Bacillus anthracis and Anthrax

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Edited by

Nicholas H. Bergman

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This book is dedicated to the scientists throughout the world who have devoted their careers to studying Bacillus anthracis *and other biothreat agents. Their commitment to understanding the biology of these organisms in the face of technical, financial, and political obstacles has given us the foundations of biodefense, and for this, society owes them its deep gratitude.*

Contents

Preface

Looking back over the past decade, it is clear that our understanding of *Bacillus anthracis* and anthrax has improved dramatically in recent years. Much of this is due to technical advances that were beneficial to microbiology in a very broad sense. Genome sequencing, improved animal models, and efficient methods for genetic manipulation, for instance, have made it possible to address questions in microbiology that were inaccessible only a few years ago. In addition, the attention given to *B. anthracis* because of its potential as a bioterror weapon has brought both more funding and more researchers to the study of anthrax, and this has also played a big role in accelerating research in this field.

Although the rapid progress made in anthrax research over the past few years has certainly been welcomed by the research and public health communities, it has also meant that in many areas of *B. anthracis* biology and pathogenesis, our knowledge extends well beyond what is reported in previous reference volumes. In developing this book, my aim was to address this issue, and to bring together a collection of reviews that would provide scientists and health professionals with a current and comprehensive reference on both *B. anthracis* and anthrax.

I am extremely grateful to the authors who contributed to the book—they represent some of the most accomplished researchers in the anthrax field, and their expertise and effort is clear in the chapters they have written. I am also grateful to the editorial staff at John Wiley & Sons for their advice and support, and specifically to Karen Chambers for her help in the earliest stages of this book's conception. Finally, I thank my friend and colleague Dr. Karla Passalacqua, for the many useful discussions as this book was being planned.

Contributors

Joel A. Bozue
Bacteriology Division
United States Army Medical Research Institute of Infectious Diseases

Katherine A. Carr
Department of Microbiology and Immunology
University of Michigan Medical Center

Leonard A. Cole
Division of Global Affairs
Rutgers University
Center for Law and Justice

Christopher K. Cote
Bacteriology Division
United States Army Medical Research Institute of Infectious Diseases

Carlos del Rio
Department of Pathology and Laboratory Medicine
Emory University School of Medicine

Adam Driks
Department of Microbiology and Immunology
Loyola University Medical Center

Nathan Fisher
United States Army Medical Research Institute of Infectious Diseases

Jonathan D. Giebel
Department of Microbiology and Immunology
University of Michigan Medical Center

Ian J. Glomski
Department of Microbiology
University of Virginia

Jeannette Guarner
Department of Pathology and Laboratory Medicine
Emory University School of Medicine

Philip C. Hanna
Department of Microbiology and Immunology
University of Michigan Medical Center

Brian K. Janes
Department of Microbiology and Immunology
University of Michigan Medical Center

Theresa M. Koehler
Department of Microbiology and Molecular Genetics
University of Texas–Houston Health Science Center

Stephen H. Leppla
Bacterial Toxins and Therapeutics Section
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Michael Mallozzi
Department of Microbiology and Immunology
Loyola University Medical Center

Mahtab Moayeri
Bacterial Toxins and Therapeutics Section
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Gleb Pishchany
Department of Microbiology and Immunology
Vanderbilt University Medical School

Anne Quesnel-Hellmann
Groupe interactions hôte-agent pathogène
Département de biologie des agents transmissibles
Centre de Recherches du Service de Santé des Armées

Conrad P. Quinn
National Center for Immunization and Respiratory Diseases
Division of Bacterial Diseases
Meningitis and Vaccine Preventable Diseases Branch
Microbial Pathogenesis and Immune Response Laboratory
Centers for Disease Control and Prevention

Jason M. Rall
Department of Microbiology and Molecular Genetics
University of Texas-Houston Health Science Center

David A. Rasko
Department of Microbiology and Immunology
University of Maryland School of Medicine
Institute for Genome Sciences

Timothy D. Read
Division of Infectious Diseases
Department of Human Genetics
Emory University School of Medicine

Elke Saile
National Center for Immunization and Respiratory Diseases
Division of Bacterial Diseases
Meningitis and Vaccine Preventable Diseases Branch
Microbial Pathogenesis and Immune Response Laboratory
Centers for Disease Control and Prevention

Sean V. Shadomy
Bacterial Zoonoses Branch
Centers for Disease Control

Eric P. Skaar
Department of Microbiology and Immunology
Vanderbilt University Medical School

Scott Stibitz
Division of Bacterial, Parasitic and Allergenic Products
Center for Biologics Evaluation and Research
Food and Drug Administration

Jean-Nicolas Tournier
Groupe interactions hôte-agent pathogène
Département de biologie des agents transmissibles
Centre de Recherches du Service de Santé des Armées

Peter C.B. Turnbull
Salisbury, UK

Susan L. Welkos
Bacteriology Division
United States Army Medical Research Institute of Infectious Diseases

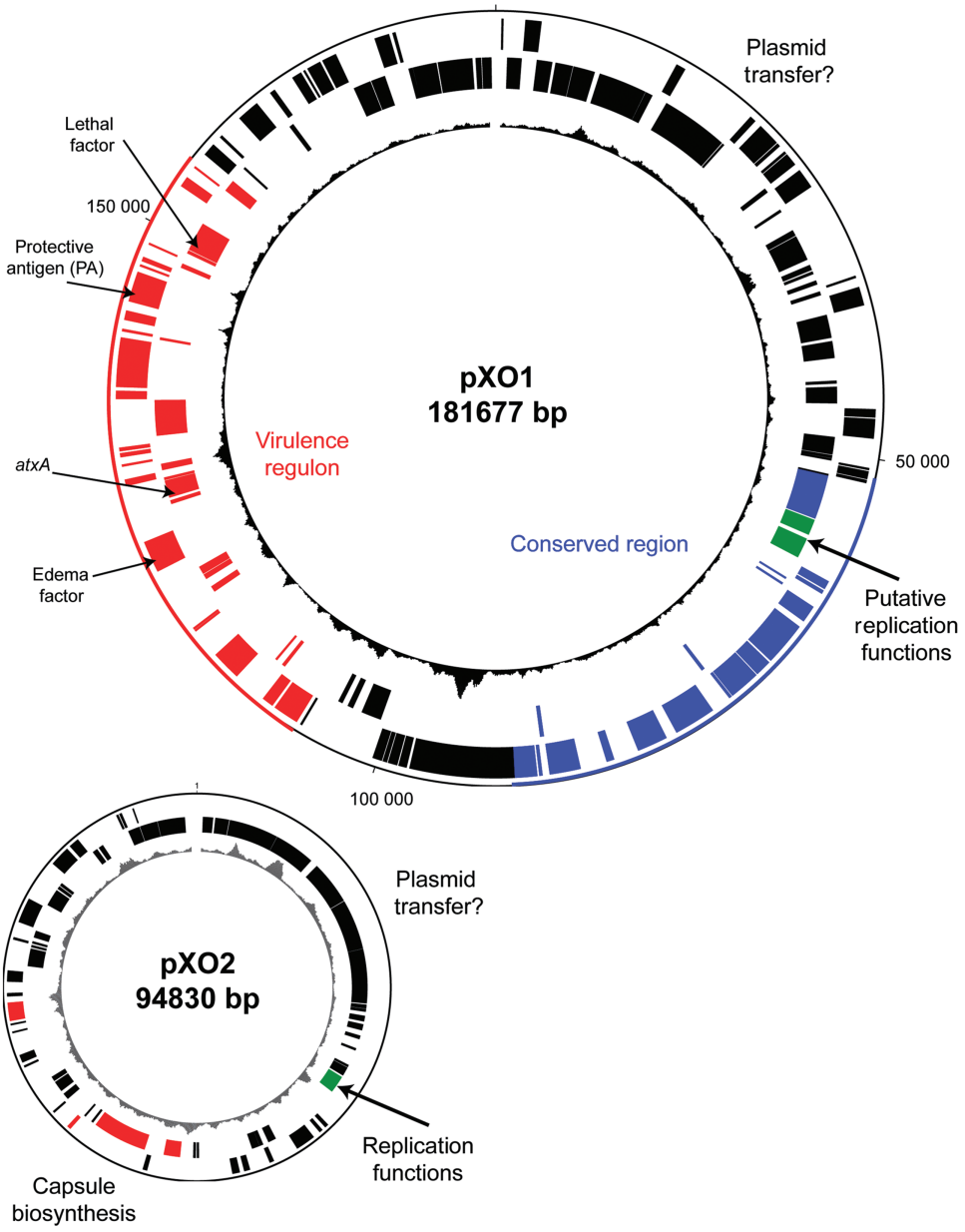


Figure 6.1 Identification of the virulence, conserved, and replication regions of the pXO1 and pXO2 plasmids. Multiple plasmid comparisons have indicated that there is a conserved region in pXO1 that is shared with many other *B. cereus* plasmids (indicated by blue). Putative replication regions are highlighted in green. Virulence related factors are highlighted in red. The regions in black are conserved in some other *B. cereus* group plasmids but not all.

