oligomeric complexes after recognition of their cell targets, a process leading to transmembrane-pore formation and, ultimately, to cell death. The resultant transmembrane channels (estimated diameter 8 Å) are mainly permeable to divalent cations. Recently, fluorescence microscopy experiments have been performed to elucidate the mechanism of membrane insertion of the γ-hemolysin complex. This study

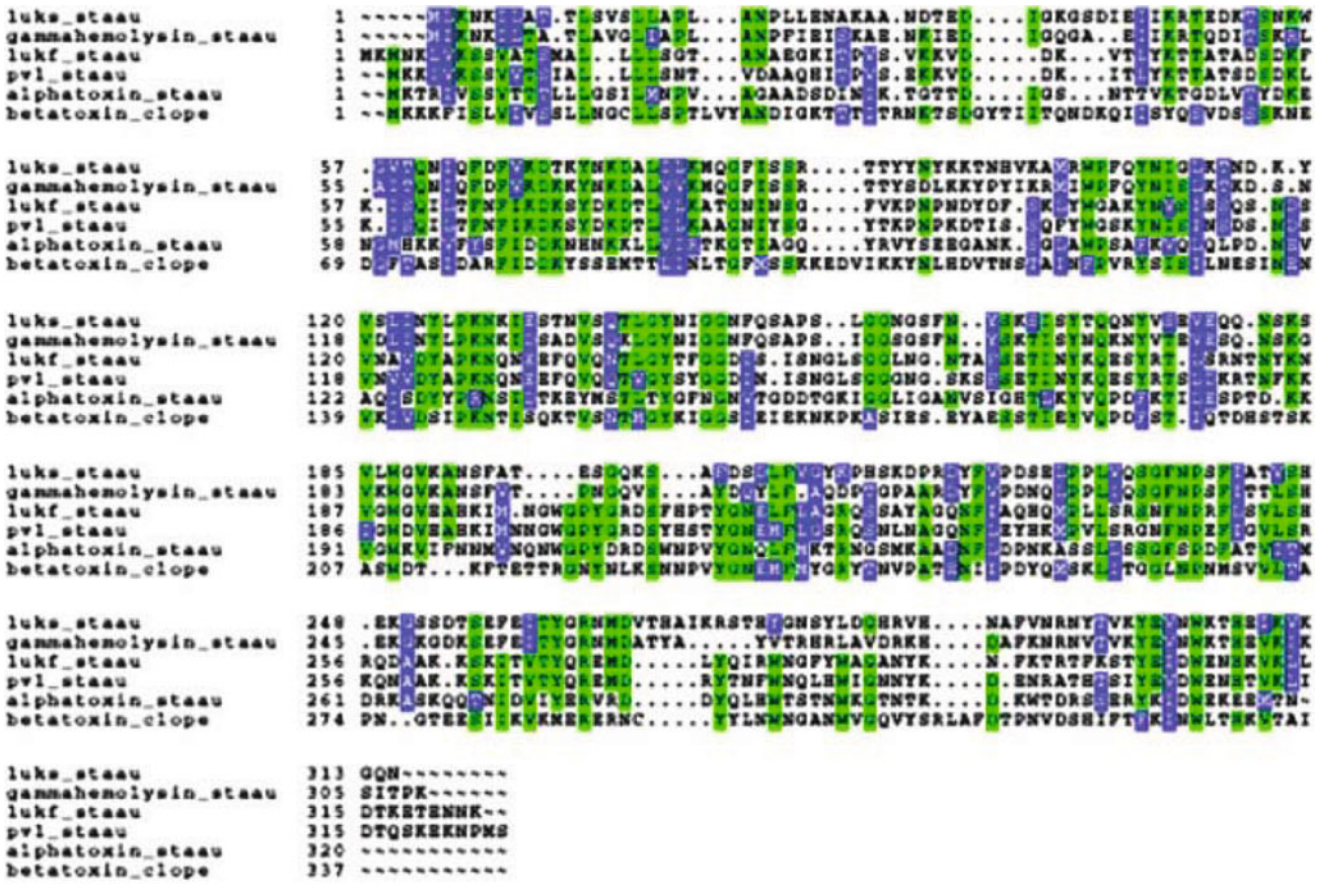

Fig. 22.14

Multiple sequence alignment of proteins belonging to the family of small pore-forming toxins. *Green* and *blue* stand for amino acid identity and similarity, respectively

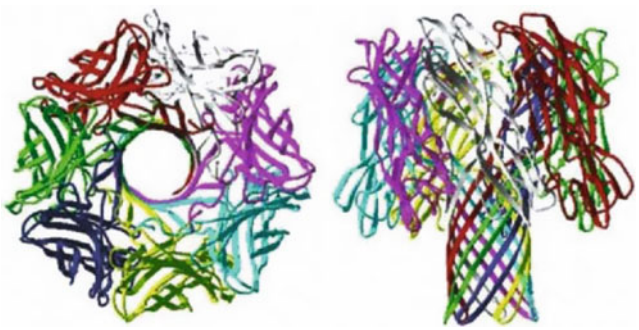

Fig. 22.15

***Top* and *side* views of the heptameric complex of alpha-toxin; each monomer is represented here with a different color (see *Fig. 22.1*, panel 5)**

shows that the three cooperative stages (dimer-dimer interaction, single pore assembly, and aggregation of pores) enhance the efficiency of assembly of oligomeric pores (Nguyen et al. 2003).

As representative of this class of bicomponent toxins, consideration is given the X-ray structure of the LukF protomer (Olson et al. 1999; *Fig. 22.17*), which has been solved at a 1.90-Å resolution. The superposition of this monomer with that of α-toxin shows that the core structures are very similar despite the relatively low primary sequence identity (32 %); nevertheless, a conformational change has affected the region of the glycine-rich stem domain, which appears in this case as a compact β-sheet folded against the body of the structure.

From a structural point of view, in contrast to a wide range of bacterial and insect toxins that utilize α-helices to perturb or penetrate the bilayer, these pore-forming toxins (members of an emerging family of proteins) can be defined by their use of bilayer-spanning antiparallel β-barrels instead.

Since the initial discovery of the first small pore-forming toxins, the number of these proteins has grown to include several members, among which are the recently identified hemolysin II (HlyII), and cytotoxin K (CytK) of *Bacillus cereus*, implicated in necrotic enteritis (Lund et al. 2000; Hardy et al. 2001; Miles et al. 2002).

RTX Toxins

Escherichia coli hemolysin (HlyA) is a 110-kDa protein, which can be considered as the prototype of a class of pore-forming

Fig. 22.16
Structure of the monomer of *S. aureus* α-toxin; the stem domain involved in pore formation protrudes outside of the core of the structure

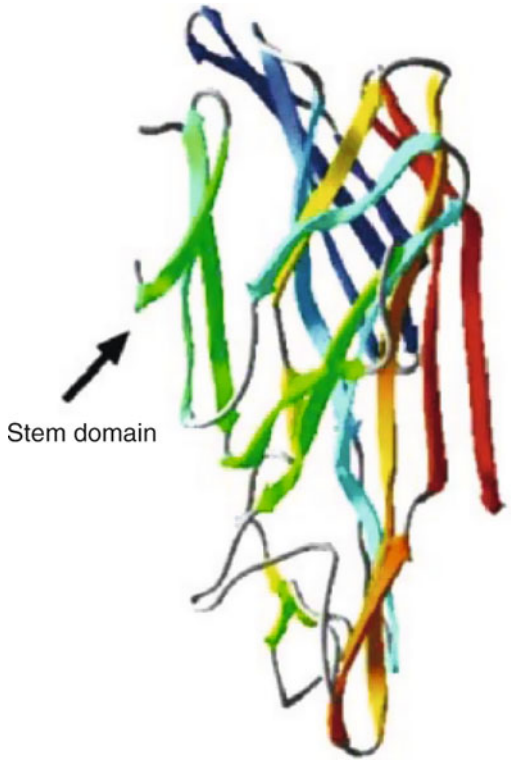

Fig. 22.17
Crystallographic structure determined for the protomer of toxin LukF; the glycine-rich, stem domain is in this case folded against the main body of the structure

toxins mainly produced by gram-negative bacterial pathogens (Felmlee et al. 1985; Welch 1991). This well-represented family includes a large number of calcium-dependent cytolysins known as RTX toxins, which are produced by different genera of Enterobacteriaceae and Pasteurellaceae. Characterized by the presence of a conserved repeated glycine- and aspartate-rich motif of nine amino acids, these cytolysins have multiple calcium-binding sites essential for function (Felmlee and Welch 1988).

The toxin is encoded by four genes, one of which, *hlyA*, encodes the 110-kDa hemolysin. The other genes are required for its posttranslational modification (*hlyC*) and secretion (*hlyB* and *hlyD*). The four genes are found in a very limited number of *E. coli* clonal types and can be sometimes located on transmittable plasmids (Smith and Halls 1967). To give an idea of the level of toxicity associated with *hlyA* gene product, when nonhemolytic strains of *E. coli* are transformed with recombinant plasmids encoding the hemolysin, the transformants (in rodent models of peritonitis) are 10-fold to a 1,000-fold more virulent than the parental strains. The receptor-binding domain of HlyA has been recently mapped (Cortajarena et al. 2003).

Other members of this class of RTX proteins include the adenylate cyclase/hemolysin of *Bordetella pertussis* (CyaA; Glaser et al. 1988), the Apx-I, II and III hemolysins from *Actinobacillus pleuropneumoniae* (Maier et al. 1996; Chang et al. 2000), and the leukotoxins of *A. actinomycetemcomitans* (LtxA; Korostoff et al. 1998; Henderson et al. 2003; Kachlany et al. 2000) and of *Pasteurella haemolytica* (LktA; Chang et al. 1987; Wang et al. 1998).

Although a remarkable level of primary structure similarity can be detected among this group of toxins (20–60 % identity), nevertheless, they differ in host cell specificity and seem to adopt diverse mechanisms for cellular damage (Frey and Kuhnert 2002).

The synthesis and secretion of RTX toxins involve the participation of at least five different gene products; the organization of the five genes is very similar (*Fig. 22.18*, panel A), with the exception of B. pertussis bifunctional adenylate cyclase/hemolysin, where all five (*cyaC*, *A*, *B*, *D*, and *E*) are found together (Glaser et al. 1988; Barry et al. 1991; *Fig. 22.18*, panel B); for the other family members, in fact, four of the genes are encoded within a single operon, whereas the fifth gene is located approximately 1 kb downstream (Welch and Pellett 1988; Wandersman and Delepelaire 1990).

The activation process performed by HlyC on HlyA ultimately results in the acquired capacity of HlyA to bind target cells; this activation involves proteolytic processing and posttranslational acylation, as well as binding of Ca^{+2} ions to the repeated domain.

Membrane-Perturbing Toxins

δ-Toxin or δ-hemolysin is secreted into the medium by *S. aureus* strains at the end of the exponential phase of growth. It is a 26-

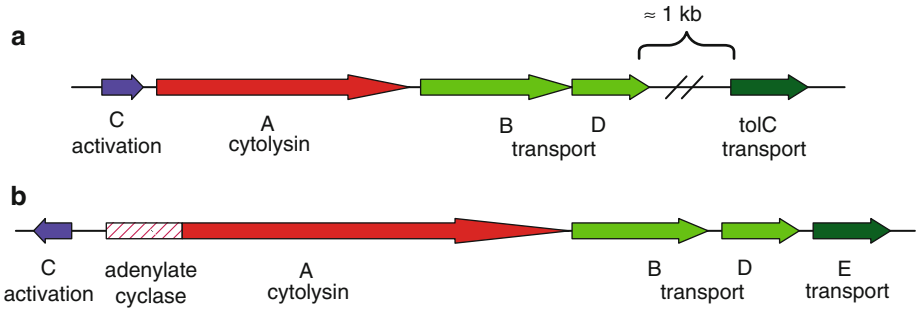

Fig. 22.18

Schematic representation of the genetic organization of RTX determinants; the genes encoding the Hly, Lkt, Aalt, and Hpp proteins are organized in the same fashion, as illustrated in *panel A*, whereas the genes involved in synthesis and secretion of adenylate cyclase/hemolysin of *B. pertussis* display a somewhat different organization (*panel B*)

amino-acid peptide (MAQDIISTIGDLVKWIIDTVNKFTKK) that has the general structure of soap with a nonpolar segment followed by a strongly basic carboxy-terminal peptide. The peptide has no structure in aqueous buffers but acquires an alpha-helical structure in low-dielectric-constant organic solvents and membranes. The α-helix has a typical amphipathic structure, which is necessary for the toxin to interact with membranes. The toxin binds nonspecifically parallel to the surface of any membrane without forming transmembrane channels. At high concentration, the peptide self-associates and increases the perturbation of the lipid bilayer that eventually breaks into discoidal or micellar structures. Interestingly, melittin, which is also a 26-amino-acid lytic peptide produced by *S. aureus*, has no sequence homology with δ-toxin but has identical distribution of charged and nonpolar amino acids. These toxins are active in most eukaryotic cells. Cells first become permeable to small solutes and eventually swell and lyse, releasing cell intracellular content (Matsuzaki et al. 1997).

Recent data have demonstrated that δ-hemolysin insertion is strongly dependent on the peptide-to-lipid ratio, suggesting that association of a critical number of monomers on the membrane is required for activity. The peptide appears to cross the membrane rapidly and reversibly and cause release of the lipid vesicle contents during this process.

Other Pore-Forming Toxins

Additional members of this class of β-barrel, channel-forming toxins include aerolysin of *Aeromonas hydrophila* (Parker et al. 1994; Rossjohn et al. 1998) and the closely related alpha-toxin of *Clostridium septicum* (Ballard et al. 1995), the anthrax toxin protective antigen PA of *Bacillus anthracis* (Petosa et al. 1997; Wesche et al. 1998), and the HlyE pore-forming toxin produced by pathogenic *E. coli*.

Aerolysin and Alpha-Toxin (AT). Aerolysin (*Fig. 22.1*, panel 7) is mainly responsible for the pathogenicity of Aeromonas hydrophila, a bacterium associated with diarrheal diseases and wound infections (Altwegg and Geiss 1989; Fivaz et al. 2001). It is secreted as a 52-kDa protoxin that is proteolytically cleaved into a 25-residue carboxy-terminal peptide and a 48-kDa active protein. Like other functionally related toxins, aerolysin changes its topology in a multistep process from a completely water-soluble form to a membrane-soluble heptameric transmembrane channel (ca. 1.5 nm in diameter) that destroys sensitive cells by breaking their permeability barriers.

Proaerolysin is a dimer in solution as well as in the crystal form (van der Goot et al. 1993; Parker et al. 1994); four structural domains characterize the monomer (*Fig. 22.19*).

In the structure of the dimer, the position of domain 1 appears to be stabilized by contacts with domain 1 of the other monomer, resulting in a very strict interaction of the two (*Fig. 22.20*).

Domain 4 is characterized by an amphipatic β-barrel structure, which is responsible for membrane insertion of the final complex. In fact, oligomerization is an essential step in channel formation, and it seems to precede membrane insertion. A model has been suggested for the entire process; it assumes that proaerolysin approaches the target cell as a water-soluble, hydrophilic dimer which, once concentrated on the surface of the target cell, binds to the receptor; subsequent proteolytic cleavage would cause dimer dissociation and oligomerization. This would ultimately result in an exposure of the hydrophobic region of the toxin and thus in membrane penetration.

Clostridium septicum AT is a channel-forming protein that is an important contributor to the virulence of the organism. Recent data have proved that this toxin, like aerolysin, binds to glycosylphosphatidylinositol (GPI)-anchored protein receptors. Furthermore, AT is also active against *Toxoplasma gondii* tachyzoites. Toxin treatment causes swelling of the parasite endoplasmic reticulum thus providing the first direct evidence that α-toxin is a vacuolating toxin (Ballard et al. 1995; Gordon et al. 1999). Recently, based on the available crystal structure of aerolysin, a molecular model of the membrane-spanning domain of AT has been generated (Melton et al. 2004).

Anthrax Protective Antigen (PA). Anthrax protective antigen (PA; *Fig. 22.1*, panel 17) is one of the three components of the anthrax toxin complex secreted by *Bacillus anthracis*, which also includes the edema factor (EF) and the lethal factor (LF;

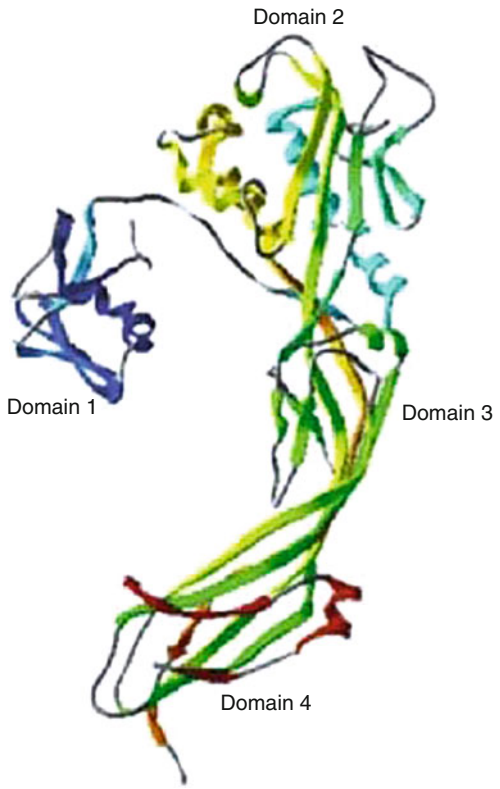

Fig. 22.19
X-ray structure of aerolysin toxin of *Aeromonas hydrophila*; the four domains are indicated; in particular, domain 1 clearly protrudes outside of the main body of the structure

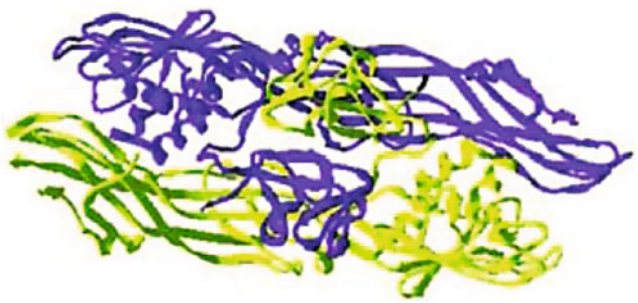

Fig. 22.20
Structure of the dimer of aerolysin and interaction between the two first domains

Brossier et al. 2000; Collier and Young 2003). Whereas EF and LF are responsible for the toxic activity, PA can be considered as the receptor-binding domain for two distinct A subunits, which are in turn EF and LF. The three subunits are encoded on a plasmid and are synthesized and secreted independently. Once on the host cell surface, PA needs a proteolytic activation to form a membrane-inserting heptamer through which EF and LF can be translocated (Klimpel et al. 1992; Milne and Collier 1993; Milne et al. 1994; see *Fig. 22.37* for the mechanism of action). The monomer is mainly constituted by antiparallel β-sheets and

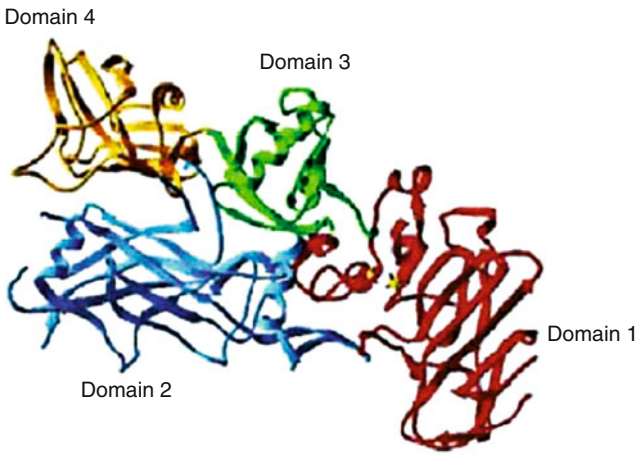

Fig. 22.21
X-ray structure of *Bacillus anthracis* protective antigen PA. The four structural domains are indicated by different colors. The two cysteines present in domain 1 are colored in *yellow*

contains four functional domains (*Fig. 22.21*). The crystallographic structure has revealed how PA can be assembled into heptamers and has suggested how some of the domains can undergo pH-driven conformational change.

Domain 1 (red) contains two Ca^{+2} ions (yellow) and the cleavage site for proteolytic activation; domain 2 (cyan) is the heptamerization domain and is implicated in membrane insertion; domain 3 (green) has an unknown function, whereas domain 4 (yellow) is for receptor binding. Given its ability to promote the translocation of many heterologous proteins, PA is being evaluated as a general protein delivery system (Leppla et al. 1999).

Escherichia coli HlyE. *Escherichia coli* produces a novel pore-forming toxin HlyE (*Fig. 22.1*, panel 8), which is completely unrelated to the *E. coli* hemolysin HlyA of the RTX family (Reingold et al. 1999; Wallace et al. 2000). Nevertheless, sequence comparison studies confirm the presence of highly homologous toxins in other pathogenic organisms such as *Salmonella typhi* and *Shigella flexneri* (these orthologs display 92–98 % identity to HlyE). This observation suggests that HlyE could be the prototype of a new family of HlyE-like hemolysins specific for gram-negative bacteria.

This new class of pore-forming toxins forms cation-selective water-permeable pores (25–30 Å in diameter); the channel formation could be either part of a mechanism for iron acquisition by the bacterial cell, or it may promote bacterial infection by killing immune cells and causing tissue damage (Ludwig et al. 1999).