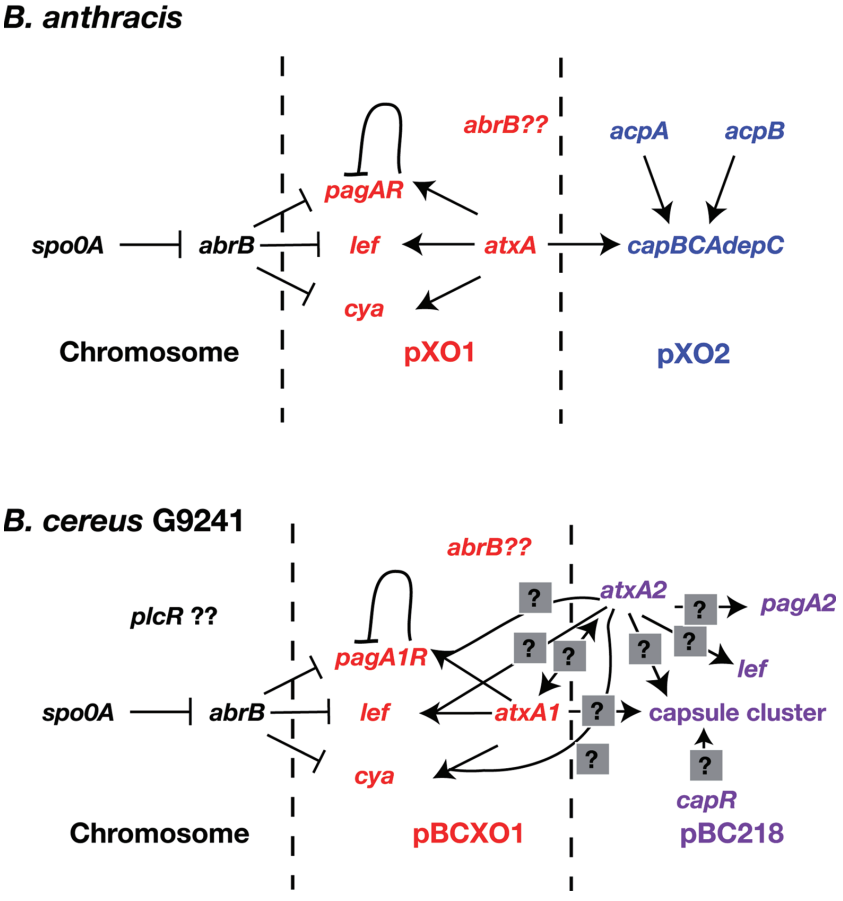
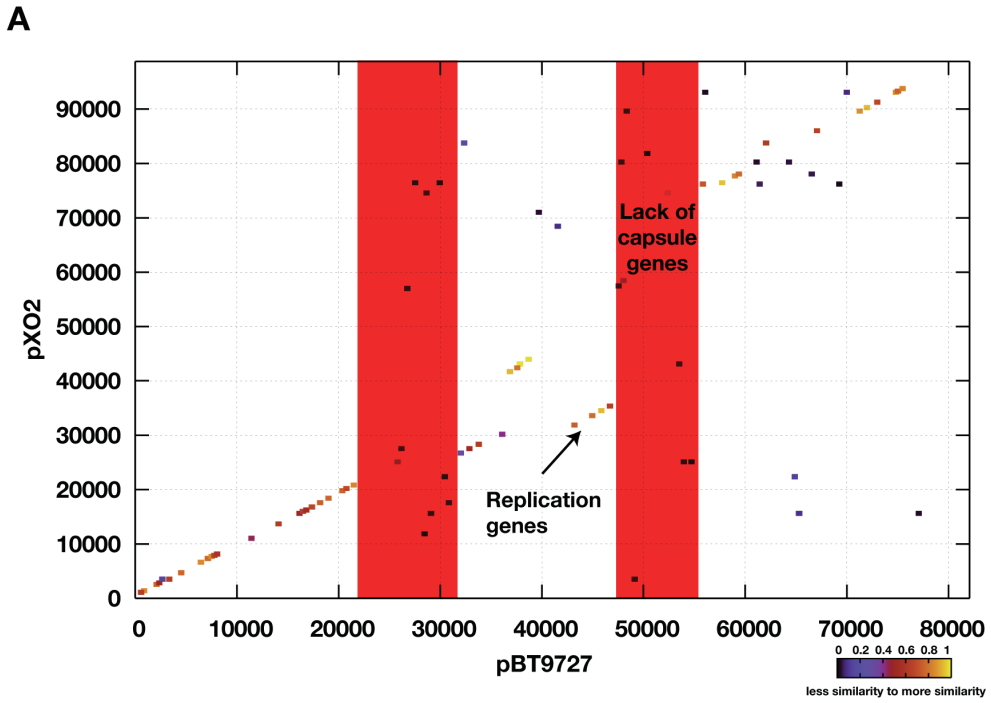


Figure 6.2 Potential cross talk between the chromosome features of *B. anthracis* and in *B. cereus* G9241. The gene features colored in red are from the pXO1 and pXO1-like plasmid; features in blue are encoded on pXO2; and features in purple are encoded on pBC210. In *B. anthracis* the AtxA peptide activates the expression of the pXO1 encoded tripartite lethal toxin as well as the capsule loci on pXO2. Additional features, AcpA and AcpB encoded on pXO2, also positively regulate the expression of the capsule. In *B. cereus* G9241 a pXO1-like plasmid is present; however, the pXO2 plasmid is absent and an alternative plasmid is present, pBC210. pBC210 encodes an additional copy of *atxA*, named *atxA2*, but it is unclear if the gene product regulates the same genes and in the same way as the pXO1 encoded AtxA. The pBC210 plasmid also encodes divergent virulence genes with unknown functional and regulatory activities.



B

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pXO2        TGGTTAATTTTTAATTGTCCACTCTGCCAATACATAGT-ATATCTACGATACGTGGTTTGG
pBT9727     TGGTTAATTTTTAATTGTCCACTGTGCCAATACATAATTATATCTACGATACATCTTTCTG
pAW63       TGGTTAATTTT-AATTGTCCACTCTGCCAATACATAATTATATCTTCGATACATGGTTAGC
pAMB        GGCTGAAAATA-AAACCCGCACTATGCCATTACAT--TTATATCTATGATACGTGTTTGTT
             * * **  *  **     **** ***** *****  * ******  ***** *  **
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Figure 6.3 Examination of the homology between pXO2-like plasmids. Panel A contains the PSR plot comparing pBT9727 and pXO2, demonstrating that the plasmids are highly conserved except for the plasmid region with encoding the capsule biosynthesis genes and one other region. Panel B is a detailed view of the nucleic acid sequence of the proposed plasmid origin in this group of pXO2-like plasmids. The gray box is the conserved origin and the arrow indicates the nick site.

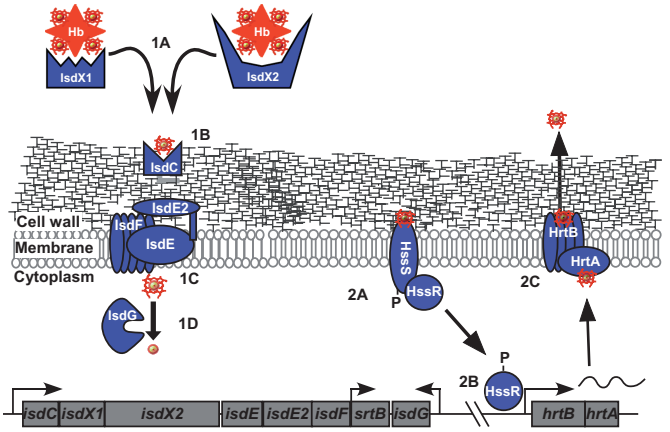

Figure 7.2 Heme homeostasis of *B. anthracis*. (See text for full caption.)

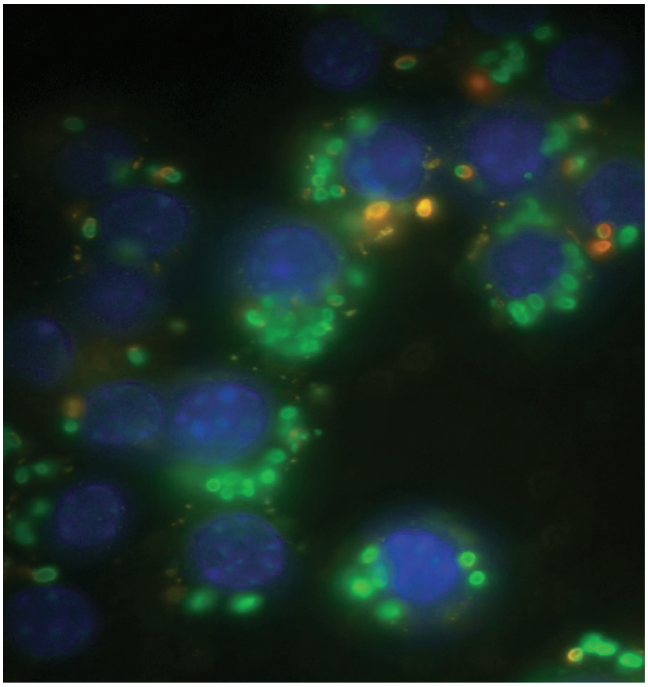

Figure 10.1 Phagocytosis of *B. anthracis* Ames strain spores by macrophages as determined by immunofluorescence microscopy. (See text for full caption.) Reprinted with permission from *Microbes and Infection*, 10, Cote, et al., Early interactions between fully virulent *Bacillus anthracis* and macrophages that influence the balance between spore clearance and development of a lethal infection, 613–619, Copyright Elsevier (2008).

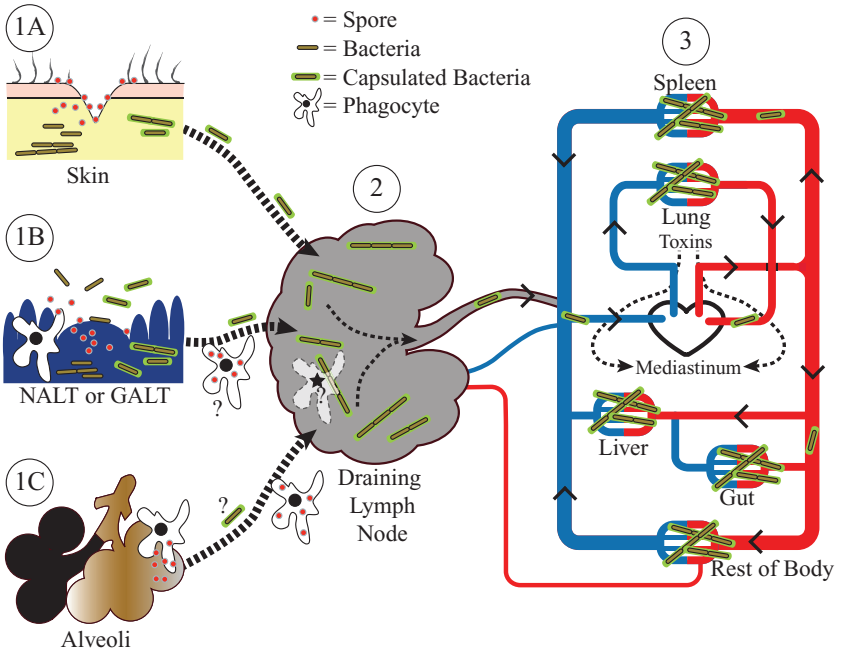

Figure 12.1 A model for *B. anthracis* dissemination through a host.

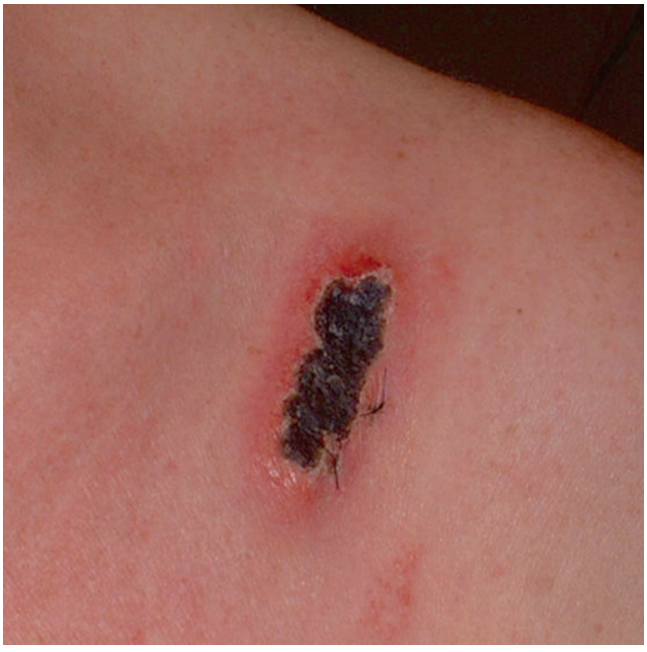

Figure 13.1 Cutaneous anthrax lesion in shoulder.

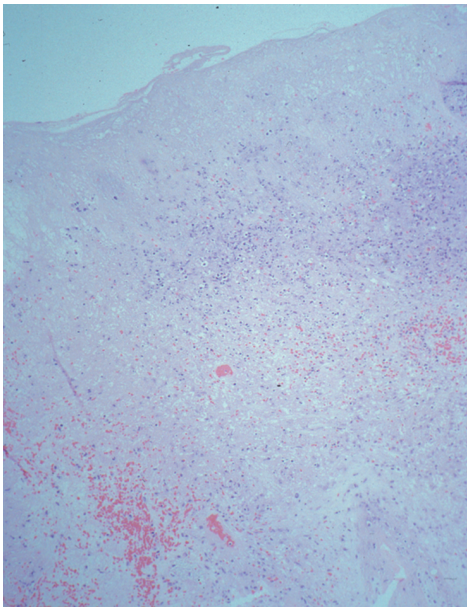

Figure 13.3 Histopathology of cutaneous anthrax showing necrosis of epidermis, inflammation in upper dermis and focal hemorrhage (hematoxylin and eosin stain, original magnification 25×).

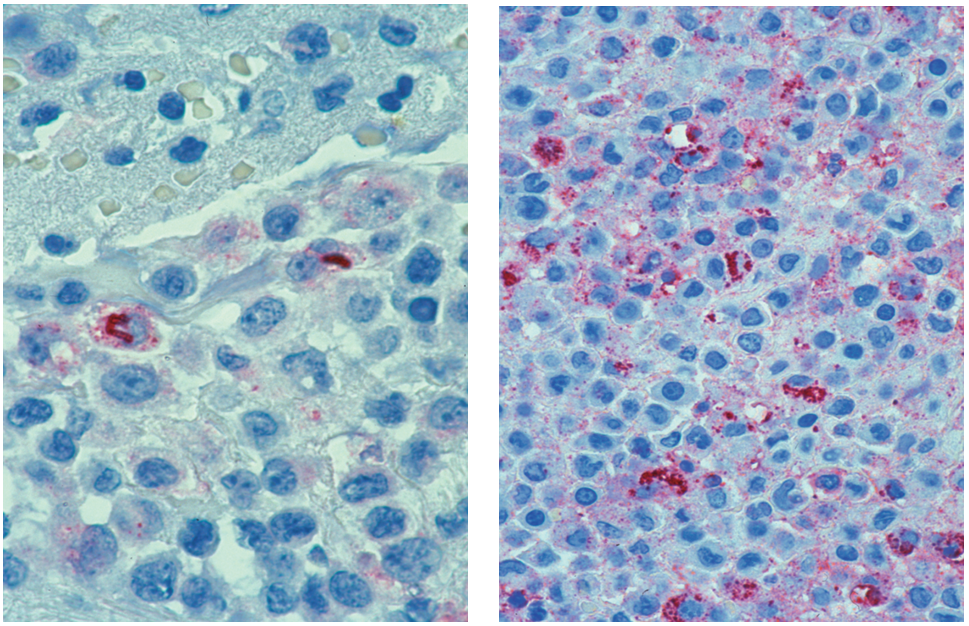

Figure 13.4 Cell block prepared from pleural fluid of a patient with inhalational anthrax showing bacilli and granular antigen staining (immunohistochemical assay using monoclonal antibodies against cell wall (a) and capsule (b), original magnifications 250× (a) and 157.5× (b).

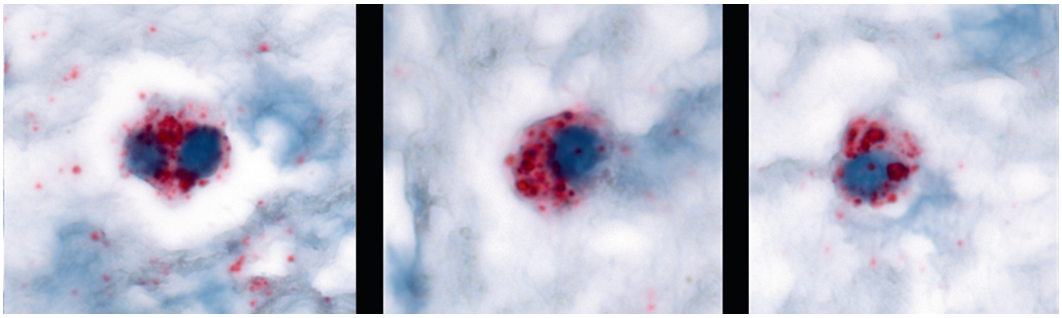

Figure 13.5 Cells in pleural fluid 10 days after initial hospitalization showing granular antigen staining for *B. anthracis* (immunohistochemical assay using a monoclonal antibody against the capsule, original magnification 250×).

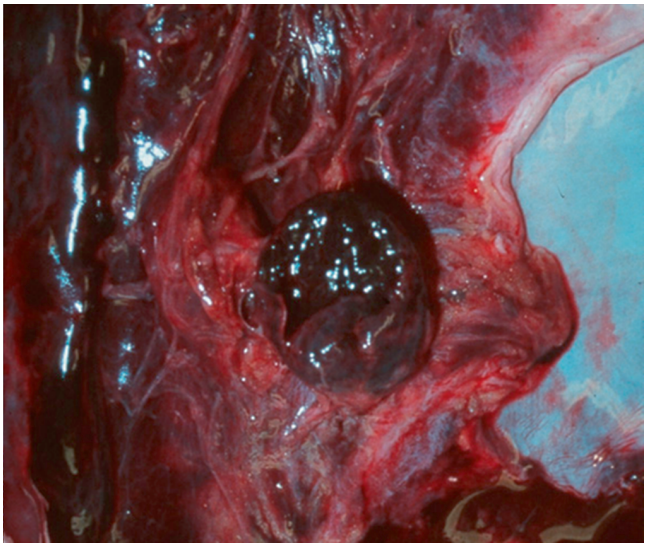

Figure 13.6 Edematous and hemorrhagic mediastinal lymph node.

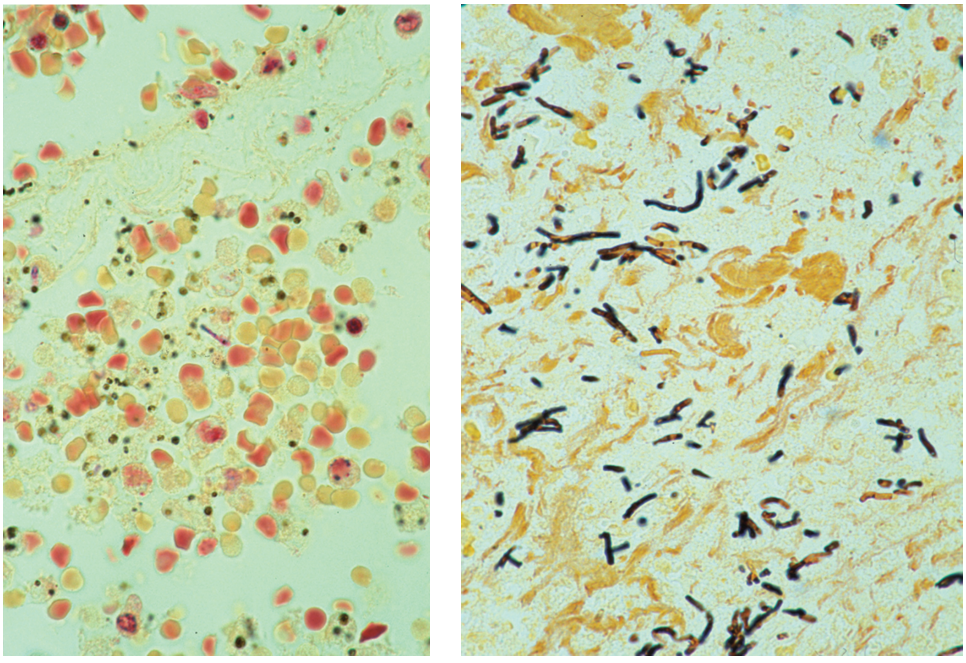

Figure 13.7 Gram stain and Steiner silver impregnation stain of same area of lymph node showing sparse gram-positive bacilli (a) and abundant silver staining organisms (b) (original magnification 250×).

Chapter 1

Anthrax from 5000 BC to AD 2010

Peter C. B. Turnbull and Sean V. Shadomy

FROM ANCIENT TIMES TO THE 19TH CENTURY

Historical Names of Anthrax

Anthrax (Latin, a carbuncle) is derived from the Greek ἀνθραξ (anthrax) meaning coal and referring to the characteristic black eschar in human cutaneous anthrax. Other older names for the disease, such as "malignant pustule" or "black bane," and names in other languages, such as charbon (French) and carbonchio (Italian), similarly reflect these features. Yet other names reflect other manifestations of the disease in humans and/or animals or its sources of infection, such as woolsorter's/ragpicker's/Bradford disease and the German equivalent Hadernkrankheit (rag disease), splenic fever, Milzbrand (German, meaning "spleen fire"), Siberian plague, Lodiana fever, and Pali plague in India, and many more. The many names in many languages reflect the historical and widespread recognition of the numerous features of anthrax before it was understood that they were all manifestations of a single etiological agent.

The earliest application of the name "anthrax" to the afflictions caused by *Bacillus anthracis* is uncertain. "Bloody murrain" was probably the most common term for the disease in animals in early English language texts, and carbuncle—or malignant carbuncle to distinguish it from other carbuncular manifestations—was the term used for the cutaneous infection in humans. From a book of 1766, Viljoen (1928) cites "Visit to your servant girl suffering from a considerable anthrax and found several furuncles on the back; cured same" but believes that "anthrax" at that time was a common term embracing any severe localized dermatitis and this was not a *B. anthracis* infection. According to Swiderski (2004), physicians attending George Washington diagnosed as "an anthrax" "a very large and painful tumor" which developed on his left thigh about 6 weeks after his inauguration as first president of the United States in 1789. However, that description and the description of "anthrax, or carbuncle" in the American edition of *The Surgeon's Vade-Mecum* (1813) similarly appear unlikely to have been *B. anthracis* infections.