The crystal structure of HlyE has been solved (Wallace et al. 2000; *Fig. 22.22*).

The toxin has an elongated shape characterized by a four-helix (A–D) bundle topology with each helix approximately 70–80 Å long. Two predicted hydrophobic domains have been identified on the primary sequence: both are located at the extremities of the molecule, one being mainly composed of a short β-hairpin (β-tongue) folded between the third and fourth helices of the main bundle, and the other consisting of the C-terminal end (magenta) of helix B (cyan).

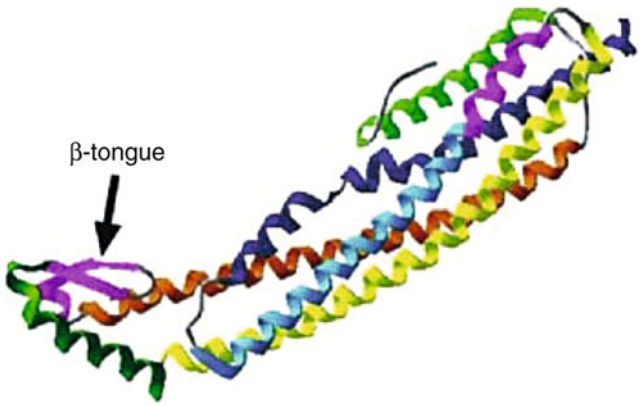

Fig. 22.22
X-ray structure of *E. coli* HlyE cytolysin. Two hydrophobic domains are present at the extremities of the α-helical bundle (colored in *magenta*)

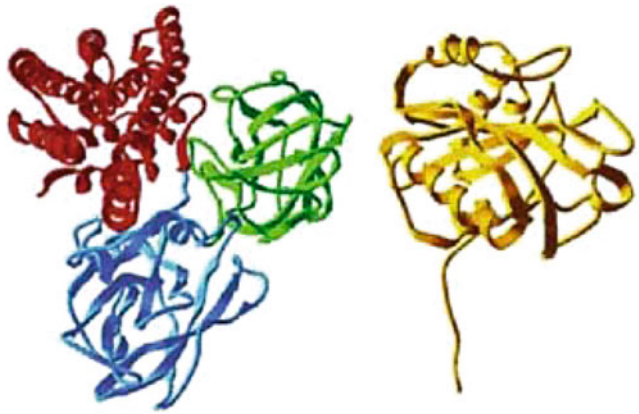

Fig. 22.23
Comparison of X-ray structures determined for representatives of the cry and cyt families of insecticidal δ-endotoxins: CryIA (*left panel*) is organized in three structural domains, whereas CytB (*right panel*) is a single-domain globular protein

The precise mechanism of HlyE oligomerization to form the final transmembrane pore is at the moment unknown; nevertheless, the first step involves a process of dimerization of two HlyE molecules that pack in a head-to-tail fashion burying the two hydrophobic patches against each other. Electron microscopy experiments have led to a model of channel formation in which the possible oligomer topology is that of an octameric complex, and the β-tongue domain is primarily responsible for interaction with the membrane.

Insecticidal Toxins

The class of insecticidal proteins, also known as δ-endotoxins, includes a number of toxins produced by species of *Bacillus thuringiensis*. These exert their toxic activity by making pores in the epithelial cell membrane of the insect midgut (Hofte and Whiteley 1989; Du et al. 1999).

δ-Endotoxins form two multigenic families, *cry* and *cyt*; members of the cry family are toxic to insects of Lepidoptera, Diptera, and Coleoptera orders (Hofmann et al. 1988), whereas members of the cyt family are lethal specifically to the larvae of Dipteran insects (Koni and Ellar 1994). The insecticidal toxins of the cry family are synthesized by the bacterium as protoxins with molecular masses of 70–135 kDa; after ingestion by the susceptible insect, the protoxin is cleaved by gut proteases to release the active toxin of 60–70 kDa (Drobniewski and Ellar 1989). In this form, they bind specifically and with high affinity to protein receptors and create channels 10–20 Å wide in the cell membrane. This subgroup includes several toxins (CryIA, CryIIIA, CryIV, CryV, etc.), whereas the only proteins so far characterized that belong to the cyt are CytA and CytB (Koni and Ellar 1993; 1994).

Three-dimensional (3D) structures determined for members of the two families show that the folding of these toxins is entirely different. As representative of the two families, consideration is given to the structures of CryIA (◉ *Fig. 22.1*, panel 9; Grochulski et al. 1995) and CytB (Li et al. 1996; ◉ *Figs. 22.1* [panel 10] and ◉ *22.23*), which share more than 39 % sequence identity, suggesting an overall similar folding of the corresponding 3D structures.

The CryIA toxin is a globular protein composed of three distinct (but closely packed) domains connected by single linkers: domain 1 is totally α-helical, domain 2 consists of three antiparallel β-sheets and two short α-helices, and domain 3 is a β-sandwich. On the other hand, CytB (also a globular protein) is composed of a single domain with α/β architecture. The molecular mass of the protoxin is in this case only 30 kDa.

The region of CryIA, which has been associated with receptor binding, maps within a loop of domain 2, whereas domain 1 has been shown to be responsible for membrane insertion and pore formation (Martens et al. 1995); this notion is strongly supported by the high structural similarity between the domain 1 of CryIA and that of CryIIIA to the pore-forming domains of colicin A and diphtheria toxin, both composed of helical bundles (Cabiaux et al. 1997; Duche et al. 1999). Conversely, in the case of the CytB/A, the model that has been proposed for the channel formation is based on a β-barrel structure.

Because they are toxic to several species of insects, δ-endotoxins have been formulated into commercial insecticides, and these insecticides have been used for more than three decades. Recently, Lepidoptera-specific toxin genes have also been used to engineer insect-resistant plants (Christov et al. 1999).

Very recently, a novel crystal protein produced by *B. thuringiensis* has been identified. This toxin (BT) is noninsecticidal and nonhemolytic, but has strong cytocidal activity against various human cells. Its amino acid sequence has little homology with the other known insecticidal toxins,

suggesting that BT might belong to a new group of *Bacillus thuringiensis* crystal toxins (Ito et al. 2004).

Toxins Acting on Intracellular Targets

See *Tables 22.1* and *22.2* for a summary of the principal features of toxins described in this section.

The group of toxins with an intracellular target (A/B toxins) contains many toxins with different structures that have only one general feature in common: they are composed of two domains generally identified as "A" and "B." The A domain is the active portion of the toxin; it usually has enzymatic activity and can recognize and modify a target molecule within the cytosol of eukaryotic cells. The B domain is usually the carrier for the A subunit; it binds the receptor on the cell surface and facilitates the translocation of A across the cytoplasmic membrane (*Fig. 22.2*, panel 2). Depending on their target, these toxins can be divided into different groups that act on protein synthesis, signal transduction, actin polymerization, and vesicle trafficking within eukaryotic cells.

Toxins Acting on Protein Synthesis

These toxins are able to cause rapid cell death at extremely low concentrations. Two ADP-ribosylating bacterial proteins (see also the section "ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity") are actually known to belong to this class of toxins: diphtheria toxin (DT) of *Corynebacterium diphtheriae* (Pappenheimer 1977; Collier et al. 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; Gray et al. 1984b; Wick et al. 1990). Both display their toxic activity by transferring the ADP-ribose moiety to a posttranslationally modified histidine residue of the cytoplasmic elongation factor 2 (EF2) of eukaryotic cells (Brown and Bodley 1979; Van Ness et al. 1980). This reaction leads to the formation of a completely inactive EF2-ADP-ribose complex, which ultimately results in inhibition of protein synthesis and cell death. From the biochemical point of view, the two toxins have a similar size, a signal peptide and disulfide bridges, and both are produced in iron-depleted medium. Nevertheless, they show a completely different amino acid composition and bind different cell receptors. In addition, Shiga toxin is another protein that exerts its toxic activity by interfering with protein synthesis.

Diphtheria Toxin. This toxin (DT; *Fig. 22.1*, panel 11) is a 535-amino acid polypeptide that is secreted into the growth medium by strains of toxinogenic *Corynebacterium diphtheriae*, and the polypeptide sequence is encoded by a lysogenic bacteriophage. Biosynthesis is regulated by an iron-binding protein and proceeds only in the absence of iron (Qiu et al. 1995; Ding et al. 1996). The toxin is synthesized as a single polypeptide chain that is subsequently cleaved into two fragments, A and B of 21 and 37 kDa, respectively (Pappenheimer 1977).

From the functional point of view, three separate domains (C, T, and R) are seen in the crystallographic structure of DT.

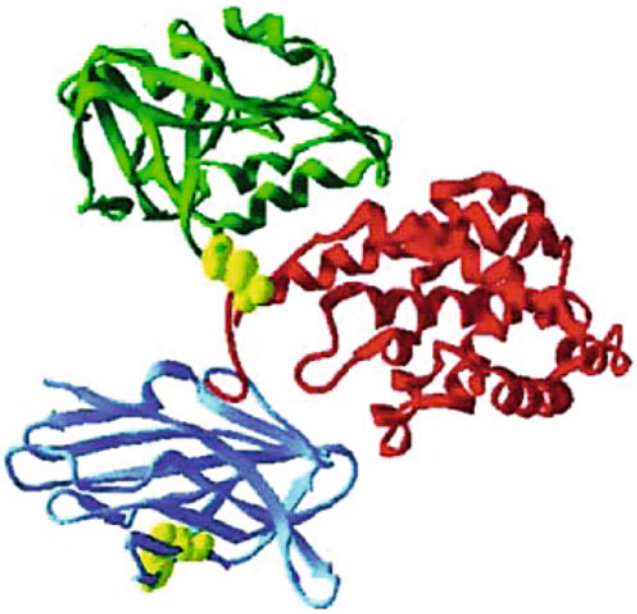

Fig. 22.24
X-ray structure of diphtheria toxin. The three functional domains are indicated with different colors: the catalytic domain C is *green*, the translocation domain T is *red*, and the receptor-binding domain R is *cyan*. The two disulfide bridges are colored *yellow*

The catalytic domain (C) entirely corresponds to the A subunit, whereas the translocation domain (T) and the carboxy-terminal receptor-binding domain (R) are contained in fragment B (Choe et al. 1992; Bennett and Eisenberg 1994).

From the structural point of view, the C domain (residues 1–191) has an α+β structure, the receptor-binding domain is a flattened β-barrel with a jelly-roll-like topology, whereas the translocation domain T (residues 201–384) consists in nine helices, two of which may participate in the pH-triggered membrane insertion. The molecule contains four cysteines and two disulfide bridges: one joins fragment C to fragment T and the other is contained within fragment R (*Fig. 22.24*).

Although the toxicity of DT is entirely due to the enzymatic activity carried on by fragment A (*Fig. 22.25*), fragment B is absolutely required for the cell intoxication process.

After secretion from *Corynebacterium diphtheriae*, the toxin binds to the DT receptor and is internalized by receptor-mediated endocytosis. In the endosome, the acidic environment triggers a conformational change of the B subunit that exposes the hydrophobic regions of the T domain, allowing the interaction with the endosomal membrane and the translocation of the amino-terminal catalytic domain C across the membrane to the cytosol. According to a recent model, the A subunit of DT is able to cross the endosomal membrane, making use of a metastable transmembrane domain, which has also been identified (Wolff et al. 2004). The toxin receptor is the heparin-binding, epidermal growth factor (EGF)-like precursor (Naglich et al. 1992; Hooper and Eidels 1995) that is present in most mammalian cells; nevertheless, the receptors of murine cells

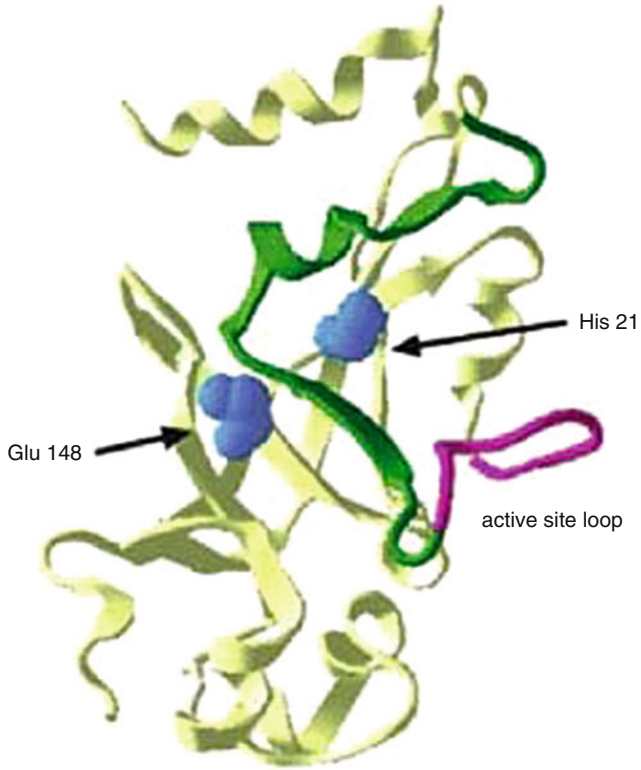

Fig. 22.25
Crystal structure of the isolated catalytic domain of diphtheria toxin. The scaffold of the enzymatic cleft is *green*, and the two described catalytic residues are *blue*. The "active-site loop" is represented here in the "closed" conformation

contain a few amino acid substitutions that make rodents insensitive to DT.

Diphtheria toxin is one of the most potent bacterial toxins: in vitro experiments have shown that a single molecule of the enzymatically active fragment A is by itself able to kill one eukaryotic cell (Yamaizumi et al. 1978).

Biochemical and mutagenesis studies have greatly contributed to the understanding of structure-function relationships and to the mapping of the catalytic residues. In particular, His-21 has been mutagenized with a number of different residues and has been found to be essential for catalysis (Papini et al. 1989; Blanke et al. 1994); in fact, some activity was maintained only when Asn replaced His. In a similar manner, Glu-148 was identified as an active-site residue by photoaffinity labeling experiments with nicotinamide adenine dinucleotide (NAD; Carroll et al. 1985) and subsequent site-directed mutagenesis studies; in this case, not even a conservative substitution with Asp could be possible without complete loss of activity (Tweten et al. 1985). Whereas the possible function for His-21 could be that of maintaining the integrity of the active-site pocket, Glu-148 is likely to be involved in the interaction with the upcoming substrate molecule. Later, crystallographic data confirmed and extended the experimental observations, and added a number of other important residues to the list of the catalytic ones.

A very important step in the elucidation of the mechanism of enzymatic activity has been the determination of the crystal structure for the complex of diphtheria toxin with NAD (Bell and Eisenberg 1997). Upon the addition of NAD to nucleotide-free DT crystals, a significant structural change affects the region encompassing residues 39–46. This portion of the C domain constitutes a mobile loop that becomes disordered after the formation of the complex. The best hypothesis to explain this observation is that NAD enters the cavity upon displacement of the mobile loop, which is then made available for the recognition and binding of the acceptor substrate EF-2. This would explain why DT recognizes EF-2 only after NAD has bound (see the section "A Common Structure of the Catalytic Site" in this chapter).

Detoxified diphtheria toxin has been used in the formulation of a vaccine against toxinogenic strains of *Corynebacterium diphtheriae* (Porro et al. 1980; Rappuoli 1983).

Pseudomonas aeruginosa Exotoxin A. This exotoxin (PAETA; *Fig. 22.1*, panel 12) is a 66-kDa single-chain protein that inhibits protein synthesis (by a mechanism of action identical to that of DT) in eukaryotic cells by catalyzing the transfer of the ADP-ribosyl moiety of oxidized NAD onto elongation factor 2 (Brown and Bodley 1979; Van Ness et al. 1980; Gray et al. 1984b; Wick et al. 1990; see the section "ADP-Ribosylating Toxins" in this chapter). Exotoxin A is the most toxic of the proteins secreted by the opportunistic pathogen *Pseudomonas aeruginosa*, having an LD_{50} of 0.2 mg upon intraperitoneal injection into mice. Secreted in the supernatant as an enzymatically inactive proenzyme, this toxin must undergo structural alteration to be able to perform its ADP-ribosylating activity.

According to X-ray crystallography (Allured et al. 1986; Li et al. 1995), the molecule can be divided into three functional domains. The receptor-binding domain I binds to the ubiquitous α2-macroglobulin receptor of eukaryotic cells, thus initiating receptor-mediated endocytosis. This domain is composed primarily of antiparallel β-structure and is arranged in two noncontiguous regions that encompass residues 1–252 (Ia) and 365–399 (Ib), respectively. Domain II maps within amino acids 253–364, is composed mostly of hydrophobic α-helices, and mediates the translocation of the enzymatically active carboxy-terminal domain III (residues 400–613) to the cytosol of infected cells. Furthermore, it has been shown that for domain III to be functional, a specific proteolytic cleavage at residue 280 of domain II is needed.

Genetic studies based on the expression of mutated forms of the exotoxin A gene in *E. coli* have confirmed these functional assignments. In fact, whereas deletion of domain Ia results in nontoxic, enzymatically active molecules that cannot bind the cells, deletions in domain II give rise to molecules that bind to the cells, are enzymatically active, but are not toxic; finally, deletions or mutations in domain III result in enzymatically inactive molecules (Siegall et al. 1989). To become active, the PAETA toxin requires an intracellular furin-mediated proteolytic cleavage to generate a 37-kDa C-terminal fragment that is then translocated to the cytoplasm to reach the EF2 target (Inocencio et al. 1994).

By using a fluorescence resonance energy transfer approach, the mechanism of interaction between ExoA and its substrate EF has been studied, showing that the binding is strongly dependent on the pH. Furthermore, the finding that EF-2 bound to GDP or GTP is still recognized by ExoA shows how adaptable this toxin is in ADP-ribosylating its substrate.

In particular, mutational analysis affecting the last five residues at the carboxy-terminus of the enzymatic domain resulted in complete loss of cytotoxicity; this segment (Arg-Glu-Asp-Leu-Lys, REDLK) closely resembles the KDEL motif that is a well-defined endoplasmic reticulum retention sequence and that has also been found at the C-terminus of other ADP-ribosyltransferases such as cholera toxin and heat-labile enterotoxin of *E. coli* (Chaudhary et al. 1990). It has been postulated that the sequence REDLK may be a recognition signal required for entry of the ADP-ribosylation domain of PAETA into the cytosol. Four disulfide bonds are present in the structure, but all of them are confined to the portion of exotoxin A that is not required for enzymatic activity.

Photoaffinity labeling experiments have identified Glu-553 as an active-site residue; substitution of this residue with any other amino acid, including the closely related Asp, decreased the enzymatic activity by a factor of 1,000 (Douglas and Collier 1990). In a similar manner, experiments of site-directed mutagenesis on His-440 led to molecules with a severely reduced cytotoxic activity, thus suggesting an important role for this residue in the reaction (Han and Galloway 1995).

The crystal structure of the catalytic domain has been recently solved both in the isolated conformation and in the presence of an NAD analog (β-methylene thiazole-4-carboxamide adenine dinucleotide; β-TAD; Li et al. 1995; *Fig. 22.26*). Comparison of the two structures shows that the major difference resides in the new conformation of the loop 458–463, which appears to be displaced by ligand binding; displacement of this loop from the active-site cleft could be an essential step allowing entrance and correct positioning of the NAD molecule during the enzymatic reaction.

Given the potent lethal activity, the catalytic domain of exotoxin A has been widely used for the construction of fusion proteins with cell-binding domains specific for tumor cells or other types of dangerous cells. So far, nucleotides encoding domain I have been replaced by sequences encoding interleukin (IL) 2, IL-6, and T-cell antigen CD4. These fusion molecules are promising candidates for the treatment of arthritis and allograft rejection (PAETA-IL2), acquired immune deficiency syndrome (PAETA-CD4), and other diseases (Chaudhary et al. 1987, 1988; Siegall et al. 1988; Ogata et al. 1989; Baldwin et al. 1996; Mori et al. 1997).

Shiga Toxin. This toxin (SHT; *Fig. 22.1*, panel 13), also known as "verotoxin," is the key virulence factor produced by *Shigella dysenteriae*, the pathogen responsible for the most severe forms of dysentery in humans (Kozlov et al. 1993). Shiga toxin is the prototype of a family of closely related bacterial protein toxins (Shiga-like toxins), also produced by certain strains of *E. coli* responsible for hemorrhagic colitis (Karmali et al. 1988).

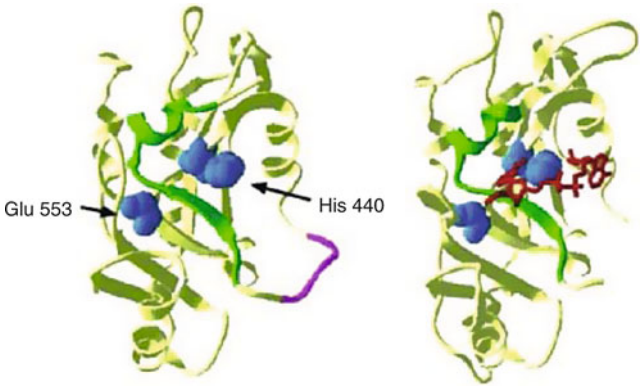

Fig. 22.26
Comparison of exotoxin A crystal structures in the absence (*left panel*) and in the presence (*right panel*) of the ligand (in *red*). The active site residues are shown in *blue*. The loop (when present) is colored in *magenta* (see *Fig. 22.1*, panel 12)

From its 3D structure (Fraser et al. 1994), it is possible to recognize this protein as belonging to the class of A/B bacterial toxins, which consist of an enzymatic A subunit associated with a B domain binding to specific cell surface receptors. The A subunit bears the enzymatic activity and is thus responsible for toxicity; like *Pseudomonas aeruginosa* exotoxin A and diphtheria toxin of *Corynebacterium diphtheriae*, SHT has an effect on protein synthesis, and in particular, by means of its *N*-glycosidase activity, it is able to depurinate a specific adenosine of ribosomal RNA and stop protein synthesis in the target cell (Endo et al. 1988). The catalytic subunit is composed of two regions, A1 and A2, and like many other bacterial protein toxins, it needs to be activated by proteolytic cleavage. Fragment A2 has an α-helical structure and is noncovalently linked to the B domain (*Fig. 22.27*). Interestingly, its primary structure displays a notable similarity to chain A of ricin, a plant toxin that also shares the same enzymatic function acting on the same substrate (Katzin et al. 1991).