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

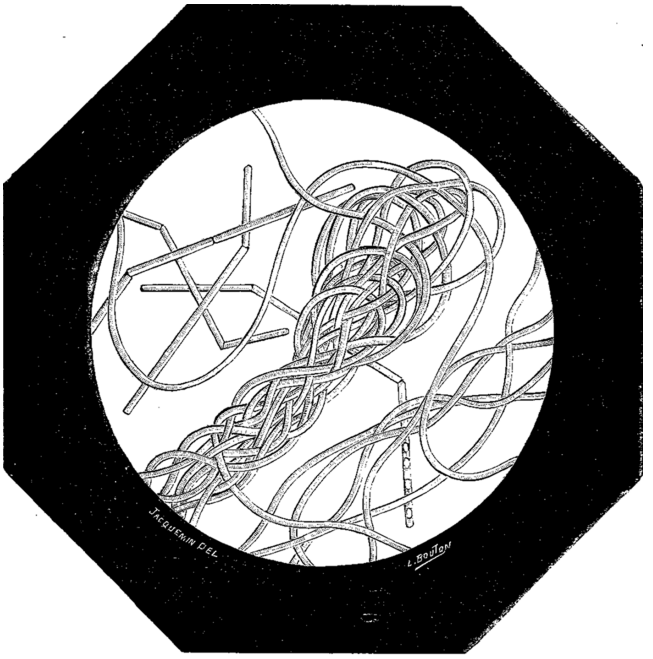

Figure 1.1 From a culture of anthrax blood in chicken broth at 24–48 h. (From Ch Chamberland's celebrated book *Charbon et Vaccination Charbonneuse d'après les travaux de Mr Pasteur*, 1883.)

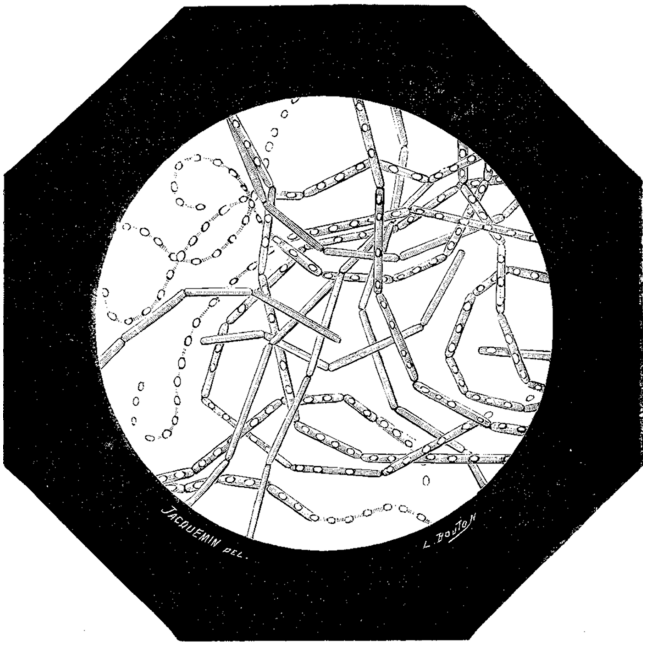

Figure 1.2 The same culture after several days, with spores now apparent. (From Ch Chamberland's celebrated book *Charbon et Vaccination Charbonneuse d'après les travaux de Mr Pasteur*, 1883.)

From Ancient Egypt to 1892

The allegedly long history of anthrax features in numerous papers and articles. Most scholarly among those readily accessible today are probably the papers of Klemm and Klemm (1959) and Blancou (2000) which, together, supply a brief but comprehensive review of earlier literature attesting to the historical familiarity with the disease through the ages. Klemm and Klemm (1959) suggest anthrax originated in early Egypt and Mesopotamia, where agriculture was established some 5000 years BC, and then address the feasibility of the frequently cited statement that the fifth plague of Egypt in the time of Moses (ca 1250 BC)—"a grievous murrain affecting cattle, horses, asses, camels, oxen and sheep"—was the earliest instance of systemic anthrax on record. As Ebedes (1981) points out, no other disease kills such a wide spectrum of species. Others also consider the sixth plague—boils breaking out in sores on man and beast—to have been cutaneous anthrax. Ebedes (1981) suggests these lesions affected only the Egyptians because only the Egyptians would have handled the carcasses of affected animals, the Israelites being forbidden to touch dead animals. Not everyone concurs with these hypotheses; Morens (2002), for example, considers the evidence that the fifth Pharaonic plague in the biblical book of Exodus was anthrax to be weak.

Klemm and Klemm (1959) and Blancou (2000) also summarize the evidence that early Greece was familiar with anthrax as depicted by Homer in his "Iliad" (ca 1230 BC), Hippocrates' writings (ca 400 BC) and the plague of Athens in 430 BC (see also Morens and Littman, 1992), Aristotle in his *History of Animals* (ca 333 BC), Plutarch (ca AD 120), and Galen (ca AD 200); that it was described in Hindu literature of around 500 BC and that, based on the writings of Livius (ca 460 BC), Virgil (70 BC–90 BC), and Vegetius (ca AD 400), the Romans were well acquainted with it. Dirckx (1981) had no doubt that the Norican plague (now Bavaria) in Virgil's third Georgic (ca 32 BC) was anthrax. Klemm and Klemm (1959) even suggest that anthrax may have contributed to events that led to the fall of Rome. According to Dong (1990), anthrax has featured in Chinese animal husbandry for millennia, being especially well described in the Jin and Sui dynasties (AD 500–600) by Ge Hong in his *Handbook of Prescriptions* and Ch'ao Yaun Fang in the *General Treatise on the Etiology and Symptomatology of Diseases*.

Klemm and Klemm (1959) believe that records of the occurrence of anthrax in post-Roman Europe begin with references to what is likely to have been this disease in the *Hippiatrika* (horse medicine), a tenth-century collection of veterinary writings, and the eleventh century *The Medicine of Quadrupeds* Blancou (2000) summarizes records of the death of a clan chief in Ireland in 1030 from what may well have been anthrax; of Arab authors in the twelfth and thirteenth centuries describing anthrax-like signs in cattle; the description in 1250 by German Emperor Frederick II's chief veterinarian of what appears to have been anthrax in horses; a description by one Pietro di Crescenzi (Italy) of what is thought to have been anthrax in sheep in the late 1200s; and further descriptions of probable anthrax in animals and humans in 1316, 1523, 1673, and 1745. D. E. Salmon (after whom *Salmonella* species were named) informs us that "anthrax was frequently confounded with the rinderpest, but is described with sufficient precision to identify outbreaks of it in epizoötic form in 996 A.D. and 1090 in France; in 1552 at Lucca, Italy; in 1617 at Naples, where numbers of human beings died from eating the flesh of animals affected with the disease" (Salmon, 1896). He further documents references to the disease in animals in Venice in 1598, extensive outbreaks in Germany, Hungary, and Poland in 1709–1714, its extensive spread in the early 1800s in Russia, Holland, and England, and again in Russia during the mid-1800s when, in 1864, "more than 10,000 horses and nearly

1000 persons perished from the disease." In Novogrod (Russia), between 1867 and 1870, 528 people and 56,000 cattle died of anthrax (Klemm and Klemm, 1959; Koch, 1877). Some 60,000 people were reported to have died in the 1617 Naples epidemic, and 15,000 people allegedly died from anthrax in San Domingo (now Haiti) within 6 weeks in 1770 (Higgins, 1916; Morens, 2002).

In America, anthrax was first introduced into Louisiana at the time of French settlement in the early 1700s. Evidence is that it had spread to Kentucky by 1819 and to Philadelphia by 1836 reaching New York, New England, and California in the second half of the1800s (Hanson, 1959; Klemm and Klemm, 1959). The appearance in 1819 of the disease in horses, cows, sheep, and humans in contact with infected animals was clearly documented by Kentucky physician Dr. J. Kercheval in 1824 (Hanson, 1959). Purdom (1954), presumably unacquainted with Kercheval's report, claimed it was the "unenviable distinction" of Philadelphia to be the first city in the United States in which a recorded case of human anthrax was described in 1834 following skinning of cattle that had died of the disease. More human cases were reported in Louisiana in 1830, with further reports over "the next several years" of the disease in animals and humans in Texas, Wisconsin, New York, Mississippi, Vermont, Massachusetts, and California (Brachman, 1965).

In Africa, recently described as the "cradle of anthrax" (Smith et al., 1999), explorer Dr. Andrew Smith described the clinical form of anthrax in man and domestic animals in 1836, naming the disease "bloodzichte" or "quatsie." That it was already endemic before the arrival of Europeans is clear from missionary Robert Moffat's description in 1842 of what appears to have been anthrax as "… endemial, which assumes the form of a carbuncle, and carries off many cattle, and as the natives will on no account abstain from eating the dead meat … always accompanied by considerable swelling attended with great stupor, though … little pain." The "horsesickness" described by famous explorer David Livingstone in 1857, also observed in zebras, was almost certainly anthrax (De Vos and Turnbull, 2004; Viljoen, 1928). It was clearly well recognized as a livestock disease by the 1870s (Gilfoyle, 2006) although still being confused with other diseases (Viljoen, 1928).

Clinical (as opposed to historical) descriptions of the cutaneous disease in humans were first given by Maret in 1752 and by Fournier in 1769, and Chabert gave a clear description of the disease in animals in 1780 (Wilson and Miles, 1964). Fournier recognized that anthrax could be transmitted to humans through the handling of animal hair and wools (Martin, 1975).

Order of Events in the Nineteenth Century

The causative agent of anthrax was established in the nineteenth century, but there are some discrepancies in the more readily accessible historical reviews as to the precise order of events and the appropriate credits. The reports and events in the first 75 years of the nineteenth century would clearly benefit from being carefully revisited by a proficient scholar with appropriate linguistic skills in at least French and German as well as English. Wilson and Miles (1964) and Blancou (2000) credit Barthélémy in 1823 as the first to demonstrate transmissibility by injecting a horse and a sheep with blood from a horse that had died of anthrax, this being repeated by Leuret in 1824. Contenders for being the first to associate anthrax with the presence of rod-shaped bodies in the blood of animals that had died from the disease are Brauell, Pollender, Davaine, and Rayer between 1855 and 1859, with Delafond apparently being the first to call these bacteria in 1860. Cohn (1872)

believed these to belong to the spore-forming *Bacillus* group and accordingly named them *B. anthracis*.

Davaine, in a series of papers in 1863–1864, showed how anthrax could affect a range of species, demonstrating that the disease could be transmitted to sheep, horses, cattle, guinea pigs, and mice by the subcutaneous inoculation of infected but not of normal blood. Finding this same bacillus in a malignant pustule in 1864, Davaine and Raimbert established the etiological connection between the disease in humans and animals for the first time (Wilson and Miles, 1964). Demonstration that infectivity was lost on passage of infective fluid through a clay filter was attributed to Tiegel and Klebs in 1864 by Wilson and Miles (1964), and to Davaine in 1873 by Klemm and Klemm. The first recorded observation of the intestinal form of anthrax was made by Wahl in 1861 and the first to recognize that woolsorter's disease was the inhalational manifestation of the disease was Bell in Bradford, England, in 1879 (Martin, 1975).

Generally undisputed is that the final proof of the bacterial cause was established by Robert Koch (1877), who detailed the sporulating characteristics of *B. anthracis*, the ability of the spores to survive long periods *in vitro* and to reproduce the disease after such periods when injected into animals. While giving credit to Koch for the observation of spore formation in the anthrax bacillus, Louis Pasteur pointed out in a communication to Koch in 1883 that 7 years previously he had noted the phenomenon in the microbe causing silkworm disease (might this have been *B. thuringiensis*?) and in consequence claimed priority for the discovery of this process (Mason, 1937). Certainly, Pasteur was working on anthrax in 1877 using his fermentation techniques to culture the bacterium from the blood and reproducing the disease with such a culture.

The observation by Koch in the 1870s that a piece of spleen from a mouse that had died of anthrax inserted under the skin on the back of a frog induced phagocytosis of the anthrax bacilli by frog leucocytes led to Metchnikoff's remarkable studies in the early 1880s on phagocytosis and what became known as opsonization in the blood or lymph of immunized animals (Metchnikoff, 1884). Metchnikoff also laid the foundation for studies on the pathogenesis of anthrax with early explanations for differing resistance in various species and apparently being the first to observe the capsule of *B. anthracis*—although he did not relate the capsule to resistance to phagocytosis.

Weichselbaum noted the methylene blue staining characteristic of the capsule in 1892 (M'Fadyean, 1904). M'Fadyean (1903) then immortalized this in the simple methylene blue capsule staining procedure which became, and remains today, the primary rapid diagnostic test for animals suspected of having died of anthrax.

Correct Diagnoses?

The many authors giving a brief history of anthrax in an introduction to a paper on some other aspect of the disease rarely raise the question as to whether the historical references were really anthrax. In the terminology prior to about 1850 for example, murraine (from Latin *morire*, to die), peste, and plague for animals, or peste, plague, and charbon for humans, were nonspecific and were applicable to other diseases such as rinderpest in animals, or *Yersinia pestis* plague, and smallpox in humans. In general, however, descriptions of sudden death in animals, frequently with more than one species involved, accompanied by hemorrhage from the orifices, gelatinous edema, and swollen lymph nodes or, in the case of humans, typical cutaneous lesions or deaths associated with animals that have died with the correct symptoms, can reasonably be accepted as having been anthrax.

Pasteur and the First Anthrax Vaccines

It is well-known that one of the most colorful parts of the history of anthrax is the story of Louis Pasteur's animal anthrax vaccine. This story, and how Pasteur's first report of successful protection of animals against deliberately induced disease in 1881 was actually preceded by the reports in 1880 of Greenfield in England and Chauveau and Touissant in France, has been covered in detail by this author elsewhere (Turnbull, 2010).

TWENTIETH-CENTURY ANTHRAX

Global Incidence

A wealth of information on the understanding of anthrax current at the outset of the 1900s lies in the three-volume (U.K.) Report of Departmental Committee on Anthrax (Report of the Departmental Committee, 1918). From this it is clear that, while animal and human case rates were beginning to be well recorded in the early years of the twentieth century in industrial countries such as Britain, Germany, and the United States, figures on the incidence of the disease in endemic countries were unavailable, despite efforts at determining these through both the British consuls and buyers of animal products in such countries, many of which were part of the then British Empire. It was well recognized, however, that, well into the century, anthrax was widely prevalent in many parts of eastern and southern Europe, central Asia, India, and much of Africa at least. For many of the countries in these regions, the knowledge was based on the high proportions of contaminated consignments among imports. Occasionally, figures are cited, some dramatic, such as >26,000 and >43,000 deaths among animals in Russia in 1913 and 1914, respectively (Eurich and Hewlett, 1930; Report of the Departmental Committee, 1918), 13,000 human cases, again in Russia, in 1924 (Pijper, 1926), and more than a million sheep dying of anthrax in Iran in 1945 (Report of the Committee of Inquiry on Anthrax, 1959).

Some of the best available data of the early 1900s are those for South Africa (Viljoen, 1928), where it rose from a disease of relative insignificance in 1905 to the most serious of the scheduled contagious livestock diseases by 1923. Viljoen (1928) attributes this largely to increased movement of animals and animal products with developing railway and road networks, the increase in stock ownership, careless owners, and failure of veterinary supervision to keep pace with these movements.

The association between the incidence in livestock in industrialized northern countries and importation of animal products became particularly apparent at the time of the two world wars. In Germany, 7181 cases in 1914 compared with just 743 in 1919 and 699 outbreaks in 1939 in the United Kingdom had fallen to 95 by 1946, rising again to 407 in 1951 and 1245 in 1956 with the reestablishment of importation of bone, meat, and blood meals (Wilson and Miles, 1964).

The First 30 Years

Isolation and Identification

The polychrome methylene blue capsule stain diagnostic test of M'Fadyean (1903) already referred to above, though simple, was probably the first highly significant milestone of the twentieth century. The role of the capsule in the virulence of anthrax came to light in the

early part of the century with Morihana in 1921 reporting the loss and reestablishment of virulence by cultures induced to lose and regain their capsules (Eurich and Hewlett, 1930).

Eurich and Hewlett's (1930) review of the literature at that time shows that it was also replete with a miscellany of seemingly bizarre observations. Papers in the 1920s variously reported "large spherical and lemon-shaped elements" in cultures on gelatin and glycerin agar, bent and hook-shaped bacilli in fish liver broth cultures, gram-negative coccobacillary forms, capsules of unusual size around bacilli grown on "brain agar" at 42–43ºC, and formation of gonidia in the filaments of *B. anthracis* cells grown in broth cultures. Stable asporogenous mutants and rough and smooth colony types were also described, but it is hard to know if and how these corresponded to equivalents produced or recognized today.

Research at this time also focused on methods of isolation from both animal and environmental specimens and the best techniques for correct identification. The logic behind methods utilized today to isolate *B. anthracis* from environmental samples heavily loaded with other environmental *Bacillus* species is little changed from that in the 1920s, and it was already being appreciated that conventional biochemical and serological identification tests were unable to differentiate *B. anthracis* from closely related "anthracoid" *Bacillus* species. In the terminology of the time, "Serological races such as exist among pneumococci and meningococci" could not be identified. The one important serological test of the time, Ascoli's precipitin reaction of 1911 for determining whether animal products derived from animals that had died of anthrax, depended not on the specificity of the antiserum for *B. anthracis* but rather on the fact that *Bacillus* antigen in animal tissues could only be from *B. anthracis*. Seemingly, this test is still used in certain eastern European countries (WHO, 2008).

Immunity, Therapy, and Prophylaxis

It was well recognized at the outset of the 1900s that early diagnosis of cutaneous anthrax in humans was essential for successful treatment, which took the form of thermocautery, chemical caustics, surgical incision or excision, wet antiseptic dressings, or injection of antiseptic solutions, and which were increasingly supported and eventually wholly replaced by the administration of antiserum as the other treatments fell into disrepute as actually being dangerous in addition to being extremely painful and disfiguring (Regan, 1921; Report of the Departmental Committee, 1918). Regan (1921) gives the credit for the original production of suitable antiserum to Marchoux in France and Sclavo in Italy, both in 1895. It was equally well recognized that there was no means of diagnosing internal anthrax in the early stages, and claims of instances of recovery could not be supported by laboratory evidence; death was regarded as the almost invariable outcome. Joint problems, arthritis, and skin eruptions were adverse effects sometimes seen with the use of antiserum, but it was regarded as "not dangerous to life."

Natural immunity was also a topic of interest at this time. The existence of bactericidal ("anthracocidal") activities in the sera of some species were evidently known to Metchnikoff, Roux, and Marchoux in the 1890s but were subsequently shown not to be directly related to the relative susceptibility or resistance of a particular species to infection (Kolmer et al., 1920). Keller (cited without reference by Eurich and Hewlett, 1930) stated that in humans, anthracocidal activity appears in the serum in the seventh to ninth week of life. Penna et al. (1917) reported successful treatment of a large series of human anthrax cases by subcutaneous injection of normal bovine serum, and Zehetmayr (1922; cited by Eurich and Hewlett, 1930) later demonstrated that a subcutaneous injection of normal ox

serum exercised a protective effect against an injection of anthrax bacilli at the same site but not against bacilli injected at a distant site unless very large doses of serum are given. However, the efficacy of normal animal serum therapy could not be confirmed and was questioned by other investigators (Pijper, 1926; Regan, 1921).

While it was recognized that early diagnosis of signs of cutaneous anthrax in at-risk occupations was important for reducing mortality rates, it was admitted that it was impossible to diagnose internal anthrax before death, although it was believed that monitoring the opsonic index might be of value here (Report of the Departmental Committee, 1918).

As covered in a separate chapter on anthrax vaccine history (Turnbull, 2010), the possibility that acquired immunity to anthrax was the result of antibodies to a toxic "aggressin" dates back to 1889–1890. The aggressin and "protective antigen" in extracts of skin lesions and edematous fluids from animals with anthrax infections were further subjects of studies in the first decades of the 1900s (Bail, 1904; Bail and Weil, 1911; Salisbury,1926; all cited by Lincoln and Fish, 1970). These studies laid the foundation for the later work leading to the human anthrax vaccines of today in the United States and in the United Kingdom.

Chemotherapy was also being trialed in the first decades of the 1900s. Eurich, following a recommendation by Ehrlich, tried using the arsenical salvarsan for the treatment of anthrax in 1910, adopting it formally in 1911, and later attesting to its success in his own and others' hands (Eurich, 1933; Eurich and Hewlett, 1930). Also, based on Ehrlich's studies on dyes as chemotherapeutic agents, argochrome (methylene-blue-silver) was used with apparent success in a small number of human cases, and acriflavine at a dose of 7 mg/kg in a 1% solution was used with apparent success in a number of sheep (Möslinger, 1924 and Burke and Rodier, 1923; both cited by Eurich and Hewlett, 1930). Especially noteworthy is that in his landmark paper on his discovery of penicillin, Fleming (1929) showed that *B. anthracis* was susceptible to penicillin—although it was another 15 years before it was used to treat the disease (see below).