This domain displays an overall organization which is very similar to that of the corresponding receptor-binding subunits of the ADP-ribosyltransferases cholera toxin and heat-labile enterotoxin LT of *E. coli*, all formed by five identical protomers which assemble into the final ring-like structure of the B oligomer (*Fig. 22.28*). The B subunit of Shiga toxin has been demonstrated as a powerful vector for carrying attached peptides into cells for intracellular transport studies and for medical research (Hagnarelle et al. 2002).

Upon binding of verotoxin to its receptor (globotrialosylglyceramide, Gb) on the surface of a eukaryotic cell (Cohen et al. 2000), the toxin is internalized by receptor-mediated endocytosis and is transported to the Golgi and to the endoplasmic reticulum, from which the A subunit is translocated to the cytoplasm, where it can gain access to the ribosomal target. Numerous recent studies have shown that Shiga toxins trigger programmed cell death signaling cascades

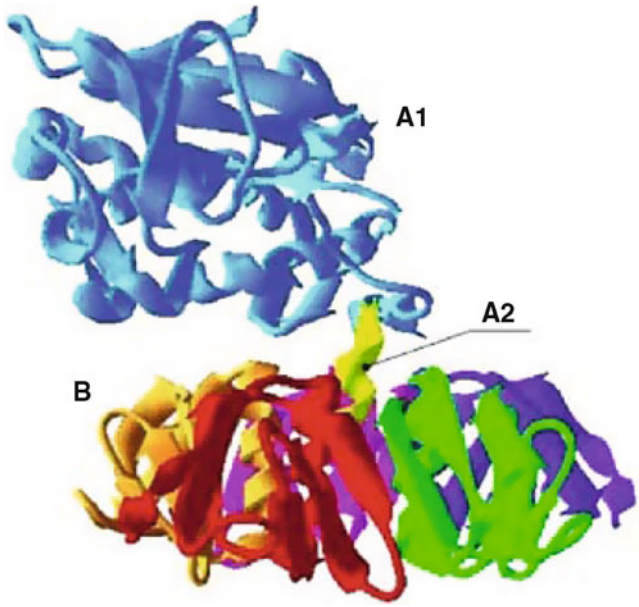

Fig. 22.27
Three-dimensional structure of Shiga holotoxin. The A subunit is distinguished between A1 (*blue*) and A2 (*yellow*), whereas the receptor-binding domain B has different colors for the five monomers

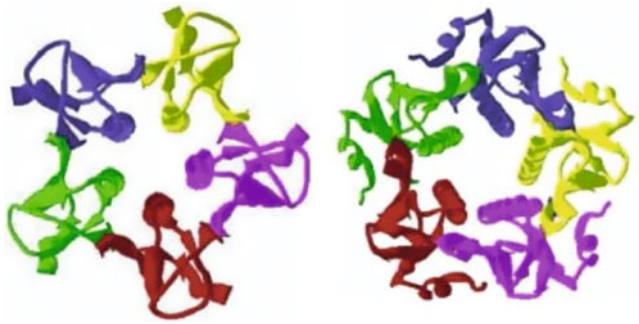

Fig. 22.28
Bottom view of the B subunit of Shiga toxin (*left panel*) in comparison to the B subunit of *E. coli* LT (*right panel*)

in intoxicated cells. The mechanisms of apoptosis induction by these toxins are newly emerging, and the toxins may signal apoptosis in different cells types via different mechanisms (Cherla et al. 2003).

Toxins Acting on Signal Transduction

Signal transduction is an essential mechanism for the survival of any living organism. In eukaryotic cells, signals received from the outside stimulate receptors on the cell surface and are subsequently transmitted across the cell membrane mainly using two types of mechanism: (1) tyrosine phosphorylation of the cytoplasmic portion of the receptor, which initiates a cascade of intracellular signaling events, and (2) modification of a receptor-coupled GTP-binding protein that transduces the signal to various enzymes which respond with the release of secondary messengers such as cyclic AMP (cAMP), inositol triphosphate, and diacylglycerol; accumulation of these products alters the normal equilibrium of the cell and thus provokes malfunction and death.

Pertussis Toxin

This toxin (PT; ◉ *Fig. 22.1*, panel 14) is a protein of 105 kDa released into the extracellular medium by *Bordetella pertussis*, the etiological agent of whooping cough. It belongs to the A/B class of ADP-ribosylating toxins and is composed of five distinct subunits, named "S1" through "S5," where S4 is present in two copies in the final oligomer. The genes encoding for the five monomers of pertussis toxin are organized into an operon structure (Locht and Keith 1986; ◉ *Fig. 22.29*) and contained within a chromosomic DNA fragment of approximately 3,200 base pairs.

Interestingly, the genes coding for S2 and S3 share a 75 % similarity (67 %, if calculated from S2 and S3 gene products at the amino acid level), suggesting a common evolutionary origin for the two sequences, possibly because of gene duplication.

The five subunits are independently secreted into the periplasmic space, where the toxin is assembled and then released in the culture medium by a specialized type IV secretion apparatus (Covacci and Rappuoli 1993a; Weiss et al. 1995). Subunit S1 represents the enzymatically active domain A, which is totally responsible for the toxicity, whereas the pentamer S2-S3-$(S4)_2$-S5 constitutes the receptor-binding domain B (◉ *Fig. 22.30*).

The A domain acts on eukaryotic cells by ADP-ribosylating their GTP-binding proteins, and specifically it transfers an ADP-ribose group to a cysteine residue located in the carboxy-terminal region of the α-subunit of many G proteins such as G_i, G_o, and transducin (Katada et al. 1983; West et al. 1985); G_s which has a tyrosine residue in place of the cysteine is not a valid substrate for PT. The consequence of ADP-ribosylation is the uncoupling of G-proteins from their receptors which results in an alteration of the response of eukaryotic cells to exogenous stimuli and thus in a variety of in vivo phenotypes, such as leukocytosis, histamine sensitization, and increased insulin production (Sekura 1985). Conversely, the most interesting activity displayed by PT in vitro is the observed change in cell morphology in Chinese hamster ovary (CHO) cells (Hewlett et al. 1983).

The B domain is a nontoxic oligomer that binds the receptors on the surface of eukaryotic cells and allows the toxic subunit S1 to reach its intracellular target proteins through a mechanism of receptor-mediated endocytosis, likely following a mechanism of retrograde transport through the Golgi apparatus. The importance of the Golgi localization of pertussis toxin

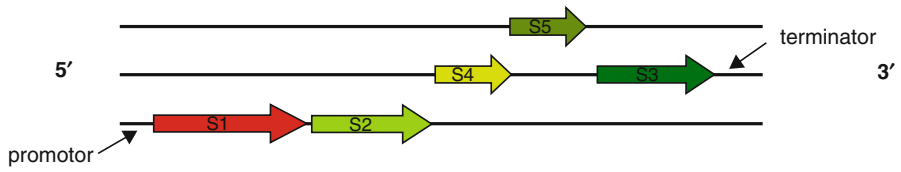

Fig. 22.29
Schematic representation of the genetic organization of the open reading frames (ORFs) coding for the five subunits of pertussis toxin

Fig. 22.30
Three-dimensional structure of the pertussis holotoxin. *Left panel*: side view of the intact holotoxin; *right panel*: *bottom* view of the receptor-binding domain. Each subunit is colored accordingly to the corresponding genes as represented in *Fig. 22.29*

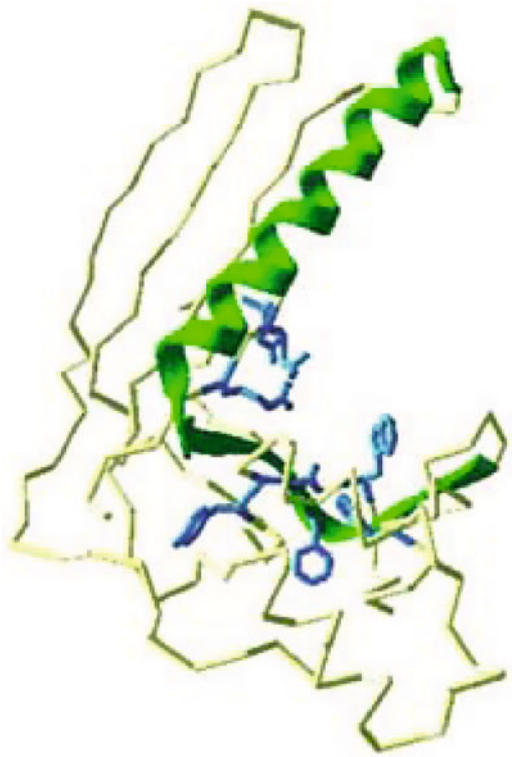

Fig. 22.31
Crystal structure of the wild type S1 subunit of pertussis toxin. The scaffold of the enzymatic cleft is represented as a *green* ribbon, whereas the rest of the molecule is in *pale yellow* carbon trace representation. Residues proved to be essential for activity by means of site-directed mutagenesis are represented with side chains and are colored in *blue*

for the S1-dependent ADP-ribosylation of G proteins was investigated, employing Brefeldin A (BFA) treatment to disrupt Golgi structures. This treatment completely blocked the pertussis toxin ADP-ribosylation activity of cellular G proteins, therefore indicating that retrograde transport to the Golgi network is a necessary prerequisite for cellular intoxication (el Baya et al. 1997). In CHO cells, the PT receptor has been shown to be a high-molecular weight glycoprotein that binds the B oligomer through a branched-mannose core containing *N*-acetylglucosamine (Sekura 1985). In contrast to the other ADP-ribosyltransferases, where the enzymatically active domain A mediates all the toxic activities, PT possesses other nonlethal activities (such as a mitogenic activity on T cells), which are mediated exclusively by the receptor-binding domain B (Tamura et al. 1983). The active site of pertussis toxin is structurally homologous to the active sites of other ADP-ribosylating toxins. This aspect will be described in the section "ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity" in this chapter.

Pertussis toxin plays a central role in the pathogenesis of whooping cough and in the development of protective immunity against reinfection. For this reason, the role of many residues of S1 has been tested by site-directed mutagenesis to produce nontoxic mutants of the toxin to be used as vaccines. The minimal region still enzymatically active is constituted by amino acids 4–179 of S1 subunit (Pizza et al. 1988; Cieplak et al. 1988; *Fig. 22.31*), and it is within this fragment that many mutations have been designed and analyzed for activity. In particular, Arg-9, Asp-11, Arg-13, Trp-26, His-35, Phe-50, Glu-129, and Tyr-130 were found to be essential for enzymatic activity and, when replaced with other residues, the toxicity was reduced to levels of about 1 %; nevertheless, none of the single amino acid mutants were completely devoid of toxicity.

The most successful mutant contains in fact two amino acid substitutions: Arg-9/Lys and Glu-129/Gly (PT-9K/129G; *Fig. 22.32*). This mutant is structurally identical to the wild type but is completely nontoxic and has been used for the construction of an acellular vaccine against pertussis. This vaccine has been extensively tested and has been shown to induce protection from disease (Pizza et al. 1989; Rappuoli 1997).

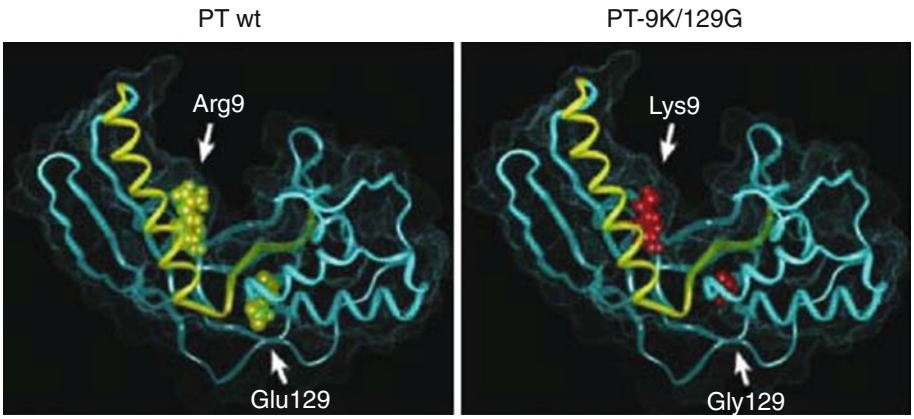

Fig. 22.32
X-ray representation of the wild type pertussis toxin (*left panel*) and of the double mutant 9K/129G (*right panel*). The catalytic cleft is colored in *yellow*, whereas the mutated residues are in *red*

Cholera Toxin and Heat-Labile Enterotoxin

Cholera toxin (CT) and *E. coli* heat-labile enterotoxins (LT-I and LT-II) share an identical mechanism of action and homologous primary and 3D structures (Dallas and Falkow 1980; Spicer et al. 1982; Sixma et al. 1991; *Figs. 22.1* [panel 15] and *22.33*). The CT is produced by *Vibrio cholerae* (the etiological agent of cholera), whereas LT-I and LT-II are produced by enterotoxigenic strains of *E. coli* (ETEC) isolated from humans with traveler's diarrhea, from pigs (LT-I), or from food (LT-II; Seriwatana et al. 1988). The two toxins belong to the class of ADP-ribosylating toxins and are organized in an AB_5 architecture, where the B domain is a pentamer which binds the receptor on the surface of eukaryotic cells, and domain A bears the enzymatic activity and is thus responsible for toxicity (Holmgren 1981; Moss and Vaughan 1988). Both the A and B subunits of CT and LT are synthesized intracellularly as precursor proteins which, after removal of the leader peptide and translocation across the cytoplasmic membrane, assemble in the periplasmic space to form the final AB_5 complex. While *V. cholerae* exports the CT toxin into the culture medium, LT remains associated to the outer membrane bound to lipopolysaccharide (LPS; Horstman et al. 2002). The corresponding genes of CT and LT are organized in a bicistronic operon and are located on a filamentous bacteriophage and on a plasmid, respectively (So et al. 1978).

The A subunit (*Fig. 22.34*, left panel) is a 27-kDa monomer composed of a globular structure and linked to the B domain by a trypsin-sensitive loop and a long α-helix, which inserts inside the core of the B pentamer thus anchoring the two subunits. For full activity, the A subunit needs to be proteolytically cleaved and reduced at the disulfide bridge between cysteines 187 and 199 to give two fragments: the enzymatic subunit A1 and the linker fragment A2 (Lai et al. 1981). Whereas in cholera toxin the proteolytic process is performed during biosynthesis by an endoprotease (Booth et al. 1984), in the case of LT, it occurs by extracellular processes; in both cases, the reduction is thought to take place at the surface of the target cell.

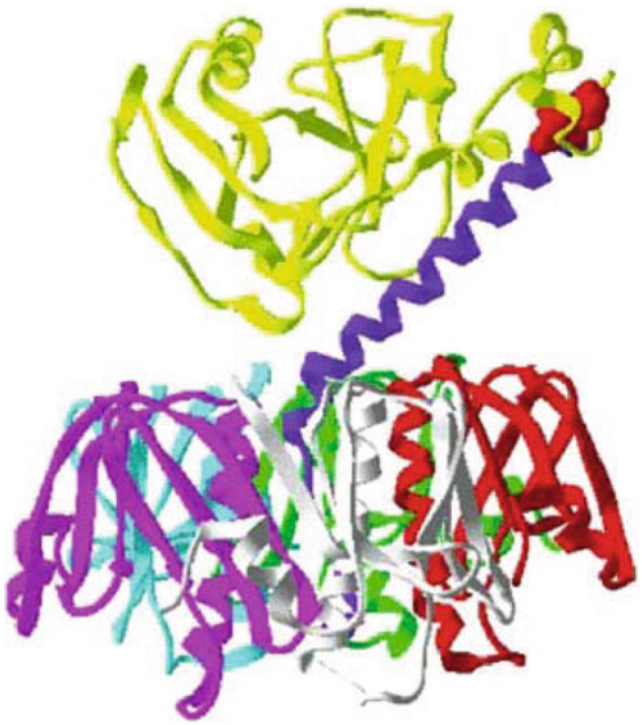

Fig. 22.33
X-ray structure of heat-labile enterotoxin LT of *E. coli*. The catalytic domain A1 is *yellow*, the linker domain A2 is *blue*, and the five monomers of the B subunit are all represented in different colors

The enzymatically active domain A binds NAD and transfers the ADP-ribose group to an Arg residue located within the central portion of several GTP-binding proteins such as G_s, G_t, and G_{olf}. Upon ADP-ribosylation of G_s, in particular, the adenylate cyclase is permanently activated, causing an abnormal intracellular cAMP accumulation, which in turn alters ion transport and thus is the main reason for the toxic effects (Field et al. 1989a, b).

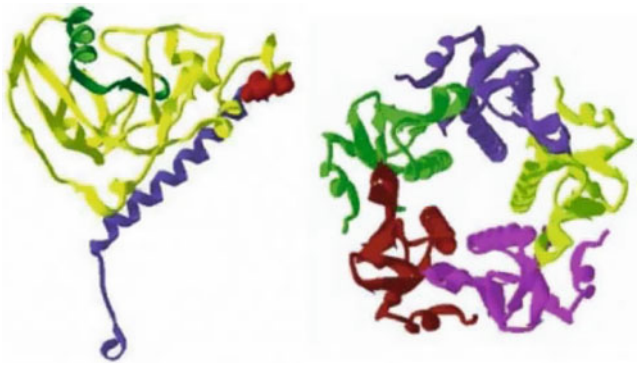

Fig. 22.34
***Left* side: *front* view of the catalytic A subunit, with the toxic moiety A1 in *pale green* and the linker domain A2 in *violet*; cysteines 187 and 199 involved in the disulfide bridge are *red*. *Right* side: *bottom* view of the pentameric receptor-binding domain B**

A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with 20-kDa guanine-nucleotide binding proteins, known as "ADP-ribosylation factors" (ARFs; Tsai et al. 1988; Moss and Vaughan 1991). After receptor binding, the holotoxins are internalized and undergo retrograde transport through the Golgi to the endoplasmic reticulum (ER). Recent studies show that both A and B subunits move together from the cell surface into the ER, and this depends on the B subunit binding to ganglioside GM1. The KDEL motif in the A2 chain does not appear to affect retrograde transport, but slows recycling of the B subunit from ER to distal Golgi stacks. Specificity for GM1 in this trafficking pathway is shown by the failure of the *E. coli* type II toxin LTIIb that binds ganglioside GD1a to concentrate in lipid rafts, enter the ER, or induce toxicity. These results show that the B subunit carries the A1 chain from cell surface into the ER where they dissociate, and that a membrane lipid with strong affinity for lipid rafts provides the dominant sorting motif for this pathway (Fujinaga et al. 2003). In the ER, the A1 chain of the CT unfolds and enters the cytosol by a process termed "retro-translocation." Upon entering the cytosol, the A1 chain rapidly refolds, binds ARF, and induces toxicity (Lencer et al. 1995). The B subunits persist in the Golgi and are subsequently degraded.

The exact localization of the ARF-binding site is still unknown, but it has emerged from recent studies that the two domains (the NAD-binding and ARF-binding) are independent and located in different regions of the A domain (Stevens et al. 1999).

When the toxins are released in the intestine during infection, the major consequence is intestinal fluid accumulation and watery diarrhea (also typical symptoms of the diseases; Holmgren 1981).

The B domain (*Fig. 22.34*, right) is composed of five identical subunits (each 11.5 kDa) that are arranged in a symmetric shape around a central pore inside which the C-terminal portion of the catalytic domain (A2) is inserted (Sixma et al. 1991, 1993). Their secondary structure consists predominantly of two three-stranded antiparallel β-sheets, a short N-terminal helix, and a long central helix. Although still well conserved in terms of quaternary structure, CT and LT B domains have a lower degree of primary sequence homology than the corresponding A domains. Interestingly, the B subunit of LT-II, although maintaining a conserved structure, lacks any sequence homology with the corresponding B domains of CT and LT-I (Domenighini et al. 1995).

In addition to their function as receptor-binding domains and as carriers of the toxic moieties, the B subunits possess specific biological activities such as induction of apoptosis of CD8+ and CD4+ T cells (Truitt et al. 1998) and the property to function as potent mucosal adjuvants (Xu-Amano et al. 1994). This feature has been extensively used to develop mucosal vaccines against cholera and ETEC infection, and to induce a mucosal response also against the other antigens used.