Public Health Measures for Industrial Anthrax

J. H. Bell's milestone discovery in 1879 in Bradford, England, that woolsorter's disease was inhalation anthrax resulted over the next few years in numerous enquiries, codes of practice, and regulations in Britain for the wool and hair industries, first given legal force in 1897 and reinforced subsequently by the Wool, Goat and Camel Hair Processes Regulations 1905, the Horsehair Regulations 1907, the East India Wool Regulations 1908, the Anthrax Order 1910, the Anthrax Prevention Act 1919, the Anthrax Prevention Order (Shaving Brushes Order) 1920, the Hides and Skins Regulations of 1921, and so on. It was this dangerous aspect of industrial anthrax that led to the disease receiving rapidly increasing attention in the first decades of the twentieth century with the concerns of importing countries, such as the United States, Britain, and Germany, being transmitted to the countries in Africa, Asia, and the Middle East exporting contaminated products of animal origin.

The initial codes and regulations for the British wool and hair processing industries were based on the perceived role of dust in transmitting infection. When no obvious reduction in incidence accompanied dust suppression, removal of bloodstains from the material before further processing was recommended by the Bradford Anthrax Investigation Board in 1908. However, analysis by the Departmental Committee on Anthrax (Report of the Departmental Committee, 1918) revealed this also had had little impact on case rates, nor

did attempts at improving personal hygiene among the workforce. Furthermore, it proved impossible to implement reduction of the incidence of anthrax in the livestock of the exporting countries. The ultimate conclusion was that the problem was not one that could be addressed by further regulations at the factory level and that disinfection, carried out not by the factories themselves, but in dedicated government disinfection stations, was the way forward. From this arose the Duckering Process involving sequential treatment in baths of an alkaline soap solution and 2–2.5% formalin at 102–105ºF followed by drying in hot air. The process was named after Elmhirst Duckering, a member of the Disinfection Sub-Committee, but should probably have been called the Delépine process after Professor Sheridan Delépine, Director of the Public Health Laboratory, Manchester, who designed and tested the procedure (Report of the Departmental Committee, 1918). Disinfection by this process was made a requirement for wool and hair of specified origins under the Anthrax Prevention Act 1919, and the further Orders of 1921 and 1935. The intention was for disinfection stations to be set up in the relevant exporting countries based on the British Government Wool Disinfection Station constructed in Liverpool, but in the end the Liverpool plant was the only one constructed. It operated for 50 years from 1921 to 1971. Interestingly enough, it was the conclusion of Parliamentary Committee of Inquiry in 1959 (Report of the Committee of Inquiry on Anthrax, 1959) that only a small part of the fall of incidence of anthrax in the wool and hair industries could be ascribed to the working of the Disinfection Station and that unspecified other factors had contributed to the reduction in cases.

Such a process was never implemented in the United States on account of both economic and political reasons. Economically, it was estimated that it would raise the cost of processed materials by 5 to 10 cents a pound; politically, it would result in loss of commerce to ports that did not have disinfecting stations (Steele, 1959). Consequently, control of industrial anthrax in the United States was dependent on mechanical and chemical approaches to factory hygiene.

Bioaggression

The early decades of the 1900s also saw the first use of the deliberate infection with anthrax as an act of aggression with, during World War I, Germany targeting horses being raised in neutral countries to supply to Germany's enemies (Wheelis, 1999). In the years immediately following World War I, little concern appears to have been attached to biological weaponry outside of France, and it was only at the urging of Poland that such weapons were included in the 1925 Geneva Protocol (Geissler and van Courtland Moon, 1999). France, recalling the arrest in 1917 of a German agent attempting to infect French cavalry horses with anthrax (and glanders) went through a period of intense biological weapon research in the period 1921–1926, which included *B. anthracis* among several other pathogens (Lepick, 1999). Then France joined other signatories of the 1925 Geneva Protocol and reduced the program to "technical monitoring."

1930 to Sverdlovsk (1979)

The 1930s

With the 1925 Geneva Protocol in place, interest in anthrax fell back to being one of controlling the disease in livestock. Research in this decade was largely concerned with a search for an improvement on the Pasteur vaccine. As has been covered elsewhere

(Turnbull, 2010), the signal name of the decade was Max Sterne who, in 1937, formulated the livestock vaccine still in use in most countries of the world (WHO, 2008).

Also with a view to controlling the disease in livestock was some concern to understand how the disease was being transmitted and, therefore, how this could be prevented. Clark (1938) reasoned that most cases of bovine anthrax in South Africa were contracted by pica (chewing bones), rather than grazing, biting flies, or drinking contaminated water. The significant role of imported animal products continued to be monitored assiduously among trading countries. In the then Dutch East Indies, Kraneveld and Mansjoer (1939) and Kraneveld and Djaenoedin (1939, 1940) believed that biting flies (*Tabanus* spp.) played an important role in the spread of anthrax in that region and demonstrated this experimentally in horses and guinea pigs—further demonstrating that concurrent trypansome infection in guinea pigs did not alter their sensitivity to anthrax infection. Kraneveld and Mansjoer (1939) also established that there was no multiplication phase in the tabanid vector.

The capsule of *B. anthracis* remained the subject of some interest at this time with credit going to Ivanovics and colleagues for its identification as a polypeptide of D-glutamic acid, also showing its poor immunogenicity (Ivanovics, 1939). An interesting observation of a quellung-type reaction on the non-polysaccharide capsule was made by Bodon and Tomsic (1934) but seemingly was never followed up for practical purposes or in relation to pathogenesis. Enhanced phagocytosis of capsulated anthrax bacilli by leucocytes hyperimmunized with capsulated bacilli was demonstrated by Boari (1938).

Chemotherapy also advanced in the 1930s. By 1930, antiserum treatment was the undisputed therapy of choice, but it required large (up to a liter) doses of hyperimmune animal serum administered intravenously with inevitable undesirable side effects. According to Lincoln et al. (1964), the cures effected in the 1920s and 1930s using antisera developed in asses, sheep, and oxen (seemingly not horses) exceeded those effected by antibiotics in the 1940s and 1950s. It is interesting to note that in Penna's 1917 report, the authors state that using bovine serum is preferable to equine serum due to the lower rate of serum sickness reactions seen with the bovine serum (Penna et al., 1917). As stated earlier, Eurich and others were vouching for the value of salvarsan in the early 1900s, and the early 1930s saw several favorable reports on the effectiveness of the less toxic neosalvarsan (also developed in the laboratories of Eurich in 1912) although Gold (1942) did not find it of much value. Sulfa drugs then made their dramatic appearance in 1936, and Gold (1942), treating numerous cases from 1938 to 1941 with these, declared sulfathiazole the drug of choice as being the least toxic if not the most effective.

1940 to the 1972 Weapons Convention: Preparations for Biological Warfare

Germany had unconditionally ratified the 1925 Geneva Protocol in 1929 and, records showed subsequently, had adhered to this through World War II. However, British intelligence reports in the 1930s alleged that the Germans were developing delivery systems for anthrax spores within a substantial biological warfare (BW) program (Geissler, 1999) and accordingly established the Committee of Imperial Defence of a Bacteriological Warfare Subcommittee in 1936, which became the War Cabinet Biological Warfare Committee during the war (Carter and Pearson, 1999). Prior to the war, the Subcommittee was largely concerned with the availability of vaccines in the event of need with practical work only being authorized in 1940, 5 months after the war had begun. Most of the resulting work was with *B. anthracis*, with both offensive and defensive issues being addressed. On the offensive side, the logistics of delivery, ranging from large-scale production of spores through aerosol characteristics to dose determinations and delivery systems were

established, leading ultimately to the infamous experiments in 1942 and 1943 on Gruinard Island off the Scottish coast, the less well-known tests, also in 1942, on the beaches of Penclawdd on the Gower coast of Wales, the production and stockpiling of 5 million anti-livestock cattle cakes containing lethal doses of anthrax spores—5×10^8 spores per cake—and at least one postwar set of sea trials (Hammond and Carter, 2001).

Neither the anti-human nor the anti-livestock devices were put to use, but the beneficial by-product of the U.K. war-stimulated activities, also begun in Canada and the United States in 1943 (Avery, 1999), was the resulting research which commenced after the war in these and other countries on the pathology and pathogenesis of anthrax. At this point the number of publications on anthrax, and these aspects in particular, burgeoned, and it is impossible to do justice here to all the researchers and their excellent papers that appeared from about 1946 onward; many, however, are reviewed by Lincoln and Fish (1970).

By far the greatest numbers of papers on these topics emerged from Camp (later Fort) Detrick in the United States and Porton Down in the United Kingdom. Events at the histopathological events following inhalation of anthrax spores were studied by Young et al. in 1946 in Camp Detrick and by Barnes in 1947 in Porton with both noting that germination and multiplication commenced, not in the lung itself, but in the lymph glands, and Cromartie et al. in 1947 in Detrick defined the histopathological events following cutaneous infection, comparing these events in susceptible and resistant animals.

Druett et al. (1953) in Porton demonstrated the loss of infectivity on inhalation of particles of >5 μm carrying anthrax spores. Since naturally acquired anthrax only takes the inhalation form exceedingly rarely, the concern with potential bioaggression is seen in the emphasis on events following inhalation of spores.

Over the next two decades, a clear understanding of the nature of the toxin complex and the roles of the principal virulence factors of *B. anthracis* had been elucidated in the series of papers between 1953 and 1963 by Smith and colleagues at Porton under the overall heading "The Chemical Basis of the Virulence of *Bacillus anthracis*" together with a number of other supplementary papers and by large teams over the same time period in Detrick focusing extensively on the pathophysiological and neurological responses to anthrax infection and the toxin. The cause of death was established by Smith and colleagues in 1955 as a toxin (Keppie et al., 1955), and this team (Stanley and Smith, 1961) and Beall et al. (1962) in Detrick then established the three-component nature of this toxin. Progress on improvements in the purification of the three factors were reported by Fish et al. (1968), again in Detrick. A large proportion of the research of the period were concerned with the basis of immunity, the overall target being a vaccine for humans, and this is covered in detail elsewhere (Turnbull, 2010).

These research activities were brought to a focus point in the Conference on Progress in Understanding Anthrax convened by the Federation of American Societies for Experimental Biology, Bethesda, Maryland, in 1966 and the published proceedings in 1967. Curiously, that conference appeared to mark something of an end point to the almost frantic activity of the previous two decades with interest waning in the run up to, and after the 1972 Biological Weapons Convention—only to be rapidly rekindled by the Sverdlovsk incident in 1979 (Meselson et al., 1994).