To produce molecules suitable for these pharmacological applications but completely devoid of toxic activity, more than 50 mutant derivatives have been constructed both for the A and B subunits. Among those which completely inactivate the toxin, the best characterized are LT-K63 (*Fig. 22.35*), LT-K97, and LT-K7, all in the vicinity of the catalytic domain, and for which the 3D structures have also been determined (Merritt et al. 1995; Van den Akker et al. 1995, 1997). In the case of LT-K63 (and the corresponding CT-K63), where the wild type Ser in position 63 is substituted with a Lys, the mutated proteins are enzymatically inactive and nontoxic, either in vitro and in vivo, but are otherwise indistinguishable from the wild type. In fact, they are still able to bind the receptor and the ARFs (Stevens et al. 1987), and the crystal structure and that of wild type LT are almost perfectly superimposable except for the catalytic site, where the bulky side chain of Lys-63 fills the catalytic pocket thus making it unsuitable for NAD entrance and binding (Giannelli et al. 1997; Douce et al. 1998).

Another interesting mutant is LT-K97, where the substitution Val/Lys introduces a salt bridge between Lys-97 and the carboxylate of Glu-112, thus making it unavailable to further interactions. This observation suggests a dominant role of this glutamic acid in the enzymatic reaction.

Mutations affecting the B domain lead often to products that can no longer bind to eukaryotic receptors, as is the case of LTB-D33, which contains a glycine-to-aspartic acid substitution in position 33. These types of mutants have been found to be almost completely nonimmunogenic at mucosal surfaces, suggesting that an intact receptor-binding site is necessary not only for binding but also for immunogenicity and adjuvanticity (Guidry et al. 1997).

Clostridium perfringens Alpha-Toxin

This toxin (*Fig. 22.1*, panel 16) is the most important toxin produced by *Clostridium perfringens* and is responsible for gas gangrene or clostridial myonecrosis (Stevens et al. 1987; Flores-Diaz and Alape-Giron 2003). It plays a key role in the spread of

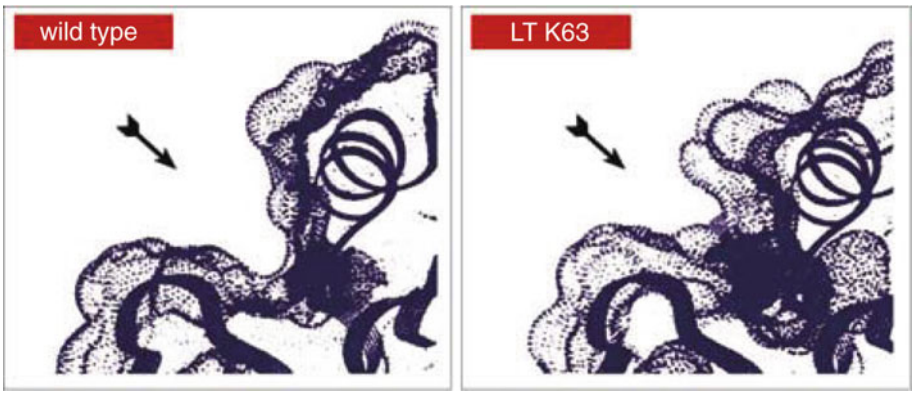

Fig. 22.35
Three-dimensional structure of the enzymatic cavity of the wild type LT (*left*) and of the mutant LT-K63 (*right*). The *arrows point* out how much a single amino acid substitution can affect the dimension of the pocket and thus the entrance of NAD

the infection either by suppressing host immune responses, triggering the release of inflammatory mediators, or causing changes in intracellular calcium levels. Specific mutants of *C. perfringens* that do not produce the toxin are unable to cause disease, and vaccination with a genetically engineered toxoid has been shown to induce protection against gas gangrene (Williamson and Titball 1993).

This virulence factor is a 370-amino acid zinc metalloenzyme that also displays phospholipase C (PLC) activity (Leslie et al. 1989); nevertheless, not all the bacterial PLCs act as virulence determinants, and therefore, this enzymatic activity is not sufficient for toxicity.

Alpha-toxin is capable of binding to mammalian cell membrane and cleaving membrane-bound phosphatidylcholine (or sphingomyelin) to produce phosphocholine and diacylglycerol (or ceramide). The reaction product diacylglycerol, which is a leukotriene precursor, is believed to be the responsible of the subsequent lethal effects.

The crystal structure of α-toxin has been recently solved (Naylor et al. 1998; ❯ *Fig. 22.36*), indicating the presence of two distinct domains in the molecule. Whereas the N-terminus is mainly organized as a globular α-helical domain that contains the active site, the β-sandwich C-terminal subunit is involved in membrane binding and shows strong structural analogy to eukaryotic calcium-binding C2 domains. A flexible linker containing a series of highly mobile residues connects the two domains.

In addition, the C-terminal subunit displays hemolytic and sphingomyelinase activities and primarily contributes to the toxin's lethal effect, even if it is completely devoid of toxic activity when used alone. Nevertheless, immunization with this domain affords full protection from disease in mouse models, thus indicating that the protective epitopes are located in this portion of the molecule (Titball et al. 1993; Nagahama et al. 2002).

Recently, other bacterial PLCs, like those from *L. monocytogenes* and *Mycobacterium tuberculosis*, have been implicated in the pathogenesis of a number of diseases (Wadsworth and Goldfine 1999; Raynaud et al. 2002).

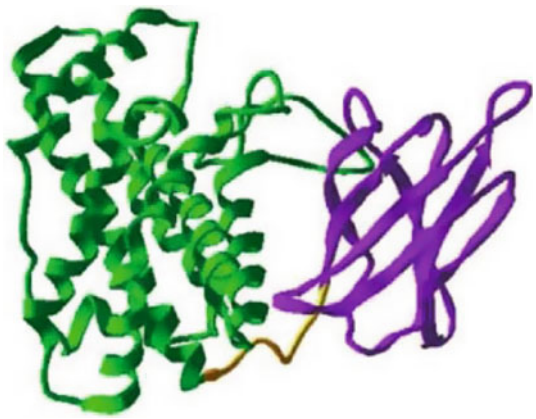

Fig. 22.36
Three-dimensional structure of *Clostridium perfringens* α-toxin. The N-terminal and C-terminal domains are *green* and *violet*, respectively, and the flexible linker is *orange*

Clostridium difficile Toxins A and B

Enterotoxin A (TcdA) and cytotoxin B (TcdB) of *Clostridium difficile* are the two virulence factors responsible for the induction of antibiotic-associated diarrhea. These toxins have molecular masses of 308 and 270 kDa, respectively, and belong to the class of large clostridial cytotoxins (Lyerly et al. 1986; Knoop et al. 1993).

The toxin genes *tcdA* and *tcdB* together with three accessory genes (*tcdC–E*) constitute the pathogenicity locus (PaLoc) of *C. difficile* (Cohen et al. 2000). Primary sequence homology between *tcdA* and *tcdB* gene products is higher than 60 % identity (von Eichel-Streiber et al. 1996).

Upon binding to eukaryotic cells and translocation across membranes via receptor-mediated endocytosis, TcdA and TcdB monoglucosylate small GTP-binding proteins such as Rho, Rac, and Cdc42 at a threonine residue (Just et al. 1995a, b; Ciesla and Bobak 1998). In most cells, *C. difficile* toxins induce

depolymerization of the actin cytoskeleton, leading to a morphology similar to that induced by C3-like transferases. While toxin B has potent cytotoxic activity in vitro, the enterotoxic activity of *C. difficile* in animals has been mainly attributed to toxin A.

From the structural point of view, they are composed of two portions: the N-terminal nonrepetitive two-thirds corresponding to the catalytic subunit and the C-terminal third characterized by a highly repetitive domain called the "clostridial repetitive oligopeptide" (CROP), identified as the site of interaction with a carbohydrate structure as well as the ligand to which neutralizing antibodies bind (von Eichel-Streiber et al. 1996). A central hydrophobic region contains several predicted transmembrane segments and is believed to function as the translocation unit.

Bordetella pertussis Adenylate Cyclase

Adenylate cyclase (CyaA) is a toxin produced by *Bordetella pertussis*, *B. bronchiseptica*, and *B. parapertussis* (Weiss and Hewlett 1986). It is essential in the early stages of bacterial colonization of the respiratory tract and can induce apoptosis of lung alveolar macrophages (Goodwin and Weiss 1990; Khelef et al. 1993).

Organized as a bifunctional protein, CyaA (177 kDa) is composed of an N-terminal cell-invasive and calmodulin-dependent adenylate cyclase domain (residues 1–400) fused to a pore-forming hemolysin (residues 401–1706; Glaser et al. 1988; Bejerano et al. 1999; see also the section "RTX Toxins"). Unlike most of the other members of the RTX family that are secreted into the supernatant, CyaA remains associated to the bacterial surface, through interactions with filamentous hemagglutinin (FHA). This toxin forms small cation-selective channels in lipid bilayer membranes and delivers into the cytosol of target cells the adenylate cyclase (AC) domain, which, upon binding to calmodulin, catalyzes an uncontrolled conversion of ATP to cAMP, thus causing intoxication and disruption of cellular functions (Ladant and Ullmann 1999). Calcium has been shown to play a fundamental role in channel formation (Knapp et al. 2003). Furthermore, it was also demonstrated that the ability of the AC domain to form pores and translocate across the membrane is strictly linked to the correct folding of an amphipathic α-helix spanning residues 509–516. Substitution of Glu-509 with a helix-breaker proline residue, in fact, significantly reduced the capacity of the toxin to undergo translocation (Osicková et al. 1999).

A very similar function and mechanism of action is that of ExoY, an adenylate cyclase produced by *Pseudomonas aeruginosa* and injected into the cytoplasm of eukaryotic cells by the type III secretion apparatus (see *Table 22.1*, and the section "Toxins Injected into Eukaryotic Cells" in this chapter). However, differently from CyaA, ExoY is not activated by calmodulin. In vivo, following infection with ExoY-expressing strains, CHO cells showed a rounded morphology, which correlated with increased cAMP levels (Yahr et al. 1998).

Anthrax Edema and Lethal Factors

Lethal factor (LF) and edema factor (EF) proteins, produced by *Bacillus anthracis*, combine with the protective antigen PA to give the lethal (PA + LF) and edema (PA + EF) toxins (Brossier et al. 2000; Collier and Young 2003; *Fig. 22.1*, panel 17). In both complexes, the PA has the pore-forming, receptor-binding activity (see the section "Other Pore-Forming Toxins" in this chapter), whereas EF and LF display, in turn, the toxic activities.

The EF and LF genes are located on a large plasmid (Robertson and Leppla 1986; Robertson et al. 1988) and encode precursors of approximately 800 residues. Cleavage of the N-terminal signal peptides yields mature EF and LF proteins with molecular masses of 88.8 and 90.2 kDa, respectively. These virulence factors enter cells by binding to proteolytically activated, receptor-bound, oligomeric PA; following receptor-mediated endocytosis, the low pH causes a conformational change in PA, allowing the translocation of EF-LF across cell membrane (Collier 1999). The EF-LF is then endocytosed and translocated from endosomes directly to the cytosol of cells, where both toxins perform their toxic activities (*Fig. 22.37*). The binding sites of EF and LF on PA have been recently mapped (Cunningham et al. 2002).

Once inside the cells, EF binds calmodulin and catalyzes an unregulated production of the second messenger cAMP, thereby perturbing the normal cell regulatory mechanisms (Goyard et al. 1989). Calcium influx is required for inducing cyclic AMP toxicity in target cells (Kumar et al. 2002).

Whereas the PA-binding domain displays a strong sequence homology to lethal factor LF, the catalytic domain is more similar to the other known adenylate cyclase CyaA toxin of *Bordetella pertussis* (Escuyer et al. 1988). On the other hand, LF cleaves the amino-terminus of the cellular mitogen-activated protein kinase kinases (MAPKK1 and MAPKK2), thus causing inhibition of the MAPK signal transduction pathway, which is key to cellular proliferation and signal transduction processes in the cell (Duesbery et al. 1998; Vitale et al. 1999).

Recently, the 3D structures of LF and EF have been solved (*Fig. 22.38*). LF comprises four domains: domain I binds the membrane-translocating component of anthrax toxin (PA); domain II resembles the ADP-ribosylating toxin from *Bacillus cereus*; domain III is inserted into domain II and seems to have arisen from a repeated duplication of a structural element of domain II; and domain IV is distantly related to the zinc metalloprotease family, and contains the catalytic center (Pannifer et al. 2001). The catalytic portion of EF is made by three globular domains. The active site is located at the interface of two domains (C_A and C_B), which together form the catalytic core, containing the catalytic residue His351. EF has been crystallized both alone and in complex with calmodulin. The differences between the two forms are induced by calmodulin, which acts by stabilizing the conformation of the substrate-binding site of EF (Drum et al. 2002). Interestingly, a remarkable level of primary sequence similarity can be detected between EF and the N-terminal, calmodulin-binding domain of *Bordetella* adenylate cyclase CyaA. In particular, His-351 is conserved between the two proteins.

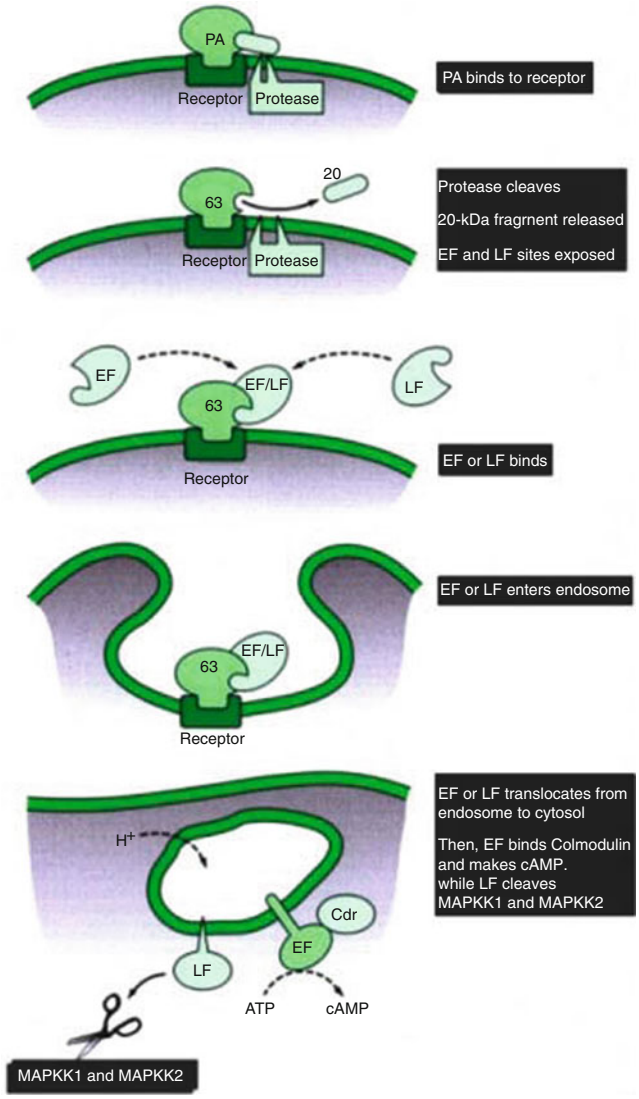

Fig. 22.37
Mechanism of PA-mediated entry and intoxication of anthrax LF and EF toxins

Once in the cytoplasm, LF acts as a zinc metalloprotease disrupting normal homoeostatic functions (Klimpel et al. 1994). The macrophage is a uniquely sensitive cell type that seems to be a vital global mediator of toxin-induced pathologies. Removal of macrophages from mice renders them insensitive to LF challenge (Hanna 1999).

In addition, LF, but not EF, is able to cause apoptosis in human endothelial cells. As a consequence, the observed endothelial toxicity contributes to vascular pathology and hemorrhage during systemic anthrax (Kirby 2004).

E. coli Cytotoxin Necrotizing Factors

Cytotoxin necrotizing factors (CNF-1 [◉ *Fig. 22.1*, panel 18] and CNF-2), single-chain proteins of 115 kDa produced by a number of uropathogenic and neonatal meningitis-causing pathogenic *E. coli* strains (Caprioli et al. 1984; De Rycke et al. 1987), are immunologically related and share 85 % identity. They also share some similarity with the dermonecrotic toxin of *Pasteurella multocida* and *Bordetella pertussis* (Schmidt et al. 1999). Both CNF1 and CNF2 toxins are encoded by a single structural gene with a low G+C content (35 %). However, whereas *cnf1* is chromosomally encoded, cnf2 is carried on a large transmissible F-like plasmid called "Vir" (Oswald and De Rycke 1990; Falbo et al. 1992).

These toxins induce ruffling, stress fiber formation, and cell spreading in cultured cells by activating the small GTP-binding proteins Rho, Rac, and Cdc42, which control assembly of actin stress fibers (Oswald et al. 1994). CNF1 induces only a transient activation of Rho GTPase and a depletion of Rac by inducing the addition of an ubiquitin chain, which is known to drive to specific degradation by the proteasome. Reduction of Rac GTPase levels induces cell motility and cellular junction dynamics allowing efficient cell invasion by uropathogenic bacteria (Doye et al. 2002). The catalytic region of CNF1 has been crystallized (Buetow et al. 2001; ◉ *Fig. 22.39*). The active site contains a catalytic triad, which is positioned in a deep pocket, thus explaining the restricted access to unspecific substrates and therefore its specificity (Schmidt et al. 1998). Very likely, some type of conformational rearrangement is required also to accomodate Rho in this narrow cavity.

Recently, a CNF1-like toxin (CNFY) has been identified also in *Yersinia pseudotubercolosis* (Lockman et al. 2002). Differently from the *E. coli* CNFs, CNFY has been shown to selectively activate RhoA (Hoffmann et al. 2004).

Bordetella Dermonecrotic Toxin

Dermonecrotic toxin (DNT) is produced by *Bordetella* species as a single-chain polypeptide chain of 1464 amino acids, which is composed of a C-terminal portion that contains the catalytic site, and of an N-terminal receptor-binding domain. DNT shares about 30 % identical residues in the catalytic domain with *E. coli* CNF1, including the catalytic Cys and His residues. DNT is a transglutaminase, which catalyzes the deamidation or polyamination at Gln63 of Rho and of the corresponding residues of Rac and Cdc42 (Horiguchi 2001). This activity causes alteration of cell morphology, reorganization of stress fibers, and focal adhesions on a variety of animal models. Recently, it has been demonstrated that the initial 54 amino acids of DNT are sufficient for cell surface recognition. However, the receptor is still unknown.

Cytolethal Distending Toxins

The cytolethal distending toxin (CDT) produced by *Haemophilus ducreyi* (HdCDT) is the prototype of a growing family of bacterial toxins that act by inducing cell enlargement followed by cell death (Cortes-Bratti et al. 1999; Frisan et al. 2003).

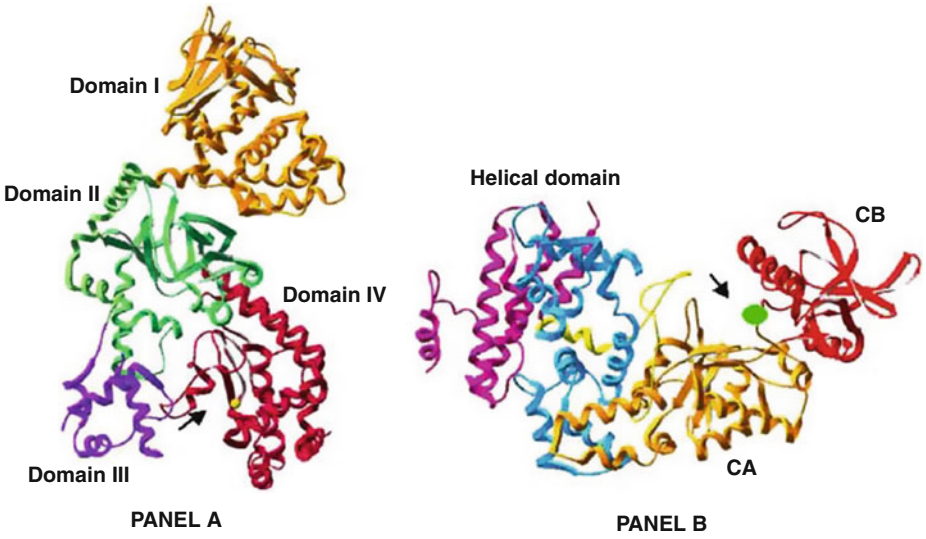

Fig. 22.38
Crystal structures of the catalytic portion of anthrax lethal factor (*panel A*) and edema factor in complex with calmodulin (*panel B*). *Panel A*. The four domains are in different colors. The zinc atom complexed by domain IV is indicated by an *arrow*

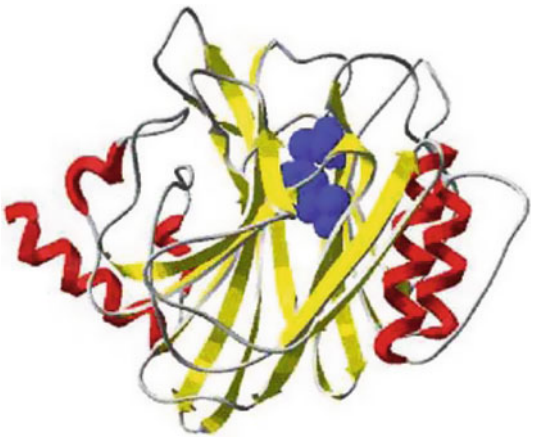

Fig. 22.39
Crystal structure of the active site of *E. coli* CNF1. The catalytic site composed by Cys866-His881 is colored in *blue*

HdCDT is a complex of three proteins (CdtA, CdtB, and CdtC) encoded by three genes that are part of an operon. Members of this family have been identified in *E. coli*, *Shigella*, *Salmonella*, *Campylobacter*, *Actinobacillus*, and *Helicobacter hepaticus* (Okuda et al. 1995; Lara-Tejero and Galan 2001; Haghjoo and Galan 2004; Pickett et al. 2004; Shenker et al. 2004; Young et al. 2004). The overall sequence similarity varies among the different members of this family of toxins. HdCDT intoxicates eukaryotic cells by causing a three- to fivefold gradual distension and induces cell-cycle arrest in the G_2 phase. It has also been shown to induce DNA double-strand breaks and formation of actin stress fibers via activation of the small GTPase RhoA. Recently, it has been shown that CdtB is the active subunit of the CDT toxin and acts as a nuclease. All the amino acids predicted to be important for nuclease activity are conserved in the CdtB of different bacteria, suggesting that the mechanism of action is the same for all CDT toxins. On the other hand, CdtA and CdtC are able to bind to the surface of HeLa cells, therefore playing a role in the delivery of the active domain to target cells (Lee et al. 2003).

Toxins Acting on the Cytoskeleton Structure

The cytoskeleton is a cellular structure that consists of a fiber network composed of microfilaments, microtubules, and the intermediate filaments. It controls a number of essential functions in the eukaryotic cell and participates in all kinds of cellular movement and transport; furthermore, the cytoskeleton is involved in processes like exo- and endocytosis, vesicle transport, cell-cell contact, and mitosis (Kabsch and Vandekerckhove 1992).

The group of cytoskeleton-affecting bacterial toxins comprises not only virulence factors that directly act on particular elements of the cytoskeleton but also proteins that perform an indirect action by affecting regulatory components, which control its organization (Aktories 1994; Richard et al. 1999). Most of them do it by modifying the regulatory, small G proteins, such as Ras, Rho, Rac, and Cdc42, which control cell shape. These toxins, which have a dramatic but indirect effect on the cytoskeleton and are described in the section "Toxins Acting on Signal Transduction," are *E. coli* CNF and *C. difficile* enterotoxins A and B. Other toxins acting on regulatory G proteins are exoenzyme S, C3, and YopE, which are described below as toxins that are directly injected into the eukaryotic cells. Other bacterial molecules that cannot be strictly considered toxins but that have a powerful ability to polymerize actin are ActA and IcsA of

Listeria and *Shigella*, respectively. These are described elsewhere in this volume (see Listeria and Relatives in Vol. 4 and The Genus Shigella in Vol. 6). Another toxin acting indirectly on the cytoskeleton is the zonula occludens toxin (Zot) produced by *V. cholerae*, a toxin with an unknown mechanism of action that modifies the permeability of tight junctions (Zot is described in the paragraph "Toxins with Unknown Mechanism of Action" in this chapter). In the following section, we consider only toxins that have the cytoskeleton as a direct target. The only toxin shown to affect directly the cytoskeleton is the C2 toxin of *C. botulinum*, which ADP-ribosylates monomeric actin, making it unable to polymerize. A second protein that has recently been described as being able to bind actin and stabilize the fibers supporting the ruffles induced by the *Salmonella* type III secretion system is SipA (described in the section "Toxins Injected into Eukaryotic Cells" in this chapter).

Representatives of both subgroups can be identified among the class of ADP-ribosylating factors that ultimately display their toxic effect on the cytoskeleton of eukaryotic cells. The family of *Clostridium botulinum* toxin C2, clostridial toxin C3 (and related proteins), and *Pseudomonas aeruginosa* exoenzyme S (Exo S) act on small GTP-binding proteins that regulate the correct functioning of the cytoskeleton, and thus have an indirect toxic effect (Coburn et al. 1999).

Clostridium botulinum Toxin C2 and Related Proteins

Clostridium botulinum toxin C2 is the main representative of a class of binary cytotoxins produced by clostridial species that predominantly act on polymerized actin microfilaments of 7–9 nm in diameter (Aktories et al. 1986; Aktories and Wegner 1992). C2 ADP-ribosylate monomeric G-actin at an arginine residue (Aktories et al. 1986; see the section "ADP-Ribosylating Toxins" in this chapter). Because this arginine (Arg-177) is a contact site between actin monomers, the binding of the ADP-ribose moiety prevents actin's polymerization.

Other members of this family are *C. perfringens* iota toxin (Stiles and Wilkins 1986; Perelle et al. 1993) and the related *C. spiroforme* and *C. difficile* ADP-ribosylating toxins (Popoff and Boquet 1988a; Just et al. 1994), which are generally classified as iota-like toxins. These binary toxins are constructed according to the A/B model architecture, but in this case, the two domains reside in separate molecules that interact to cause the toxic effect. Therefore, these toxins have an enzymatically active and toxic domain (A) and a binding component (B), which is essential for the binding at the cell surface and for the translocation inside the cell.

Clostridium botulinum toxin C2 is an extremely toxic agent, which induces hypotension, increase in intestinal secretion, vascular permeability, and hemorrhaging in the lungs. In contrast to botulinum neurotoxins, C2 does not seem to display any neurotoxic effect. The two molecules that constitute its toxic moiety are classified as C2-II (for the binding component) and C2-I (for the enzymatic component). The C2-II is a 100-kDa protein that must be proteolytically cleaved to a 75-kDa fragment before it can bind to the surface receptor; upon this interaction, a binding site for the 50-kDa C2-I component is activated and the toxic domain is taken up by receptor-mediated endocytosis (Ohishi 1987). Substrates of the C2-I toxin are β/γ-non-muscle actin and γ-smooth muscle actin, but not α-actin isoforms. Conversely, the related iota toxin of *Clostridium perfringens* has been found to ADP-ribosylate all actin isoforms (Mauss et al. 1990). The iota toxin is a binary toxin produced by *Clostridium perfringens* type E, which has been implicated in fatal calf, lamb, and guinea pig enterotoxemias (Madden et al. 1970). Structurally, it has two independent domains: Ia, which is the ADP-ribosyltransferase, and Ib, which is involved in the binding and internalization of the toxin by the cell (Stiles and Wilkins 1986). The crystallization of the C2-I component in complex with its substrate NADH has recently been achieved (Tsuge et al. 2003; *Fig. 22.40*), showing a close relationship of iota toxin with insecticidal protein VIP2 of *Bacillus cereus*.

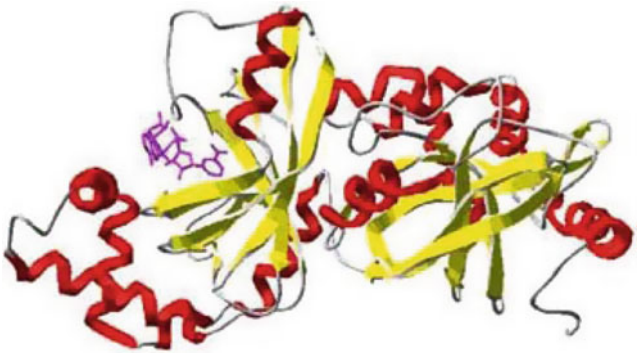

Fig. 22.40
Crystal structure of the catalytic domain C2I of *C. perfringens* C2 toxin (*red* and *yellow*) in complex with NADH (*pink*)

Clostridium difficile induces its pathogenic effects by secreting a number of potent cytotoxins; one, in particular, has been found to possess ADP-ribosyltransferase activity (CDT). CDT acts on the cytoskeleton structure by disaggregating actin filaments and thus provokes an increase of globular actin (G-actin; Popoff et al. 1988b; Gulke et al. 2001).

Another member of the group of iota-like toxins is the *Clostridium spiroforme* toxin, composed of a toxic subunit Sa and a binding subunit Sb (Popoff et al. 1989). The level of primary sequence homology detected among the enzymatic and binding components of this class of ADP-ribosylating toxin ranges from 32 % to 80 % identity, the binding domains being the better conserved. The C2 toxin is the one with the lower degree of sequence conservation, and this correlates with the fact that it does not appear to be cross-reactive with the other iota-like toxins.

Experiments of site-directed mutagenesis have helped to define for these toxins an active site very similar to those described for the better studied members of the family of ADP-ribosyltransferases (Barth et al. 1998; see the section "ADP-Ribosyltransferases: A Common Structure of the Catalytic Site" in this chapter).

Escherichia coli Lymphostatin

Lymphostatin is a very recently identified protein in enteropathogenic strains of *E. coli* (EPEC; Klapproth et al. 2000).

A leading cause of diarrhea among infants in developing countries, EPEC is also one of the few known bacterial causes of chronic diarrhea. These strains are characterized by their ability in host cells to induce cytoskeletal rearrangements that result in the formation of adhesion pedestals. This mechanism known as "the attaching and effacing effect" (Moon et al. 1983; Khoshoo et al. 1988) ultimately allows the bacterium to colonize the host for prolonged periods.

Lymphostatin also has been identified as one of the primary factors that selectively block the production of interleukin-2 (IL-2), IL-4, IL-5, and γ interferon by human peripheral cells and inhibit proliferation of these cells, thus interfering with the cellular immune response (Klapproth et al. 1995).

Lymphostatin, a very large toxin with a predicted molecular weight of 366 kDa, shares significant homology with the catalytic domain of the large clostridial cytotoxins, including toxins A and B of *Clostridium difficile*, a lethal toxin of *C. sordelii*, and a toxin of *C. novyi*. Its corresponding gene, *lifA*, with 9,669 bp, is the largest reported gene in *E. coli*. Some *lifA* mutants of EPEC have been constructed to verify the lymphocyte inhibitory factor (LIF) activity of its gene product; lysates of this mutant lacked the ability of wild type EPEC lysates to inhibit expression of IL-2, IL-4, and γ interferon mRNA and protein in mitogen-stimulated lymphocytes, while the expression of IL-8 was unaffected (Klapproth et al. 2000). Experiments of colony hybridization performed using an internal fragment of the *lifA* gene identified a similar gene present in most of the EPEC and enterohemorrhagic *E. coli* (EHEC) strains able to produce the attaching and effacing lesions on host epithelial cells, but this gene was not found in other *E. coli* and related organisms (Klapproth et al. 2000).

Toxins Acting on Intracellular Trafficking

Vesicle structures are essential in the eukaryotic cell for a number of vital processes such as receptor-mediated endocytosis and exocytosis; these are used either to internalize portions of the plasma membrane and address them to the specialized compartment, or to transport to the cell surface molecules synthesized in the ER and modified in the Golgi apparatus.

One example of exocytic pathway is that involving the release of neurotransmitters that are contained within small synaptic vesicles packed at synaptic terminals; the majority of these vesicles are bound to the cytoskeleton and are not directly available for immediate release, but some of them are present at the cytosolic face of the presynaptic membrane and are ready to release their content. However, at low calcium concentrations, only an occasional vesicle fuses to the presynaptic membrane, giving rise to a depolarization event. This event leads to the opening of calcium channels and thus to an increase of calcium concentration, which finally triggers the fusion of the neurotransmitter vesicles with the plasma membrane.

Recently, this field was greatly advanced by the identification of the eukaryotic molecules responsible for vesicle docking and membrane fusion. Three of these proteins (namely, vesicle-associated membrane protein [VAMP]/synaptobrevin, synaptosome-associated protein [SNAP-25], and syntaxin) are the specific targets of a number of neurotoxins produced by bacteria of the genus *Clostridium* (CNTs; Montecucco and Schiavo 1994; *Fig. 22.41*).

The CNT family is composed of tetanus neurotoxin (TeNT) and seven serotypes of botulinum neurotoxins (BoNT/A–BoNT/G), which are specific zinc-dependent proteases whose action finally causes the block of neuroexocytosis (Schiavo et al. 1992; Pellizzari et al. 1999; Lalli et al. 2003). The degree of sequence homology detected among this group of toxins is high, ranging from 30 % to more than 50 % identity.

Clostridium tetanii Neurotoxin

Tetanus neurotoxin (TeNT; *Fig. 22.1*, panel 19) is the unique causal agent of the pathological condition of spastic paralysis known as tetanus. This is one of the most potent toxins known so far with a 50 % lethal dose (LD_{50}) in humans of 0.1–1.0 ng/kg.

The TeNT is produced by *Clostridium tetanii* as a single-chain polypeptide of 150 kDa that, following proteolytic cleavage, is divided into fragments H (heavy) and L (light) held together by a disulfide bridge. Its overall structure is similar to that of A/B toxins, where the toxic subunit A is represented here by the light chain L, and subunit B is constituted by the H_C and H_N domains. The heavy chain is composed of fragments HC, which has recently been found to bind di- and trisialylgangliosides on neuronal cell membranes (Halpern and Loftus 1993), and HN, which is involved in the transmembrane translocation of the L chain to the cytosol (Schiavo et al. 1990; Shapiro et al. 1997). The L chain is a 50-kDa fragment containing the –HExxH– motif typical of metalloproteases. It binds zinc and specifically cleaves VAMP/synaptobrevin, a eukaryotic factor essential for membrane fusion (Rossetto et al. 1995).

The first step of intoxication is the specific binding of domain H_C of TeNT to both high and low affinity receptors exposed on the presynaptic neuronal membrane at neuromuscular junctions (Montecucco 1986); the second step is internalization of TeNT into the peripheral motoneuron and then retrograde axonal transport. The TeNT is released through the postsynaptic membrane into the synaptic space where it enters into the inhibitory interneurons of the central nervous system through receptor-mediated endocytosis (Halpern and Neale 1995). At this point, while the H_C domain is in the vesicle, the translocation domain H_N helps the catalytic light chain L to cross the vesicle membrane and gain access to the cytosolic compartment where L performs its toxic activity on VAMP/synaptobrevin (Montal et al. 1992).

Interestingly, domain H_C retains the unique transport properties of the intact holotoxin and is capable of eliciting a protective immunological response against the full-length tetanus neurotoxin (Herreros et al. 2000). A single zinc atom is

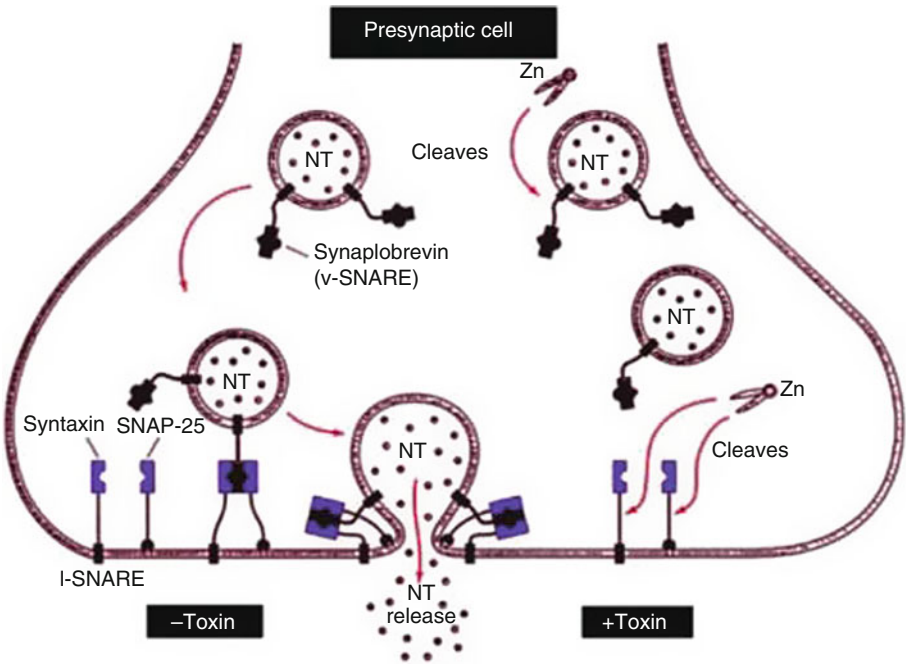

Fig. 22.41
Mechanism of action of clostridial neurotoxins

bound to the L chain of TeNT and is essential for toxicity. This specific metallo-dependent proteolytic activity is common to the other clostridial toxins and to the lethal factor (LF) of *Bacillus anthracis*.

The crystal structure of the receptor-binding fragment HC of tetanus neurotoxin has been recently determined at 2.7 Å resolution (Umland et al. 1997; *Fig. 22.42*) revealing an N-terminal jelly-roll domain and a C-terminal β-trefoil domain.

To determine which amino acids in tetanus toxin are involved in ganglioside binding, homology modeling was performed using recently resolved X-ray crystallographic structures of the tetanus toxin HC fragment. On the basis of these analyses, the amino acids tryptophan 1288, histidine 1270, and aspartate 1221 were found to be critical for binding of the HC fragment to ganglioside GT1b (Fotinou et al. 2001; Louch et al. 2002).

Although the overall sequence homology detected among clostridial neurotoxins is significant, this similarity weakens in the region encompassing the C-terminal domain; the fact that each toxin possesses its own unique receptor and is immunologically distinct from the others has been attributed to sequence divergence of this domain which, therefore, could be responsible for receptor specificities (Lacy and Stevens 1999).

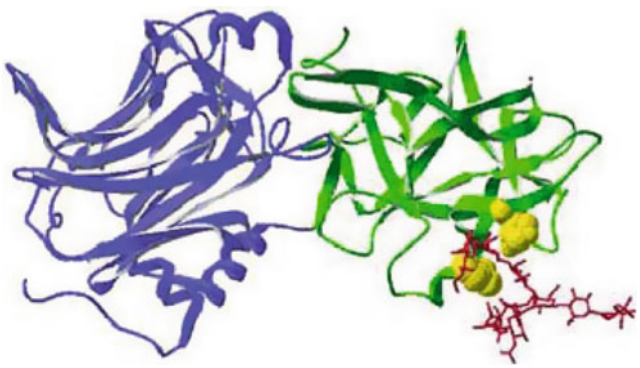

Fig. 22.42
Crystal structure of the receptor-binding domain HC of tetanus neurotoxin in complex with a ganglioside analog (in *red*). The N-terminus and C-terminus are colored in *blue* and *green*, respectively. The residues probably involved in ganglioside binding are *yellow* (see *Fig. 22.1*, panel 18)

Clostridium botulinum Neurotoxins

These neurotoxins (BoNT/A-G; *Fig. 22.1*, panel 20) are the causative agents of the flaccid paralysis typical of clinical botulism intoxication (Hatheway 1995). All of them are zinc-dependent proteases that show a strong tropism for the neuromuscular junction (Simpson 1980; Rossetto et al. 1995), where they bind to still unidentified receptors in a strictly serotype-specific manner. This binding step is followed by the entry of the toxin into the cytoplasm of the motoneurons and by specific proteolytic cleavage of intracellular targets belonging to the family of soluble *N*-ethylmaleimide-sensitive, fusion factor attachment protein receptors (SNARE). Four out of the seven botulinum neurotoxins (BoNT/B, D, F, and G) cleave VAMP/synaptobrevin, another two act specifically on SNAP-25, whereas the last one, BoNT/C, cleaves both syntaxin and SNAP-25 substrates. In all cases, the ultimate effect is the total block of acetylcholine release (Montecucco and Schiavo 1995).

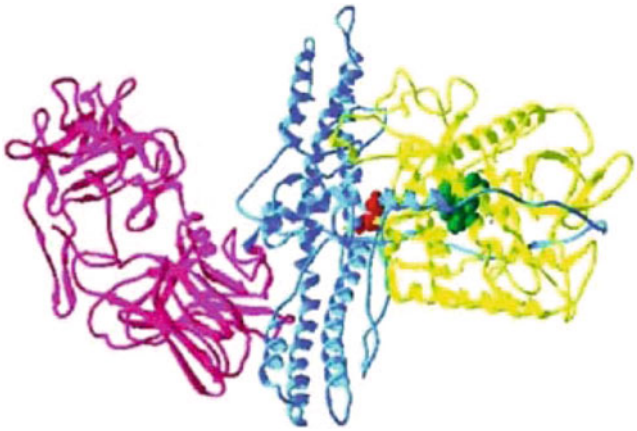

Fig. 22.43
X-ray structure of *Clostridium botulinum* neurotoxin serotype A. The 50-kDa catalytic domain (L) is colored in *yellow*, with the zinc-binding domain in *green*. The N-terminal portion of the 100-kDa subunit involved in translocation is *blue*, whereas the C-terminal receptor-binding moiety is in *magenta*. The disulfide bond linking the two 50- and 100-kDa fragments is colored in *red* (see *Fig. 22.1*, panel 20)

These toxins are generally produced as large complexes of 300–900 kDa containing additional proteins such as hemagglutinin (300 kDa) and nontoxic peptides, which are believed to act as stabilizing agents of the neurotoxins in the gut environment (Sakaguchi 1982).