Other Milestones of 1940 to Sverdlovsk

Highly significant among the early milestones of the period under consideration was the first use of penicillin to treat anthrax was in 1944 (Murphy et al., 1944), thus beginning

the long era thereafter when penicillin was the first drug of choice, replacing serum therapy and other chemotherapies in use at the time (WHO, 2008). A decade later, Henderson et al. (1956) showed the prolonged persistence of spores in the lungs of monkeys after inhalation exposure and, with penicillin as the antibiotic, formulated the approach recommended today of prolonged antibiotic administration with simultaneous vaccination in persons thought or known to have been exposed to aerosolized spores.

Recognition is also due to Brachman et al. (1962) and the Committee of Inquiry in Britain (Report of the Committee of Inquiry on Anthrax, 1959) for the in-depth understanding they supplied on the epidemiology of industrial and inhalation anthrax, Stein and Van Ness (1954) for their careful monitoring of veterinary cases and outbreaks and ecological insights (Van Ness, 1971), Knisely (1966) for the formulation of selective PLET medium which has remained the basis of detection of anthrax spores in environmental samples to the present day, and Shlyakhov et al. (1973) for the Anthraxin diagnostic skin test.

Also worthy of retrospective attention was the remarkable outbreak in humans and animals in Zimbabwe which began in 1978 and still had residual cases in 1982, with well over 10,000 human cases and countless cases in cattle and other livestock. Officially, this was put down to the breakdown in veterinary services, shortages of penicillin, and other consequences of a major insurgency occurring at the time, though opinions were expressed later that it may have resulted from deliberate release—an issue that will probably remain unresolved.

Sverdlovsk (1979) to the Anthrax Letters (2001) and Beyond

Resumption of research after the Sverdlovsk incident saw intensive and effective application of new techniques and technologies to the understanding of the pathogenesis of anthrax, with the infamous anthrax letters of late 2001 producing a second dramatic surge of research into numerous aspects of the disease. Once again, it is impossible in the space available here to give the individual credits that would ideally be desired to all the research groups and individuals behind the excellent work over the past three decades. However, it was a period when much of the work, together with the relevant credit, was well covered by periodic and readily available reviews and textbook chapters, and much of it will also form the introductory backgrounds of the chapters that follow this.

In summary, this period, which began with improved purification of the toxin components, then saw

- full structure-function characterization of the toxin components, the understanding of their "A-B" type of toxin action with the protective antigen (PA) as the binding unit and lethal and edema factors (LF, EF) as the active units, and, later, the nature of the binding site for PA, the relationships and interactions with other bacterial toxins and novel concepts for treatment of anthrax and other diseases based on the receptor-mediated endocytosis of the anthrax toxin,
- the elucidation in the early 1980s of EF as an adenylate cyclase and somewhat later the understanding of the action of LF as a protease acting on mitogen-activated protein kinase kinases at the end of the 1990s,
- elucidation of the plasmid-borne nature of the toxin and capsule genes in the early to mid-1980s, with sequences being established for the virulence factor genes in the late 1980s and early 1990s, and those of the plasmids pXO1 and pXO2 at the

end of the 1990s with finer analysis of regulatory control of these and of other important genes, such as germination operon genes, and the development of specific probes and polymerase chain reaction (PCR) primers for detection purposes,

- completion of the genome sequence by Read et al. (2003) with its profound consequences,
- steady progress in further understanding of the pathogenesis of anthrax and the nature of resistance and immunity to infection and toxin action commencing with the excellent work of Welkos and colleagues in the 1980s and focusing heavily on the role of macrophages in the 2000s,
- awareness and early characterization of other potential virulence factors, such as the S-layer, the exosporium, and anthrolysin O, and
- the breakthrough in molecular typing of *B. anthracis* for epidemiological and forensic purposes under the leadership of Keim, Jackson, and colleagues.

As in the two decades after World War II, the justification for much of the research from 1980 to the present day has been the need for an improved vaccine for administration to humans, again reviewed separately (Turnbull, 2010).

The period also saw the opportunity taken to apply the advancing technologies to the study of the natural disease in enzootic wildlife areas revealing valuable information about the natural ecology and epidemiology of the disease (WHO, 2008).

One particular sequel of significance to the anthrax letters has been a new look at approaches to diagnosis and treatment of anthrax, although this again focused heavily on inhalation anthrax as acquired by putative deliberate release scenarios.

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Chapter 2

Outer Structures of the *Bacillus anthracis* Spore

Adam Driks and Michael Mallozzi

The discovery in 1876 by Robert Koch that *Bacillus anthracis* can form a dormant, highly resistant cell type called a spore was a seminal event in the history of microbiology (Koch, 1876). In his historic paper, Koch not only described the process by which *B. anthracis* forms spores (called sporulation) but also showed that the spore is the infectious particle for the disease anthrax. The ability of the dormant spore to withstand environmental stresses that would destroy most other cells stimulated numerous studies to understand how the spore's protective structures are built and how they provide protection. The majority of these studies, especially in recent decades, have focused on *Bacillus subtilis* since it does not cause disease and has been developed as a genetic model (Sonenshein, 2000). In recent years, however, the pendulum has swung back to *B. anthracis*, and our understanding of spore formation and function, and anthrax pathophysiology, in that organism has increased significantly. This growing body of knowledge will undoubtedly affect the development of novel therapeutics. Importantly, this knowledge base, coupled with improved genetic tools, has elevated *B. anthracis* to the status of a model organism for basic studies on sporulation. Rather than compete with *B. subtilis* as a model, we can expect studies in *B. anthracis* not only to complement those in *B. subtilis*, but also to provide a new window on the evolutionary causes and ecological benefits of diversity in spore ultrastructure and function (Driks, 2007).

SPORE ULTRASTRUCTURE

Bacillus spores share a common architecture (reviewed in more detail in Aronson and Fitz-James, 1976; Driks, 1999; Driks and Setlow, 2000; Henriques and Moran, 2007; Holt and Leadbetter, 1969). The innermost compartment is the core, which houses the spore chromosome (Figure 2.1a). Moving outward, the core is first surrounded by an inner membrane and then a shell of specialized peptidoglycan, referred to as the cortex (see Chapter 3). Proteins in the core and the cortex maintain spore dormancy and protect the DNA against heat and other assaults (Setlow, 1988, 2006). The inner membrane helps protect the spore DNA and is the site of the receptors that sense germinant and, as a result,

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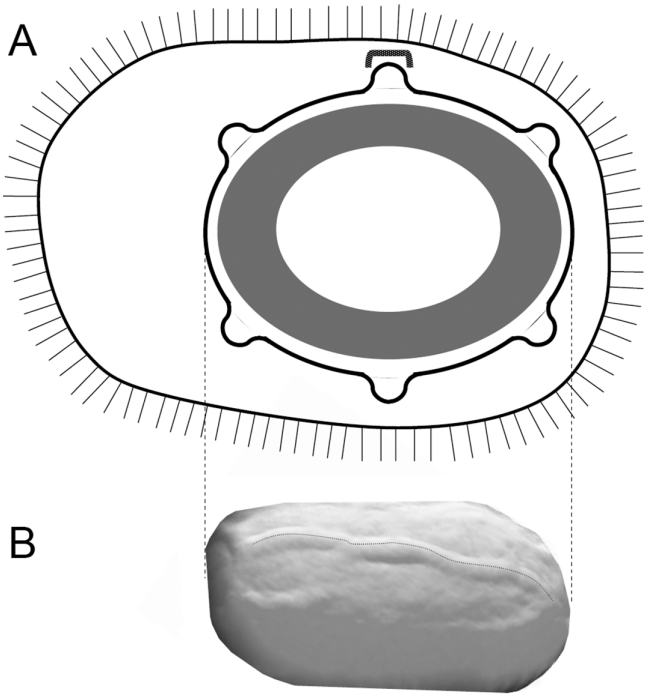

Figure 2.1 The *Bacillus anthracis* spore. (a) Cartoon showing a mature spore. The outermost structure is the exosporium, comprising a thin basal layer from which extend a series or hair-like projections, or nap. A gap, refered to as the interspace, separates the exosporium from the coat (indicated as a thick line with ridges). A bracket indicates one of the ridges of the coat. Beneath the coat is the spore peptidoglycan (known as the cortex, indicated by a thick grey band). (b) Image of the coat surface generated by atomic force microscopy. A surface ridge, extending along the long axis of the spore, is traced with a dotted line. The exosporium is not visualized in this image, probably because it is relatively soft compared to the coat. This image was generated by Dr. Ozgur Sahin, Rowland Institute at Harvard.

initiate germination, the process that ultimately leads to the resumption of metabolism (Hudson et al., 2001; Paidhungat and Setlow, 2001).

Surrounding the cortex are the structures that are the focus of this chapter: the coat and exosporium (Figure 2.1a). In contrast to the core, inner membrane, and cortex, which are morphologically and, presumably, biochemically similar in all species, the coat and exosporium vary considerably in morphology (Aronson and Fitz-James, 1976; Driks, 2002b; Holt and Leadbetter, 1969). The coat is present in spores of all *Bacillus* species examined so far. By thin-section transmission electron microscopy (TEM), the coat appears to be a darkly staining shell surrounding the cortex, and is in close contact to it (Warth et al., 1963). Its structure varies among species, primarily in its thickness. For example, in *B. subtilis* and *Bacillus sphaericus*, multiple coat layers are readily observed (Holt et al., 1975). In contrast, in *B. anthracis* (as well as in *Bacillus cereus* and *Bacillus thuringiensis*), the coat is thin, and the separate layers, while present, are much more difficult to distinguish (Aronson and Fitz-James, 1976; Giorno et al., 2007). In some species, coat architecture can be much more ornate. In *Bacillus clausii*, for example, a series of long thin fibers extend from the coat surface (Driks, unpublished observations).