The BoNTs are synthesized as inactive polypeptide chains of 150 kDa, which (following proteolytic cleavage) divide into two chains of 50 and 100 kDa that remain linked by a disulfide bridge. The catalytic function is carried by the 50-kDa fragment, the light chain L (residues 1–437), whereas the 100-kDa subunit (heavy chain, H) contains both the translocation (residues 448–872) and the receptor-binding domains (residues 873–1,295; Krieglstein et al. 1994). The crystal structure determined for the full-length polypeptide of BoNT serotype A (Lacy et al. 1998; *Fig. 22.43*) reveals a number of remarkable features, particularly related to the peculiar structure of the translocation domain. This contains, in fact, a central pair of α-helices 105-Å long and a 50-residue loop that wraps around the catalytic domain in a belt-like fashion, partially occluding the active-site pocket. This unusual loop bears the site of the proteolytic cleavage, which is required for activation of the toxin; the fact that in the protoxin, the translocation domain shields the active site explains why the catalytic activity in test tube experiments is greatly enhanced by reduction of the disulfide bond. The fold of the translocation domain suggests a mechanism of pore formation different from that displayed by other pore-forming toxins. The helices are antiparallel and amphipathic and twist around each other in a coiled-coil-like structure. In addition, the domain has two strand-like segments that lie parallel to the helical axis and are predicted to be directly involved in membrane spanning. Very recently, the X-ray structure obtained for the recombinant form of chain L of BoNT/A has shed light on a possible novel mode of substrate binding and catalytic mechanism (Segelke et al. 2004).

The highest degree of homology detected among this family of clostridial neurotoxins is concentrated in the light chain L (30–60 % identity; particularly its N-terminus), probably involved in substrate recognition, and in the central portion that contains the catalytic zinc-binding motif –HexxH– characteristic of zinc endopeptidases. The zinc atom coordinated by this pocket is required for the in vivo toxicity of BoNTs.

Years ago, medical experiments demonstrated that injection of BoNT/A is very effective in strabismus; since then, the therapeutic applications of these neurotoxins have been extended to a variety of diseases which benefit from a functional paralysis of the neuromuscular junction, and all the BoNTs are under clinical testing (Atassi and Oshima 1999; Hallett 1999).

Helicobacter pylori Vacuolating Cytotoxin Vac A

Highly pathogenic strains of *Helicobacter pylori*, the etiological agent of peptic ulcer and gastritis (Cover and Blaser 1992), produce vacuolating cytotoxin A (VacA; Papini et al. 1994; *Fig. 22.1*, panel 21). This toxin is responsible for massive growth of vacuoles within epithelial cells, and when administered to mice, VacA causes loss of gastric gland architecture, cell necrosis, and gastric ulceration (Telford et al. 1994). Synthesized as a 140-kDa precursor, VacA is secreted from the bacterium through its 45-kDa carboxy-terminal domain, using a mechanism similar to that of neisserial IgA proteases (Schmitt and Haas 1994; Fiocca et al. 1999). When purified from the culture supernatant of Type I *H. pylori* strains, the protein has a molecular weight of approximately 600–700 kDa, suggesting the idea of a multimeric complex; electron microscopy studies have in fact demonstrated the flower-shaped structure of the toxin (Lupetti et al. 1996; *Fig. 22.44*) resulting from the aggregation of either six or seven monomers, each comprising the 95-kDa amino-terminal region of the VacA precursor. Recently, a model has been proposed to show how VacA can insert into membranes forming hexameric, anion-selective pores (Kim et al. 2004).

Each monomer can be cleaved at a protease-sensitive site into two fragments of 37 and 58 kDa (p37 and p58 moieties) that may represent the A and B moieties of AB-like bacterial toxins. The 37-kDa, amino-terminal portion is highly conserved at the sequence level and is able to induce vacuoles when the *vacA* gene is placed under the control of a strong eukaryotic promoter and transfected into epithelial cells. This evidence suggests that the active site could be located in this region of the molecule, whereas the carboxy-terminal portion is likely to be devoted to receptor recognition and binding. Although VacA is exported over the outer membrane and is released from the bacteria, recent data have been presented to show that a portion of the toxin remains associated with the bacterial surface. Surface-associated toxin is biologically active and organized into distinct toxin-rich domains on the bacterial surface. Upon bacterial contact with host cells, toxin clusters are transferred to the

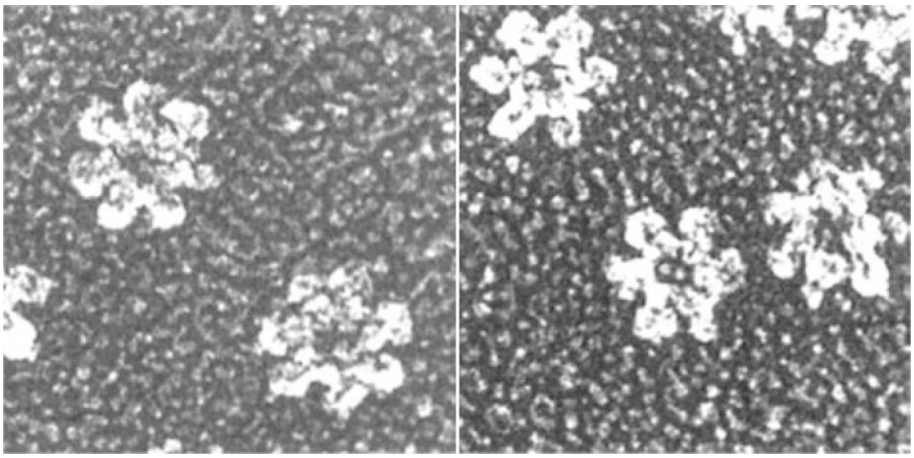

Fig. 22.44
Vacuolating cytotoxin structure: heptameric and hexameric forms of VacA as observed in electron micrographs of quick-freeze, deep-etched preparations. The oligomers are approximately 30 nm in diameter with a 10–12-nm central cavity

host cell surface via a contact-dependent mechanism, followed by uptake and intoxication (Ilver et al. 2004).

The mechanism of toxicity exploited by this virulence factor has not yet been completely elucidated. What is known is that VacA causes an alteration of the endocytic pathway, which results in the selective swelling of late endosomes or prelysosomal structures (Papini et al. 2001). The small GTP-binding protein Rab7 is necessary for vacuole formation (Papini et al. 1994, 1997). Even though it is unknown, the target of VacA action is strongly believed to be a fundamental effector in membrane trafficking.

Streptococcus pyogenes NAD^+ Glycohydrolase

NAD^+ glycohydrolase is an important virulence factor produced by group A streptococci (GAS), which is thought to enhance pathogenicity by facilitating the spread of the microorganism through host tissues. This enzyme catalyzes the hydrolysis of the nicotinamide-ribose bond of NAD to yield nicotinamide and ADP-ribose. Differently from ADP-ribosylating toxins, NAD^+ glycohydrolases possess a much higher rate of NADase activity and do not require an ADP-ribose acceptor. Interestingly, this GAS virulence factor is functionally linked to streptolysin O (SLO), a pore-forming toxin, which has been shown to be required for efficient translocation of NAD^+ glycohydrolase into epithelial cells. In contrast to the wild type GAS, isogenic mutants deficient in the expression of SLO, NAD^+ glycohydrolase, or both proteins resulted in reduced cytotoxicity and keratinocyte apoptosis. These results suggest that NAD^+ glycohydrolase modulates host cell signaling pathways and contributes to the enhancement of streptolysin O cytotoxicity (Bricker et al. 2002).

Toxins Injected into Eukaryotic Cells

See *Tables 22.1* and *22.2* for a summary of the principal features of toxins described in this section.

In the classical view, toxins were believed to be molecules that cause intoxication when released by bacteria into the body fluids of multicellular organisms. This definition failed to explain the pathogenicity of many virulent bacteria such as *Salmonella*, *Shigella*, and *Yersinia*, which did not release toxic proteins into the culture supernatant. Today, we know that these bacteria also intoxicate their hosts by using proteinaceous weapons. These bacteria intoxicate individual eukaryotic cells by using a contact-dependent secretion system to inject or deliver toxic proteins into the cytoplasm of eukaryotic cells (*Fig. 22.2*, panel 3). This is done by using specialized secretion systems that in gram-negative bacteria are called "type III" or "type IV," depending on whether they use a transmembrane structure similar to flagella or conjugative pili, respectively.

Mediators of Apoptosis

Pathogens use different mechanisms to induce or prevent apoptosis in host cells. Virulence factors produced by the pathogen can interact directly with effector molecules of apoptosis or interfere with factors involved in cell survival (Weinrauch and Zychlinsky 1999).

They include pore-forming toxins which induce cell death by altering host cell permeability, bacterial toxins (such as DT, PAETA, Shiga, and Shiga-like toxins) which induce cell death by inhibition of host protein synthesis, and type III secreted proteins of *Shigella*, *Salmonella*, and *Yersinia* which are directly delivered into host cell compartment and trigger apoptosis by altering the signal transduction pathway. This latter class of toxins will be described here in more detail.

IpaB

Shigella, the causative agent of bacillary dysentery produces IpaB. *Shigella* invades the epithelial cells by causing the cell

cytoskeleton to reorganize during bacterial entry. The bacteria are phagocytosed by macrophages and rapidly escape from phagosomal compartment to the cytosol where they induce apoptosis of the macrophages. Invasion and cytotoxicity require *Shigella* invasion plasmid antigen (Ipa) proteins, which are secreted by a type III secretion apparatus. Invasion and escape from the phagosome are dependent upon the expression and secretion of the IpaB, IpaC, and IpaD. Only IpaB is required to initiate cell death by interaction with the interleukin-1β converting enzyme, or caspase I, which is one of the effector molecules of apoptosis. The IpaB-induced apoptosis results in an inflammation that has the effect not only of clearing and possibly localizing the infection but also promoting bacterial spread in the intestinal epithelium (Hilbi et al. 1998). Protein domains directly involved in pathogenicity have recently been mapped (Guichon et al. 2001).

SipB

An analog of *Shigella* invasin IpaB, *Salmonella* invasion protein (SipB) is produced by *Salmonella* and is delivered to the host cells by a type III secretion system. In contrast to *Shigella*, *Salmonella* does not escape from the phagosome, but it survives and multiplies within the macrophages. *Salmonella* virulence genes responsible for invasion and killing of macrophages are encoded by a chromosomal operon named *sip* containing five genes (sipEBCDA; Hermant et al. 1995). The sip genes show high sequence homology with the *ipa* operon of *Shigella*, and the Sip proteins show functional similarities with Ipa proteins. Both proteins have a predominant alpha-helical structure and contain two helical transmembrane domains, which insert deeply into the bilayer (Hume et al. 2003). Similarly to IpaB, SipB also induces apoptosis by binding interleukin-1β-converting enzyme.

Necessary for *Salmonella*-induced macrophage apoptosis, SipB acts through a caspase-I-activating mechanism similar to that used by IpaB (Hersh et al. 1999). Also, SipB can complement IpaB mutants, enabling them to invade cells and escape macrophage phagosomes.

YopP, YopJ, and Related Proteins

Yersinia enterocolitica and *Yersinia pestis* produce YopP and YopJ, respectively (Straley and Bowmer 1986; Mills et al. 1997). Following contact with the host cell, *Yersiniae* deliver into the cytoplasm of eukaryotic cells, through a type III secretion system, plasmid-encoded proteins named "*Yersinia* outer-membrane proteins" (Yop). These proteins are able to induce alteration of cytoskeleton (YopE and YopT), inhibition of phagocytosis (YopH), and in the case of YopP and YopJ, induction of apoptosis (Orth 2002).

The mechanism by which *Yersinia* induces apoptosis is probably different from that described for *Shigella*, inasmuch as *Yersinia* induces apoptosis from the outside of host cells. The binding of YopJ directly to the superfamily of MAPKKs blocks both their phosphorylation and subsequent activation. These activities of YopJ are responsible for the inhibition of extracellular signal-regulated kinase, downregulation of TNF-α, and suppression of the nuclear factor kappa B (NF-κB) signaling pathways, preventing cytokine synthesis and promoting apoptosis (Orth et al. 1999). The YopJ-related proteins that are found in a number of bacterial pathogens of animals and plants, such as AvrRxv from *Xanthomonas campestris* (Whalen et al. 1993), AvrA from *Salmonella* (Hardt and Galan 1997), and y410 from *Rhizobium* (Freiberg et al. 1997) may function to block MAPKKs so that host signaling responses can be modulated upon infection. Whereas no function is known for AvrA and y410, AvrRxv is a plant pathogen virulence protein involved in the programmed cell death pathway.

Toxins Interfering with Inositol Phosphate Metabolism: SopB and IpgD

The SopB protein, secreted by *Salmonella dublin*, is a virulence factor essential for enteropathogenicity. The toxin hydrolyzes phosphatidylinositol triphosphate (PIP_3), which is a messenger molecule that inhibits chloride secretion, thus favoring fluid accumulation and diarrhea (Norris et al. 1998). Furthermore, SopB mediates actin cytoskeleton rearrangements and bacterial entry in a Rac-1 and Cdc42-dependent manner. Consistent with an important role for inositol phosphate metabolism in *Salmonella*-induced cellular responses, a catalytically defective mutant of SopB failed to stimulate actin cytoskeleton rearrangements and bacterial entry (Zhou et al. 2001).

SopB is homologous to the *Shigella flexneri* virulence factor IpgD, suggesting that a similar mechanism of virulence is also present in *Shigella*. Both proteins contain two regions of sequence similarities (motifs 1 and 2, *Fig. 22.45*) with human inositol polyphosphatases types I and II. Motif 2 contains a consensus sequence (Cys-X5-Arg) characteristic of Mg^{+2}-independent phosphatases in which the cysteine is the residue essential for catalysis. Recent studies have shown that IpgD acts as a potent inositol 4-phosphatase and is responsible for dramatic morphological changes of the host cell, ultimately leading to consistent actin filament remodeling (Niebuhr et al. 2002).

Toxins Acting on the Cytoskeleton

Pseudomonas aeruginosa Exoenzyme S. This toxin is one of several products of *Pseudomonas aeruginosa* that contributes to its pathogenicity (Woods et al. 1989; Kulich et al. 1993; *Fig. 22.1*, panel 22). It belongs to the group of ADP-ribosylating factors that lack both the receptor-binding and translocation domains, and are directly injected by bacteria into the cytoplasm of eukaryotic cells. In this case, bacteria intoxicate individual eukaryotic cells by means of a contact-dependent type III secretion system (Yahr et al. 1996).

The 49-kDa ExoS protein ADP-ribosylates the small GTP-binding protein Ras at multiple sites but

	Motif 1	Motif 2
SopB	VVTFNFGVNELALKM	AWNCKSGKDRTGMMSDE
IpgD	VAAFNVGVNELALKL	CWNCKSGKDRTGMQDAE
PTPaseI	PVLFNVGINEQQTLA	FTSCKSAKDRTAMSVTL
PTPaseII	PVLFNVGINEQQTLA	FTCCKSAKDRTSMSVTL

Fig. 22.45
Alignment of conserved motifs

preferably at Arg-41 (Ganesan et al. 1998; see the section "ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity" in this chapter). To become enzymatically active, ExoS requires the interaction with a cytoplasmic activator named "FAS" or "14.3.3" (Fu et al. 1993). When cells are transfected with the *exos* gene under the control of a eukaryotic cell promoter, a collapse of the cytoskeleton and a change of the morphology of the cells can be observed as primary consequences.

Pseudomonas aeruginosa ExoS is a bifunctional cytotoxin where the ADP-ribosyltransferase domain is located within its C-terminus portion. Recent studies showed, in fact, that when transfected or microinjected into eukaryotic cells, the N-terminus part of ExoS (amino acid residues 1–234) stimulates cell rounding. The N-terminus of ExoS (1–234) does not influence nucleotide exchange of Rho, Rac, and Cdc42 but increases GTP hydrolysis. It has also been shown that Arg-146 of ExoS is essential for the stimulation of GTPase activity of Rho proteins (Goehring et al. 1999). The GTPase-activating domain (GAP) of ExoS has been crystallized (Wurtele et al. 2001). In addition to these toxic effects performed on the cytoskeleton, other activities have been demonstrated for Exo S, such as the adhesive property on buccal cells (Baker et al. 1991) and the induction of human T lymphocyte proliferation (Mody et al. 1995). From sequence analysis, it has been possible to identify the regions of Exo S, which could be involved in NAD binding and thus constitute the common structure of the catalytic site.

Clostridium botulinum Exoenzyme C3 and Related Proteins. Produced by certain strains of *Clostridium botulinum* types C and D, exoenzyme C3 is a 251-amino acid protein that specifically ADP-ribosylates *rho* and *rac* gene products in eukaryotic cells (Moriishi et al. 1993; *Fig. 22.1*, panel 23). These substrates belong to the group of small GTP-binding proteins and seem to have a fundamental role in cell physiology and cell growth. The ADP-ribosylation process occurs at asparagine residues (Asn-41) located in the putative effector binding domains of *rho* and *rac* and thus alter their functions (Sekine et al. 1989). The enzymatic activity is identical to that of all ADP-ribosylating enzymes; however, the recently solved 3D structure has shown that the C3 exoenzyme structure can be distinguished by the absence of the elongated α-helix, which generally constitutes the ceiling of the active site cleft in the ADP-ribosylating toxins crystallized so far. Seemingly, this feature does not impair the ability of C3 either to accommodate the NAD substrate or to carry out the enzymatic reaction (Han et al. 2001; *Fig. 22.46*).

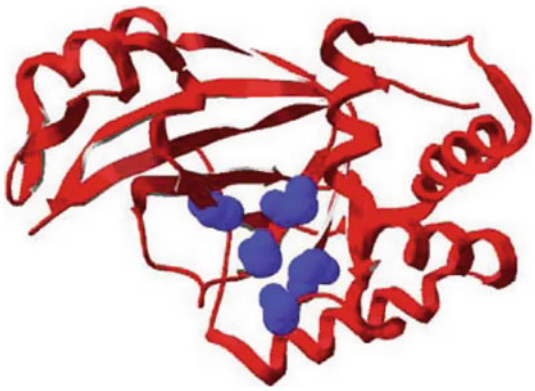

Fig. 22.46
Crystal structure of exoenzyme C3 of *C. botulinum*. The residues which constitute the catalytic site are in *blue*

This exoenzyme is the prototype of the group of A-only toxins because it apparently lacks the receptor-binding B domain and thus is unable to enter the cells; for this reason, C3 cannot be considered a real virulence factor, and still unknown is whether C3 alone is able to intoxicate the cells. Nevertheless, when microinjected into cells, it causes complete disruption of actin stress fibers, rounding of the cell body, and formation of arborescent extensions.

Other members of this family of C3-related exoenzymes have been isolated from gram-positive bacteria, such as certain strains of *Staphylococcus aureus* (Sugai et al. 1992), *Clostridium limosum* (Just et al. 1992), and *Bacillus cereus* (Just et al. 1995c). Whereas *C. botulinum* C3 and *C. limosum* exoenzyme are about 70 % homologous and immunologically related, the epidermal cell differentiation inhibitor (EDIN) produced by *S. aureus* is only 35 % homologous with C3 and shows no immunological cross-reactivity (*Fig. 22.47*). However, crystal data recently obtained for *S. aureus* C3 exotoxin (EDIN-B) have disclosed a very similar structure (Evans et al. 2003). *Bacillus cereus* exoenzyme exhibits the same substrate specificity as the other C3-like transferases (it was found to act specifically on rho proteins). Nevertheless, some differences can be observed for this toxin, such as the higher molecular weight (28 kDa) and, more importantly, the lack of immunological relationship to any other member of this family (Just et al. 1995a).

Salmonella SopE and SipA. *Salmonella typhimurium* achieves entry into cells by delivering effector proteins into the cytosol through a type III secretion system. These effectors stimulate signal pathways leading to reorganization of the cell's actin cytoskeleton, membrane ruffling, and stimulation of nuclear response to promote efficient bacterial internalization. One of the proteins that stimulate the cellular response is SopE, which is able to activate signaling pathways through Rho GTPases by stimulating GTP/GDP nucleotide exchange on proteins such as Cdc42 and Rac (Hardt et al. 1998).

These signaling events lead to the recruitment of cellular proteins such as actin and T-plastin (an actin-binding protein that bundles actin), which finally induce actin cytoskeleton

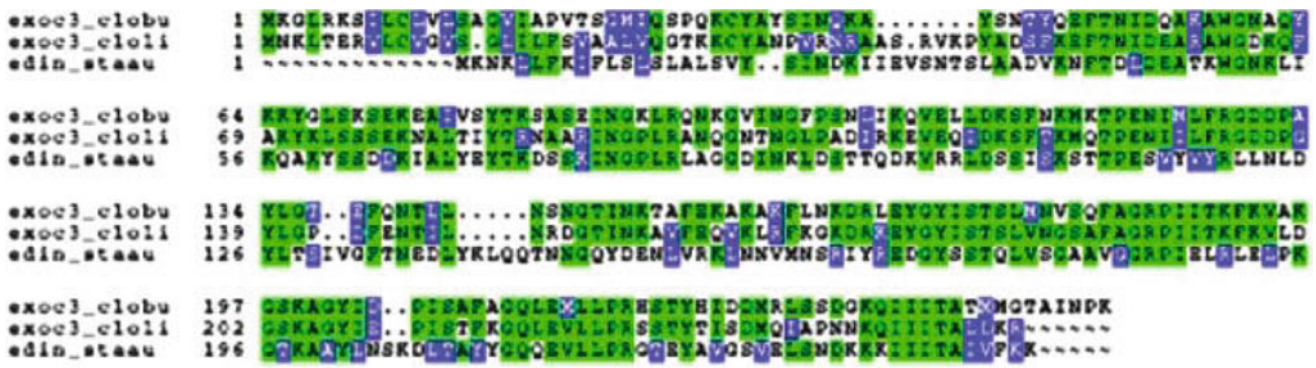

Fig. 22.47
Multiple sequence alignment of protein toxins belonging to the group of exoenzyme C3-like ADP-ribosyltransferases

rearrangement and membrane ruffling. In addition, SopE stimulates nuclear responses that induce the synthesis of proinflammatory cytokines that contribute to the induction of diarrhea.

These cytoskeletal rearrangements are further modulated by SipA, which binds directly to actin, stabilizes actin filaments inhibiting depolymerization, and forms a complex with T-plastin thus increasing its actin-bundling activity (Zhou et al. 1999a, b). SipA activities result in localized actin cytoskeleton reorganization and more pronounced extension of membrane ruffles, which facilitate bacterial uptake. The actin-cytoskeleton reorganization induced by *Salmonella* is reversible, and infected cells are able to recover their normal architecture after bacterial internalization.

Crystal structures are available for SipA (Lilic et al. 2003) and for the catalytic fragment of SopE in complex with its host cellular target Cdc42 (Buchwald et al. 2002; ❯ *Figs. 22.1* [panels 24 and 25] and ❯ *22.48*).

Shigella IPAA. The entry of *Shigella* into epithelial cells requires the Ipa proteins, which are secreted upon cell contact by the type III apparatus and act in concert. The IpaB and IpaC proteins form a complex that binds B1 integrin and CD44 receptors and induces actin polymerization at the site of bacterium-cell contact, allowing the formation of membrane extension that probably requires also the action of Cdc42, Rac, and Rho GTPases (Nhieu and Sansonetti 1999).

The translocation of IpaA into the cell cytosol probably favors *Shigella* entry. The IpaA protein binds with high affinity to the N-terminal residues 1–265 of vinculin, a protein involved in linking actin filaments to the plasma membrane. The vinculin-IpaA complex interacts with F-actin inducing subsequent depolymerization of actin filaments. Presumably, these interactions further modulate the formation on the membrane of adhesion-like structures required for efficient invasion.

Shigella internalization still occurs at low levels in the absence of IpaA, suggesting that IpaA acts in concert with other bacterial effectors to promote cell entry. Binding of the *Shigella* protein IpaA to vinculin induces F-actin depolymerization (Bourdet-Sicard et al. 1999). The IpaA and vinculin rapidly associate during bacterial invasion. Although defective for cell entry, an *ipaA* mutant is still able to induce foci of actin polymerization but differs from wild type *Shigella* in its ability to recruit vinculin and α-actinin. It has been postulated that IpaA-vinculin interaction initiates the formation of focal adhesion-like structures required for efficient invasion (Tran Van Nhieu et al. 1997).

Yersinia YopE. A protein secreted by *Yersinia* through a type III secretion system, YopE contributes to the ability of *Yersinia* to resist phagocytosis (Rosqvist et al. 1990). Following infection of epithelial cells with *Yersinia*, the microfilament structure of the cells changes, leading to a complete disruption of the actin microfilaments, which finally results in cell rounding and detachment from the extracellular matrix (Rosqvist et al. 1991). The effector YopE was recently shown to possess GAP activity toward the Rho GTPases RhoA, Rac, and CDC42 in vitro (Aili et al. 2003; ❯ *Fig. 22.1*, panel 26). Further experimentation has shown that in vivo YopE is able to inhibit Rac- but not Rho- or Cdc42-regulated actin structures (Straley and Cibull 1989). Furthermore, the structure of this toxin has recently been solved, showing a close relationship with the analogous ExoS Gap domain (Evdokimov et al. 2002) (❯ *Fig. 22.49*).

Yersinia YopT. YopT is the prototype of a new family of 19 cysteine proteases with potent effects on host cells. These include the AVr protein of the plant pathogen *Pseudomonas* and possibly YopJ of *Yersinia*. YopT cleaves the posttranslationally modified cysteine located at the C-terminal end of Rho GTPases (DKGCASS), causing the loss of the prenyl group from RhoA, Rac, and cdc42, and releasing them from the membrane (Shao and Dixon 2003). The inability of Rho to be located to the membrane causes disruption of the cytoskeleton. While the C-terminus of YopT is crucial for activity, the N-terminus of YopT is crucial for substrate binding (Sorg et al. 2003).

Shigella VirA. The invasiveness of *Shigella* is an essential pathogenic step and a prerequisite of bacillary dysentery. VirA is a *Shigella* effector protein, which is delivered into the host cell by a specialized type III secretion system. This protein can interact with tubulin to promote microtubule destabilization and membrane ruffling (Yoshida et al. 2002). With this mechanism, *Shigella* is able to remodel the cell surface and thus

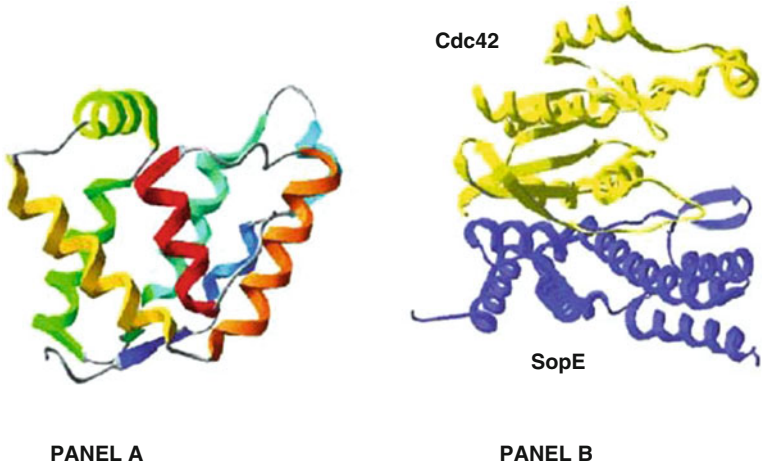

Fig. 22.48
Crystal structures of SipA (*panel A*) and of SopE in complex with Cdc42 (*panel B*)

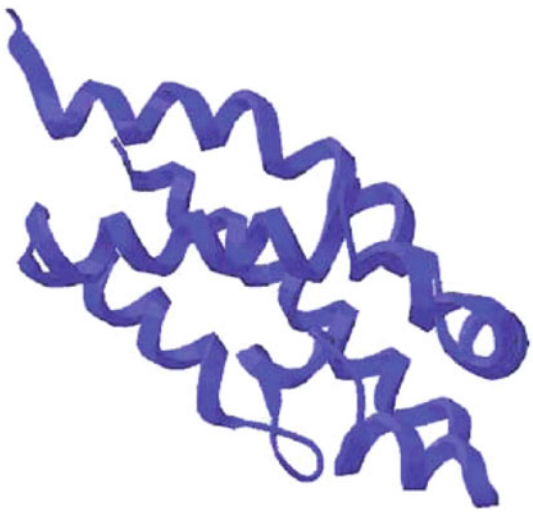

Fig. 22.49
Crystal structure of YopE

promote its entry into the host. Recent data have shown that *VirA* deletion mutants displayed decreased invasiveness and were unable to stimulate Rac1.

Toxins Acting on Signal Transduction

Yersinia YpkA and YopH. Phosphorylation is central to many regulatory functions associated with the growth and proliferation of eukaryotic cells. Bacteria have learned to interfere with these key functions in several ways. The best-known system is that of *Yersinia*, where a protein kinase (YpkA; Barz et al. 2000) and a protein tyrosine phosphatase (YopH; Zhang 1995; *Fig. 22.1*, panel 27) are injected into the cytoplasm of eukaryotic cells by a type III secretion system to paralyze the macrophages before they can kill the bacterium (Galyov et al. 1994).

YpkA is a Ser/Thr protein kinase that also displays autophosphorylating activity in vitro. In vivo experiments have shown that this protein is essential for virulence: in fact, challenge with a *YpkA* knockout mutant causes a nonlethal infection, whereas all mice challenged with wild type *Y. pseudotuberculosis* die. Recently, natural eukaryotic substrates of YpkA have been identified by using a two-hybrid assay. These belong to the class of small GTPases and comprise RhoA and Rac-1, but not Cdc42.

YopH is a modular protein where the tyrosine phosphatase domain shows a structure and catalytic mechanism very similar to those of eukaryotic enzymes. YopH acts by dephosphorylating cytoskeletal proteins thus disrupting phosphotyrosine-dependent signaling pathways necessary for phagocytosis. Host protein targets include Crk-associated substrate, paxillin, and focal adhesion kinase. In vivo, YopH inhibits phagocytosis by polymorphonuclear leukocytes (PMNs) and macrophages (Fallman et al. 1995; Ruckdeschel et al. 1996). The protein has a molecular weight of 51 kDa and is composed of an N-terminal domain important for translocation and secretion (Sory et al. 1995) and a C-terminal domain homologous to eukaryotic PTPases (Guan and Dixon 1990; Bliska 1995).

The three-dimensional structure of YopH has been solved (Stuckey et al. 1994; Su et al. 1994), revealing the presence of a catalytic domain which, despite its low level of sequence identity to the human PTP1B, still contains all of the invariant residues present in eukaryotic PTPases. Its tertiary fold is a highly twisted α/β structure with an eight-stranded β-sheet flanked by seven α-helices. Residues 403–410 form the PTPase phosphate-binding loop with the invariant Cys-403 thiol centered within the loop (*Fig. 22.50*).

EPEC Tir. A 78-kDa protein produced by enteropathogenic *E. coli* (EPEC) strains, Tir mediates the attachment of bacteria to eukaryotic cells and is essential for EPEC virulence. The Tir protein is tyrosine phosphorylated upon injection into

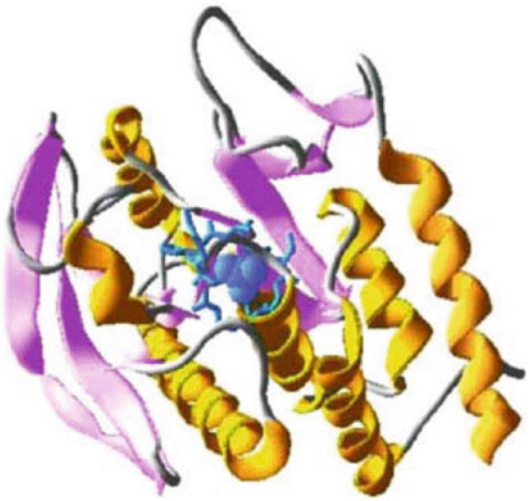

Fig. 22.50
X-ray structure of YopH. Colors have been assigned on the basis of secondary structure (*yellow* for helix and *pink* for β-sheet). The PTPase phosphate-binding loop and Cys-403 are in *blue*

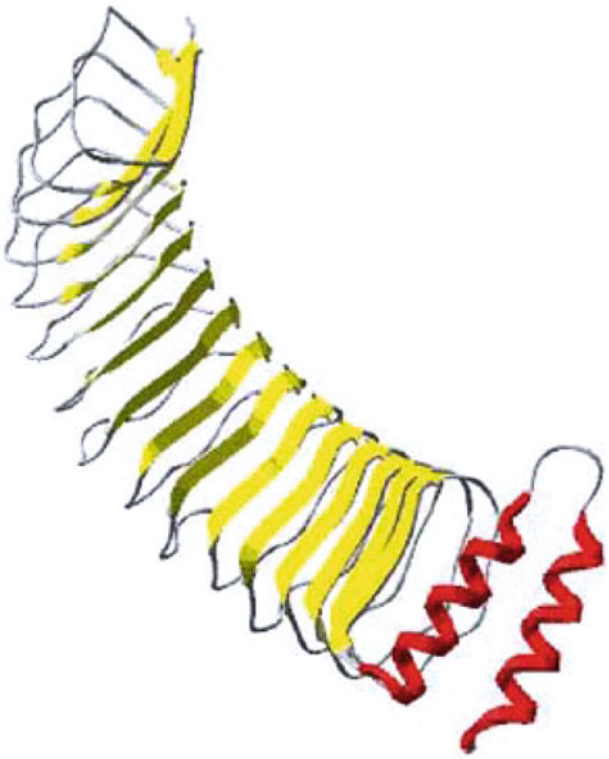

Fig. 22.51
Crystal structure of YopM effector protein of *Yersinia pestis*

eukaryotic cells by a type III secretion system. While in the host cell, it becomes an integral part of the eukaryotic cell membrane and functions as receptor for intimin, the major EPEC adhesin (Kenny et al. 1997). It is believed that, once in the host, Tir adopts a hairpin-like structure using its two putative transmembrane domains (TMDs) to span the host cell membrane. The region between the two TMDs constitutes the extracellular loop that functions as the intimin-binding domain. Following tyrosine phosphorylation, the protein mediates actin nucleation, resulting in pedestal formation and triggering tyrosine phosphorylation of additional host proteins, including phospholipase C-γ. Tir is essential for EPEC virulence and was the first bacterial protein described to be tyrosine phosphorylated by host cells (Crawford and Kaper 2002).

Helicobacter pylori CagA. Cytotoxin-associated gene A (CagA) is an immunodominant protein produced by most virulent strains of *Helicobacter pylori*, with a size that can vary from 128 to 146 kDa and which is commonly expressed in peptic ulcer disease (Covacci et al. 1993b). CagA is characterized by a central region containing an EPIYA motif, which can be repeated up to six times, increasing the molecular weight of the protein. The gene is encoded within a pathogenicity island, which also encodes the type IV secretion system necessary to inject the protein into eukaryotic cells. Once injected into the host cell, the protein is tyrosine phosphorylated at the EPIYA motif by the kinase C-Src and Lyn (Stein et al. 2002). The signal is proportional to the number of EPIYA motives present (Stein et al. 2000). The tyrosine phosphorylated CagA (CagA-P) activates SHP-2 and inactivates C-Src, leading to cortactin dephosphorylation triggering a signal transduction cascade (which results in cellular scattering proliferation, a phenotype indistinguishable from that induced by the hepatocyte growth factor [HGF]) (Covacci and Rappuoli 2000).

The long-term chronic infection and the continuous stimulation increase the risk of cancer of people infected by CagA+ *H. pylori*. CagA is the first bacterial protein linked to cancer in humans and the *cagA* gene can be considered the first bacterial oncogene.

Yersinia pestis YopM. YopM is an effector protein delivered to the cytoplasm of infected cells by the type III secretion mechanism of *Yersinia pestis*. YopM is a highly acidic protein, which is essential for virulence, but whose mechanism of action is still elusive. Differently from other effectors, this toxin has been shown to accumulate not only in the cytoplasm but also in the nucleus of mammalian cells. Recently, McDonald and colleagues have found that YopM interacts with two kinases, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1). These two kinases associate only when YopM is present, and expression of YopM in cells stimulates the activity of both kinases. These results indicate that PRK2 and RSK1 are the first intracellular targets of YopM (McDonald et al. 2003).

The X-ray structure determined for YopM has shown a modular architecture constituted by leucine-rich repeats, mainly organized in an extended β-sheet structure (Evdokimov et al. 2001; ❯ *Figs. 22.1* [panel 28] and ❯ *22.51*). This organization is very similar to that found for other important proteins, such as rab geranylgeranyltransferase and internalin B produced by *Listeria*.

Salmonella SptP. *Salmonella* protein tyrosine phosphatase (SptP) is an effector protein secreted by the type III secretion apparatus of *Salmonella enterica*. SptP is a modular protein

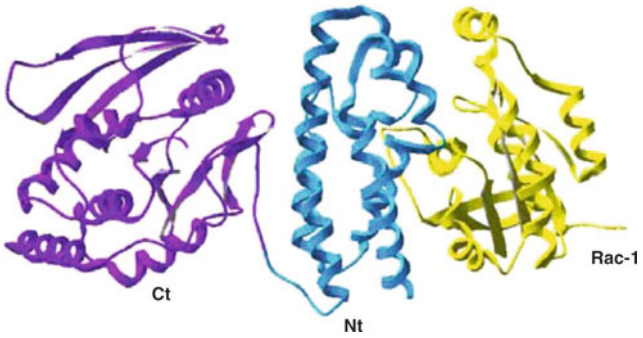

Fig. 22.52
Crystal structure of SptP in complex with Rac-1 (*yellow*). The N-term and C-term domains of SptP are colored in *cyano* and *purple*, respectively

composed of two functional domains, a C-terminal region with sequence similarity to *Yersinia* tyrosine phosphatase YopH, and an N-terminal domain showing homology to bacterial cytotoxins such as *Yersinia* YopE and *Pseudomonas* ExoS (Murli et al. 2001). Recently, it was demonstrated that this domain possesses strong GTPase-activating domain protein (GAP) activity for Cdc42 and Rac1 (Fu and Galan 1999; Lin et al. 2003). The crystal structure of SptP-Rac1 complex has shown that SptP is strongly stabilized by this interaction (Stebbins and Galan 2000; *Fig. 22.52*).

Pseudomonas aeruginosa ExoU. Several extracellular products secreted by the *P. aeruginosa* type III secretion system are responsible for virulence. Among these, the 70-kDa protein, ExoU, is responsible for causing acute cytotoxicity in vitro and epithelial lung injury. Recent studies demonstrated that ExoU has lipase activity, and that the cytotoxicity of ExoU is dependent on its patatin-like phospholipase domain. The results suggest that ExoU requires the presence of a catalytically active site Ser(142) and that a yet unknown eukaryotic cell factor(s) is necessary for its activation (Tamura et al. 2004).

ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity

ADP-Ribosylating Toxins: Main Features

The ADP-ribosylating toxins are a class of bacterial proteins that are characterized by an enzymatic domain with ADP-ribosyltransferase activity (Hayaishi and Ueda 1977; Ueda and Hayaishi 1985; Althaus and Richter 1987). During ADP-ribosylation (*Fig. 22.53*), these toxins bind NAD and transfer the ADP-ribose moiety to a specific substrate molecule, which is thus forced to undergo a dramatic functional modification. The toxic effect is totally dependent upon the enzymatic activity.

On the basis of their overall structure, ADP-ribosyltransferases can be separated into A/B toxins, binary toxins, and A-only toxins, where A is the subunit with the enzymatic activity, and B is the carrier domain involved in the recognition of the specific surface receptor and in the translocation of the toxic moiety into the eukaryotic cell. Most of the best characterized ADP-ribosylating toxins belong to the class with an A/B architecture: pertussis toxin (PT; Locht et al. 1986; Nicosia et al. 1986), cholera toxin (CT; Mekalanos et al. 1983), and *E. coli* heat-labile enterotoxin (LT; Spicer and Noble 1982; Yamamoto et al. 1984) are typical examples of this family where the A domain (called "S1" in PT) bears the enzymatic core and the B domain is an oligomer that helps the translocation across the cell membrane; the two subunits are linked together by noncovalent bonds. The genes coding for CT and LT are highly homologous (Dallas and Falkow 1980) and are organized into operons located on the chromosome of *Vibrio cholerae* and on a plasmid of *E. coli* (So et al. 1978).

Diphtheria toxin (DT; Pappenheimer 1977; Collier et al. 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; Gray et al. 1984b; Wick et al. 1990) are A/B toxins with a three-domain structure: the catalytic domain C, contained in fragment A, and the transmembrane domain T and the receptor-binding domain R, both within the B subunit.

The binary (as opposed to the A/B) toxins have a fairly similar organization, but in this case, the A and B domains are separately secreted in the culture supernatant where the B domain initially binds the receptor on the surface of the target cell and only then is able to bind the A subunit and help its translocation into the cytosol. Examples of this family of ADP-ribosyltransferases are the C2 toxin of *Clostridium botulinum* (Aktories et al. 1986), the iota toxin of *C. perfringens* (Perelle et al. 1995), the toxin of *C. spiroforme* (Popoff and Boquet 1988), the mosquitocidal toxin (MTX) of *Bacillus sphaericus* (Thanabalu et al. 1993), and the *C. difficile* transferase (Just et al. 1994).

Finally, the "A-only" toxins include Exo S of *Pseudomonas aeruginosa* (Kulich et al. 1994) and other toxins such as C3 of *Clostridium botulinum* (Nemoto et al. 1991), EDIN of *Staphylococcus aureus* (Sugai et al. 1990), and the toxins of *Bacillus cereus* (Just et al. 1995b) and of *Clostridium limosum* (Just et al. 1992). All the A-only toxins possess a still unknown mechanism of cell entry, with the notable exception of Exo S, which has been shown to be directly injected into eukaryotic cells by a specialized secretion system (Yahr et al. 1996).

With the exception of actin, all the eukaryotic proteins that are ADP-ribosylated by these toxins are GTP-binding proteins (G-proteins); these proteins are molecular switches involved in a number of essential cell functions including protein synthesis and translocation, signal transduction, cell proliferation, and vesicular trafficking (Hamm and Gilchrist 1996).

ADP-Ribosylating Toxins: A Common Structure of the Catalytic Site

Bacterial enzymes with ADP-ribosyltransferase activity include a variety of toxins with different structural organizations; the better-represented class is that comprising proteins with an A/B structure (PAETA, DT, CT, LT, and PT), where subunit A is responsible for enzymatic activity and subunit B is involved in receptor binding.

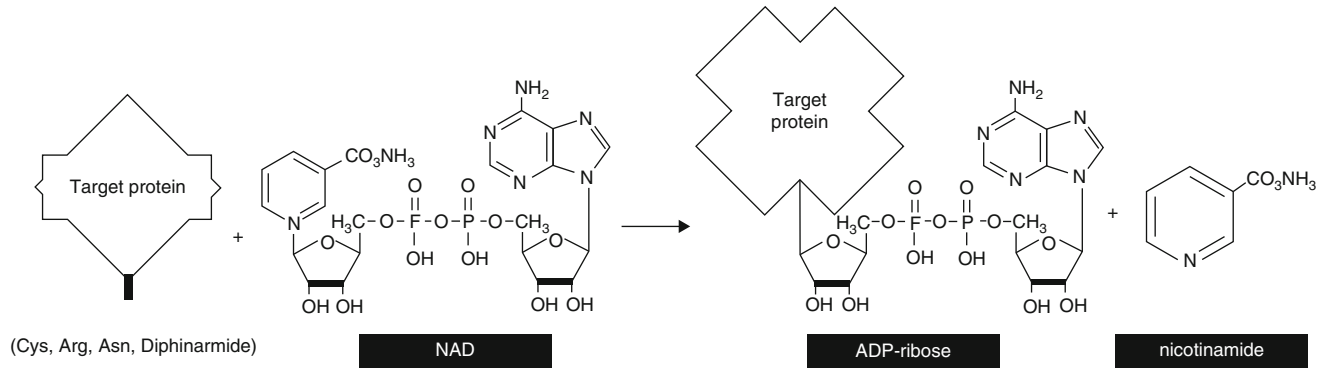

Fig. 22.53
Mechanism of ADP-ribosylation reaction catalyzed by ADP-ribosyltransferases

Other toxins, termed "binary toxins" (*Clostridium botulinum* toxin C2 and related proteins) are still composed of the two functional domains A and B. However, they reside on different molecules and need to interact to acquire activity. Finally, there is a group of ADP-ribosylating toxins that do not possess the receptor-binding domain B at all and are thus named "A-only toxins." This group includes *Clostridium botulinum* exoenzyme C3 and related proteins, which are unable to invade the cells, and toxins which are directly injected into eukaryotic cells (ExoS) by means of a specialized secretion apparatus.

From primary sequence analysis, it is possible to identify two main groups of homology (*Fig. 22.54*): the DT-like group, mainly composed of DT and PAETA, and the CT-like group comprising the remaining ADP-ribosyltransferases.

Although some homology is present among the members of the CT group, no overall significant and extended sequence similarity can be detected to justify the observed common mechanism of catalysis; nevertheless, biochemical experiments of photoaffinity labeling and studies of site-directed mutagenesis had previously demonstrated for most of the toxins that the presence of a glutamic acid is so important for catalytic activity, even a conservative substitution with an aspartate could not be tolerated without loss or drastic decrease of toxicity (Douglas and Collier 1987; Wilson et al. 1990; Lobet et al. 1991; Antoine et al. 1993).

On the basis of these experimental data and on the crystallographic structures which are now available for LT (Sixma et al. 1991), CT (Zhang et al. 1995), PT (Stein et al. 1994), DT (Choe et al. 1992), and PAETA (Allured et al. 1986), a common catalytic site could be identified which, despite the low level of sequence homology, is almost perfectly superimposable for all them (Domenighini et al. 1994).

In terms of tertiary structure, the active site is a cleft formed by a β-strand followed by a slanted α-helix that has a different length in the various toxins (spanning from 12 residues for DT, PAETA, and LT, to 21 in the case of PT). The β-strand and the α-helix represent, respectively, the lower and upper face of the cavity in which the nicotinamide ring of NAD is anchored during the enzymatic reaction (Region 2 of *Fig. 22.54*).

Although all the toxins share this similar folding in the region of the active site, at the amino acid level, the only residue which is well conserved among all the representatives of the CT and DT groups is a glutamic acid (Glu-148 of DT, Glu-553 of PAETA, Glu-112 of CT and LT, and Glu-129 of PT), which corresponds to the core of Region 3 (*Fig. 22.54*). These residues retain an equivalent spatial position and orientation residing in a short β-strand flanking the external side of the cavity (*Fig. 22.55*). With the exception of the conserved glutamate, the consensus sequence generated for Region 3 differs between the two groups of toxins. In the DT family, in fact, it is composed of the catalytic Glu followed by an aromatic and a hydrophobic residue, whereas in the CT group, the consensus can be extended to a few neighboring residues (*Fig. 22.45*). On the basis of alignment of C2-I with iota toxin and with the other ADP-ribosyltransferases, the catalytic glutamate was identified (Glu-389 of C2) and its function experimentally confirmed by site-directed mutagenesis (Barth et al. 1998). In the case of *Pseudomonas aeruginosa* Exo S, the equivalent Glu has been mapped at position 381 (Liu et al. 1996).

Another well-conserved residue is His-21 of DT that can be aligned to His-440 of PAETA, and with the conserved Arg-7 of CT and LT, and Arg-9 of PT (Burnette et al. 1988, 1991; Papini et al. 1990; Lobet et al. 1991; Han and Galloway 1995). The segment comprising this residue is termed "Region 1" (*Fig. 22.54*). These amino acids are once again located in essentially identical positions within the active site, lying opposite to the glutamic acid on an antiparallel β-strand close to the internal face of the catalytic cleft.

Although several models have been proposed to explain the possible function of the conserved histidine/arginine of Region 1, that this residue does not play a direct role in catalysis seems now widely accepted; very likely it may have a function in maintaining the integrity of the active-site pocket upon formation of structurally stabilizing hydrogen bonds (Johnson and Nicholls 1994). Nevertheless, mutations at the His-440 position of PAETA, though affecting the enzymatic activity, have little or no effect on NAD binding (Han and Galloway 1995); this suggests that His-440 may not be exactly homologous to His-21 of DT or to the arginines of the CT group. In the case of the C2-I

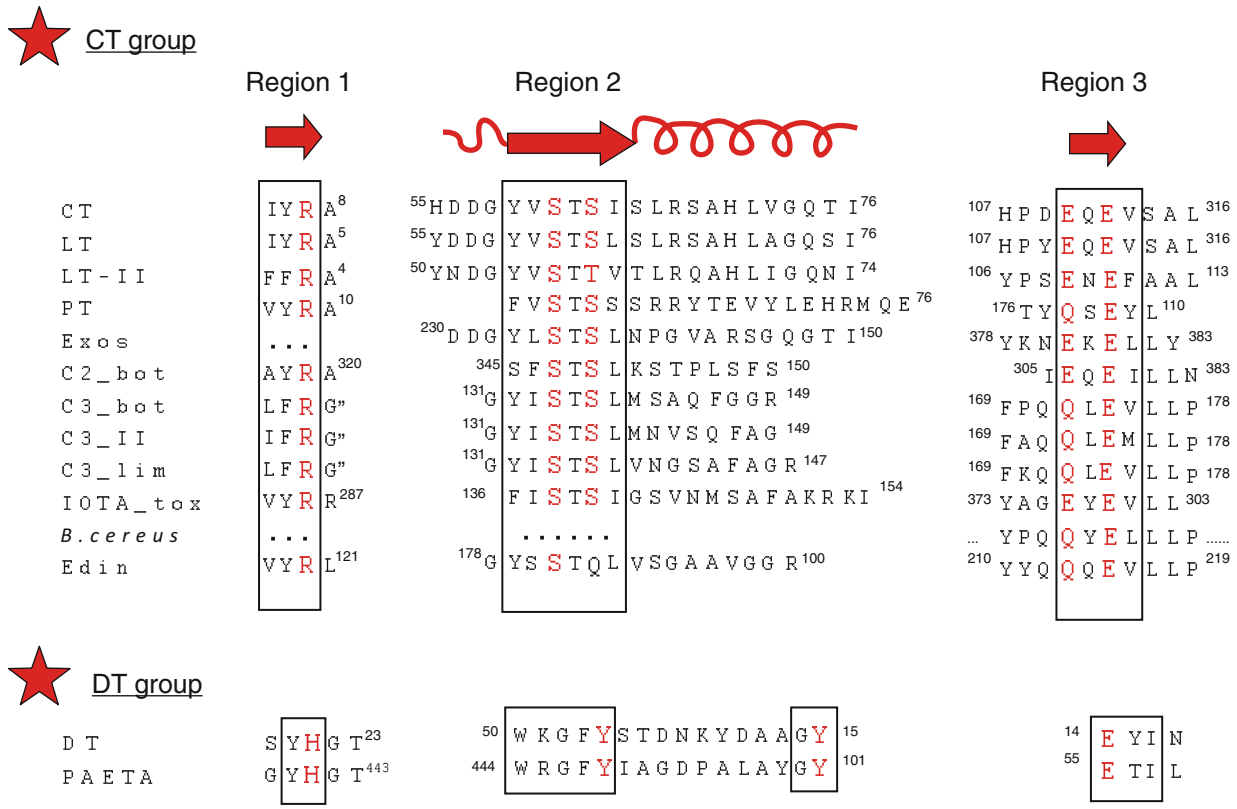

Fig. 22.54

Sequence alignment of protein segments containing Regions 1, 2, and 3 of bacterial ADP-ribosylating enzymes. The two groups of homology (DT-like and CT-like groups) are distinguished. Catalytic residues of Regions 1 and 3, and most relevant and conserved residues of Region 2 are colored in *red*; extended consensus sequences detected in the three regions are *boxed*, whereas other partially conserved residues are in *boldface*. Predicted and observed secondary structure folding is indicated for each region: Regions 1 and 3 are β-strands (*arrows*), while Region 2 is characterized by a short coil (*solid line*), followed by a β-strand and by an α-helix

component of clostridial toxin C2, site-directed mutagenesis of Arg-299 induced a dramatic reduction of transferase activity, thus suggesting an equivalent role for this residue in the conformation of the active site (Perelle et al. 1995).

Region 2 includes a number of amino acids that, while maintaining the same secondary structure in both DT and CT families (*Fig. 22.55*), result in a major sequence difference (*Fig. 22.54*). This is mainly a structural region corresponding to the core of the active-site cleft, which is devoted to the docking of NAD. The consensus sequence generated for the DT group is characterized by two conserved tyrosines spaced by ten amino acids and located on the middle portion of the β-strand and on the internal face of the α-helix, respectively. Tyr-54 and Tyr-65 of DT, and Tyr-470 and Tyr-481 of PAETA have been shown to play an important role in catalysis inasmuch as they anchor the nicotinamide ring during the reaction by creating a p pile of three aromatic rings which strengthen the overall binding of NAD and stabilize the complex (Carroll and Collier 1984; Li et al. 1995). This consensus motif can be extended to four other residues which precede the first Tyr, and to a glycine residue which is located upstream of the second Tyr.

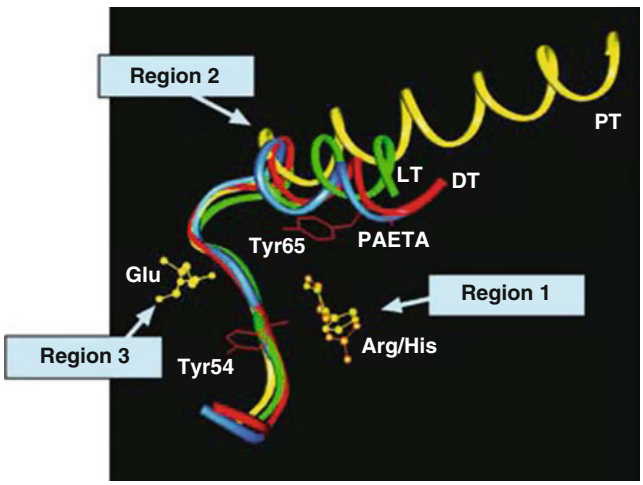

Fig. 22.55

Superimposition of the three-dimensional structures of the NAD-binding cavities (Region 2) of the bacterial toxins LT (*green*), PT (*yellow*), DT (*red*), and PAETA (*blue*). The catalytic residues carried by Region 1 (Arg/His) and by Region 3 (Glu) and common to the two regions of homology are shown. In addition, the two essential tyrosines of the DT group are colored in *red*

In PT, a similar role is likely to be played by Tyr-59 and Tyr-63, which have a similar spatial orientation and distance from each other. This observation is supported by the fact that in CT and LT, where the stacking interactions produced by the two tyrosines are lacking, the affinity for NAD is 1,000-fold lower (Galloway and van Heyningen 1987).

In the case of the CT group, Region 2 is centered on a consensus core domain characterized by the motif Ser-Thr-Ser that is observed and predicted to fold in a β-strand representing the floor of the cavity. Experiments of site-directed mutagenesis have confirmed the importance of these residues in maintaining the shape of the cavity. Substitutions of Ser-61 and Ser-63 of LT with Phe and Lys, respectively, have been shown to produce nontoxic mutants (Harford et al. 1989; Fontana et al. 1995). The core sequence of Region 2 can be extended to give the more general consensus aromatic-hydrophobic-Ser-Thr-Ser-hydrophobic.

Another amino acid that has been proposed as being important in catalysis is His-35 of PT (Xu et al. 1994) located near the beginning of the β-strand which forms the floor of the cavity, in a position equivalent to that of His-44 of LT and CT; a functional homologue, His is also present in the mosquitocidal toxin SSII-1 from *Bacillus sphaericus* (Thanabalu et al. 1991) but is absent in DT and PAETA. In the 3D structure, this residue appears to be sufficiently close to the oxygen atom of the ribose ring of NAD to interact with it and increase the electrophilicity of the adjacent anomeric carbon atom. The absence of an equivalent residue in DT and PAETA again supports the idea that the two groups of toxins perform the same enzymatic activity in a slightly different fashion.

An additional feature that is common to all ADP-ribosylating toxins is the need for a conformational rearrangement to achieve enzymatic activity.

In the native structure, in fact, the NAD-binding site of LT and CT is obstructed by a loop (amino acids 47–56) that needs to be displaced to obtain a functional NAD-binding cavity. A functionally homologous region is also present in PT where the loop comprises residues 199–207. In the case of DT, where the crystallographic data of the complex are available, the observation that the active-site loop consisting of amino acids 39–46 changes structure upon NAD binding, suggests that these residues may be important for the recognition of the ADP-ribose acceptor substrate, EF-2 (Weiss et al. 1995; Bell and Eisenberg 1996).

The recent publication of the crystallographic data of the DT-NAD complex, and the presence of common features within all ADP-ribosylating toxins, permits speculation on a possible common mechanism of catalysis (*Fig. 22.56*). The best hypothesis is that NAD enters the cavity, which is then made available for the recognition of the substrate, upon displacement of the mobile loop. Then, NAD docks at the bottom of the pocket where a small residue (the conserved serine in Region 2 of the CT group, the threonine-56 of DT, and the alanine-472 of PAETA) is required to allow good positioning. The nicotinamide moiety of NAD is then blocked in a suitable position by means of stacking interactions provided by a couple of aromatic rings

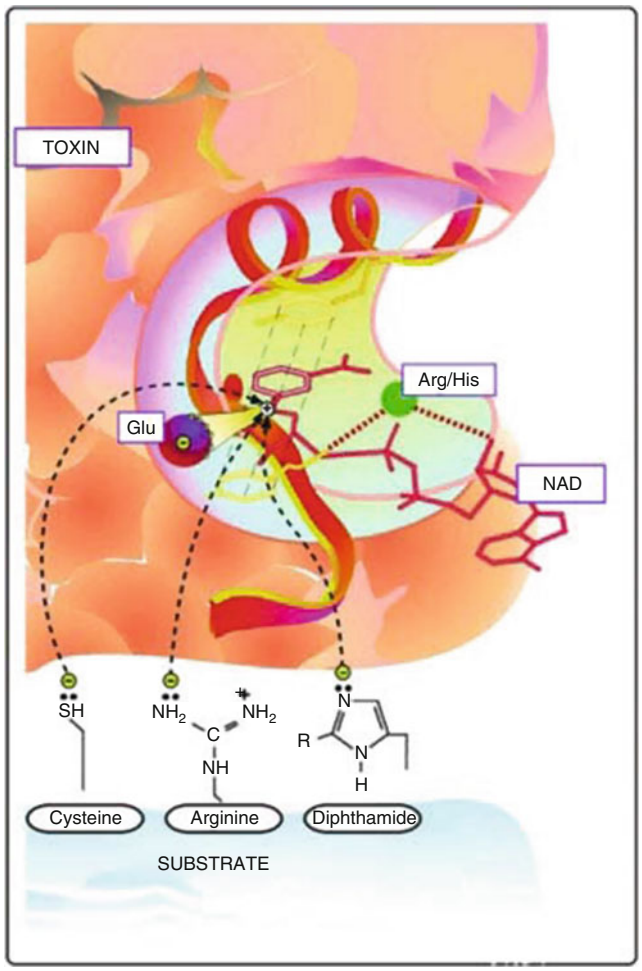

Fig. 22.56
Schematic representation of a possible common mechanism of catalysis: the nicotinamide adenine dinucleotide (NAD) molecule (*red*) is docked inside the cavity by means of stacking interactions provided by the two aromatic rings (*yellow*) that protrude from the scaffold of Region 2. The catalytic glutamic acid (*purple*) and its possible interactions with the acceptor residues of the various substrates are also reported. The Arg/His residue (*green*) provides stabilizing interactions with the backbone of the cavity and seems to be also responsible for the correct positioning of NAD inside the pocket

(Tyr-54 and Tyr-65 of DT, Tyr-470 and Tyr-481 of PAETA, and possibly, Tyr-59 and Tyr-63 of PT). In this context, the conserved arginine/histidine might display its key role in maintaining the correct shape of the active-site pocket via hydrogen bonds formed with the backbone of the structure and possibly one with the ribose moiety. The enzymatic reaction is then catalyzed by the essential glutamic acid, which is likely to stabilize a positively charged oxocarbonium intermediate of NAD, to favor its subsequent interaction with the nucleophilic residue of the incoming substrate (diphthamide in the case of DT and PAETA, arginine in the case of LT and CT, and cysteine in the case of PT).

Novel ADP-Ribosylating Toxins Detected by Genome Mining

With the advent of the Genomic Era, identification of bacterial factors possibly involved in virulence is an easier challenge. In fact, given the vast amount of information that we now possess on toxins—including sequence data—and thanks to the growing number of sequenced bacterial genomes, it is possible to proceed by homology criteria to predict novel members of important classes of bacterial toxins.

Several examples exist where computer-based methodologies have been instrumental to the identification of novel potential bacterial toxins in sequenced genomes. Among them, we will mention here the case of mono-ADP-ribosyltransferases.

Mono-ADP-ribosyltransferases (mADPRTs) constitute a class of potent toxins in bacteria, which generally play an important role in the pathogenesis of related microorganisms. Despite the poor overall conservation at the primary structure level, the catalytic subunits of these toxins show a remarkable similarity within the enzymatic cavity, so that these portions of the proteins are quite well conserved.

For these reasons, and encouraged by the availability of a growing number of sequenced bacterial genomes, a series of studies have been directed toward the computer-based identification of novel members of this family of enzymes by means of sequence-homology criteria in finished and unfinished genome sequences. As a result, more than 20 novel putative ADP-ribosyltransferases have been identified both in gram-positive and gram-negative organisms, including five from *Pseudomonas syringae*, five from *Burkholderia cepacia*, two from *Enterococcus faecalis*, and one each from *Salmonella typhi*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, *Streptomyces coelicolor*, *Bacillus halodurans*, and *Vibrio parahaemolyticus* (Pallen et al. 2001). With the exception of the protein detected in *Salmonella*, which is adjacent to an ORF protein similar to the S2 subunit of pertussis toxin, all the other genome-derived putative ADPRTs lack a predicted translocation domain. So far, none of these bacterial proteins has been tested either for their ADP-ribosyltransferase activity or for the capability of entering eukaryotic cells; however, sequence data indicate a possible role of these proteins in the pathogenesis of the corresponding microorganisms. Very recently, a new protein has been added to the list of ADP-ribosyltransferases detected by computer analysis (Masignani et al. 2003). This novel factor has been identified by means of primary and secondary structure analysis in the genomic sequence of a virulent isolate of *Neisseria meningitidis* and has been named "NarE" (*Neisseria* ADP-ribosylating enzyme). As predicted by "in silico" studies, biochemical analysis has demonstrated that NarE is capable of transferring an ADP-ribose moiety to a synthetic substrate.

Toxins with Unknown Mechanism of Action

See ❯ *Tables 22.1* and ❯ *22.2* for a summary of the principal features of toxins described in this section.

The zonula occludens toxin (Zot) is produced by bacteriophages present in toxinogenic strains of *Vibrio cholerae*. Zot is a single polypeptide chain of 44.8 kDa, which localizes in the outer membranes. After internal cleavage, a carboxy-terminal fragment of 12 kDa is excreted, and this is probably responsible for the biologic effect. Zot has the ability to reversibly alter the tight junctions of intestinal epithelium, thus facilitating the passage of macromolecules through mucosal barriers (Di Pierro et al. 2001). Zot has also been shown to act as mucosal adjuvant and to induce protective immune response in the animal model (Marinaro et al. 2003).

Hemolysin BL (HBL) is an enterotoxin produced by *B. cereus*, which is composed of three proteins (B, L1, and L2), each with a molecular mass of 40 kDa, and whose corresponding genes are located on the same operon. HBL has hemolytic as well as dermonecrotic and vascular permeability activities and is able to cause fluid accumulation in ligated rabbit ileal loops (Beecher and Wong 1997, 2000).

The bile salt hydrolase (BSH) is a protein elaborated by *Listeria monocytogenes*, which is absent from the genome of the nonpathogenic *L. innocua*. The *bsh* gene encodes an intracellular enzyme and is positively regulated by PrfA, the transcriptional activator of known *L. monocytogenes* virulence genes (Dussurget et al. 2002). Furthermore, *bsh* deletion mutants show reduced virulence and liver colonization, thus demonstrating that BSH is a toxin specifically involved in the intestinal and hepatic phases of listeriosis.

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