TEM images suggest that, in most spores, the coat has ridges or folds. This is much more clearly revealed by techniques that image surface topography, such as scanning

electron microscopy and atomic force microscopy (AFM) that document ridges and valleys on the coat surface (Figure 2.1b) (Aronson and Fitz-James, 1976; Chada et al., 2003; Holt and Leadbetter, 1969; Plomp et al., 2004, 2005a,b; Wang et al., 2007; Zolock et al., 2006). Comparisons of the species examined so far reveals some important common features to the ridges. For example, ridges usually appear to be small folds in the coat surface, most of which extend along the long axis of the spore. However, the regularity of the placement of the ridges on each spore and the exact number per spore both vary among spores in any given culture. This is consistent with the possibility that ridges form randomly and not because of some regularly positioned architectural element within the spore (Chada et al., 2003; Driks, 2004). The presence of ridges raises the possibility that the coat is flexible like the pleats of an accordion. This view is supported by observations suggesting that the ridges unfold when the spore swells during germination (Chada et al., 2003). The ridges may also unfold, at least partially, during changes in relative humidity while the spore is dormant (Driks, 2003; Westphal et al., 2003). AFM also reveals coat-surface features, at a finer scale than the ridges. High-resolution images of the *B. anthracis* coat surface show that it consists of small, so-called rodlet, features of about 27 nm in width, largely oriented along the long axis (as are the ridges) (Wang et al., 2007).

The outermost *B. anthracis* spore structure visible by electron microscopy is the exosporium, which is separated from the coat by a region called the interspace. In contrast to the spore layers just described, the exosporium is not present in all species. Although it is found, for example, in *Bacillus megaterium*, *Brevibacillus laterosporus*, *Bacillus odysseyi*, and members of the so-called *B. cereus*-group (including *B. anthracis*, *B. cereus*, and *B. thuringiensis*), it is absent from many others, including *B. clausii*, *Bacillus licheniformis*, and *B. subtilis*. In all species examined so far, the minimal exosporium structure is a thin shell, called the basal layer. Beyond this, exosporium morphology varies among species. In the case of *B. anthracis*, the exosporium also possesses a series of hair-like projections, called the nap, extending outward from the basal layer. Since the exosporia of relatively few species have been analyzed, the degree of morphological variation in this structure is unknown. In *B. anthracis*, the exosporium does not have the uniform curvature expected of an inflated car tire or the cell envelope of a coccus. Instead, exosporium curvature varies along its circumference and from spore to spore, as if the connection (whatever it may be) between the exosporium and the coat varies in length in a corresponding way around the spore perimeter. This apparent plasticity in exosporium morphology suggests that it is flexible.

High-resolution imaging of the exosporium has provided important insights into exosporium structure and function. A high-resolution three-dimensional reconstruction using electron microscopy showed that the *B. anthracis* basal layer is, most likely, a crystalline sheet consisting of a three-fold symmetric array of crown-like structures (Ball et al., 2008). Cavities of about 30 Å are present between the crown-like structures, likely allowing the passage of small molecules (such as germinants) but not large proteins. The regular geometry of the structural map suggests that relatively simple rules of assembly could govern basal layer polymerization.

Since the degree of variation in exosporium structure is greater than that of the more interior layers (Aronson and Fitz-James, 1976; Bradley and Franklin, 1958; Bradley and Williams, 1957; Driks, 2007; Hodgkiss et al., 1967; Holt and Leadbetter, 1969; Rode, 1971; Walker et al., 2007), it is plausible that exosporium function also varies among species to a greater degree than do the inner layers, such as the coat and cortex. In this regard, it is important to note that the variation among exosporia (and spore "appendages," in general) is not yet thoroughly explored (Driks, 2007). A striking example of the degree

of variation that can be found in spore outer structures is provided by *Clostridium taeniosporum*, which possesses an especially elaborate ribbon-like appendage (Walker et al., 2007). Probably, in many if not most cases, the exosporia (and other types of spore appendage) are not required for a basic level of spore resistance and, therefore, are free to evolve to facilitate adaptation to specialized niches.

THE FUNCTIONS OF THE SPORE OUTER LAYERS

Since the host is an especially important niche for *B. anthracis*, resistance to host defense is almost certainly a critical function of the spore. In fact, the ability of spores to survive an encounter with host phagocytic cells likely determines the course of the disease (Cote et al., 2008b). Given that, it is noteworthy that some phagocytic cells appear to be better at killing spores than others. For example, human neutrophils very efficiently kill phagocytosed spores (Mayer-Scholl et al., 2005). This ability to kill may be related to the observation that cutaneous anthrax (characterized by large numbers of neutrophils at the site of infection) is rarely lethal, while inhalational anthrax (with relatively few macrophages at the site of infection) is often lethal (Albrink et al., 1960; Mayer-Scholl et al., 2005). In contrast to neutrophils, the macrophage has been shown to be able to kill spores when they germinate but, paradoxically, to also promote infection by providing a space for spores to germinate and multiply (Cote et al., 2008b). How the macrophage can both kill germinating spores in some cases but promote their outgrowth and replication in others is not completely understood. At least part of the answer, however, may be that macrophage killing of spores depends on the spore number. In this view, when the number of spores per macrophage is greater than some threshold, macrophages are unable to effectively kill spores because of the relatively high toxin levels that appear soon after the completion of germination (Cote et al., 2008b).

An important area for future research is likely to be the analysis of spore resistance to dendritic cells. Recent evidence suggests that these immune cells play an important role in phagocytosing spores in the lung and transporting them to the regional lymph nodes, a key step in anthrax pathogenesis (Brittingham et al., 2005; Cleret et al., 2007). Therefore, resistance to dendritic cells could be a key factor in the disease.

Any and, perhaps, all the spore layers could plausibly contribute to defense against the host. The general roles of the core and cortex in protection against various environmental and, most likely, host stresses are relatively well understood (Moir, 2006; Moir et al., 2002; Popham, 2002; Setlow, 2003). Here, we focus on the functions of the coat and exosporium, the spore structures most likely to be involved in adaptation to specific niches.

FUNCTIONS OF THE COAT

Work in *B. subtilis* demonstrates important roles for the coat in resistance. For example, the coat acts as a sieve, protecting the spore interior from toxic degradative molecules, such as lysozyme, common in host tissues, and other enzymes that could be present in the soil and other environments (Driks, 1999). The coat also acts to protect against toxic small molecules (Setlow, 2006). The overall similarities between the *B. subtilis* and *B. anthracis* coats suggest that they share these resistance functions (Driks, 2002a). In fact, roles for the coat in resistance to lysozyme, antimicrobial chemicals such as chloroform, phenol,

hypochlorite, and diethylpyrocarbonate and UV and ionizing radiation have been demonstrated in *B. anthracis* (Bailey-Smith et al., 2005; Kim et al., 2004; Nicholson and Galeano, 2003). In *B. subtilis*, the coat protects against killing after ingestion by ubiquitous eukaryotic microbial predators, such as the protozoan *Tetrahymena thermophila* and the nematode *Caenorhabditis elegans* (Klobutcher et al., 2006; Laaberki and Dworkin, 2008). It is reasonable to suggest that the *B. anthracis* coat provides a similar type of protection, since *B. anthracis* spores may reside for significant periods of time in the soil, an environment rich in nematodes and protozoa. The ability of the coat to hinder digestion by microbes may also be useful in the mammalian host, where the same protective mechanisms may be a factor in withstanding phagocytosis.

The coat also serves a function in spore germination. In *B. subtilis*, a number of coat proteins with important roles in coat assembly have also been shown to have major effects on germination (Driks, 1999, 2002b; Henriques et al., 2004; Henriques and Moran, 2007). The large number of coat proteins that are conserved between the two species suggests that this will likely be true for *B. anthracis* coat proteins. This view is supported by studies of the cortex lytic enzyme CwlJ. In *B. subtilis*, CwlJ has been shown to be a coat protein involved in degrading the cortex peptidoglycan during germination (Bagyan and Setlow, 2002). Recently, one of two CwlJ paralogs in *B. anthracis* (CwlJ1) has been shown to have a similar role (Heffron et al., 2009). It is reasonable to conjecture that CwlJ1 in *B. anthracis* is a coat protein as well.

The coat proteins CotE and CotH also affect germination in both *B. subtilis* and *B. anthracis* (Bauer et al., 1999; Giorno et al., 2007; Naclerio et al., 1996; Zheng et al., 1988). In *B. anthracis*, *cotE* mutant spores have a surprising phenotype: germination inside a macrophage does not render them as sensitive to macrophage killing as does germinating wild-type spores. Although the reason for this relative insensitivity is unknown, a likely clue comes from the observation that *cotE* mutant spores are delayed in the resumption of metabolism in *in vitro* experiments. Possibly, this delay is due to a defect in shedding the coat and/or exosporium. If so, then the retention of outer protective layers may be responsible for resistance to the macrophage.

In contrast to a *cotE* mutant, *cotH* mutant spores are more sensitive to killing by macrophages, and, *in vitro*, appear to germinate and resume metabolism more efficiently than wild type. Unlike the wild-type spores, which have an average *in vitro* germination time of approximately 60 min, *cotH* mutant spores germinate within 15 min and do so with essentially 100% efficiency, compared to approximately 40% efficiency of wild type (Giorno et al., 2007).

In spite of the insights into coat function gained from the work just described, analyzing the role of the coat presents several conceptual difficulties. A particularly important difficulty stems from the coat's biochemical complexity. *B. subtilis* possesses about 80 coat protein species, and this is likely true of *B. anthracis* (and most other Bacilli) (Table 2.1) (Driks, 2002b; Giorno et al., 2007; Henriques and Moran, 2007; Kim et al., 2006; Kuwana et al., 2002; Lai et al., 2003). However, at least in *B. subtilis*, the majority of these proteins are dispensable for known resistance properties (Driks, 1999, 2002b; Henriques and Moran, 2007). This is likely to be true for *B. anthracis* as well (Driks, 2002a; Giorno et al., 2007, 2009; Mallozzi et al., 2008). Most probably, the coat has functions beyond those examined in the laboratory. It should also be noted that demonstrating a function for the coat in the laboratory is not equivalent to showing a role for the function in the natural environment. These conceptual difficulties point to important limitations of reductionist analysis and argue for increased attention to spore ecology and evolution (Driks, 2007).

Table 2.1 Spore Outer Structure Proteins

Spore protein name	Open reading frame (accession)	*Bacillus subtilis* protein ortholog(s)	First Found in[a]	*Bacillus anthracis* paralog(s)	Known or (putative) function	Known or (putative)[b] subcellular location
Alr (Dal-1[c])	*ba0252*	Alr, YncD	Steichen et al. (2003)	*dal-2*	Alanine racemase	Exosporium
BclA	*ba1222*	None	Steichen et al., (2003); Sylvestre et al. (2002)	*bclB*	Adherence, Exosporium fiber formation	Exosporium
BclB (ExsH[d])	*ba2450*	None	Thompson et al. (2007); Waller et al. (2005)	*bclA*	Exosporium attachment/ stability	Exosporium
BxpA	*ba2162*	None	Steichen et al., (2003)	None	Unknown	Exosporium/ Interspace
BxpC	*ba2332*	None	Redmond et al. (2004)	*ba5438*	Unknown	Exosporium/ Interspace
Cotα	*ba4266*	None	Kim et al. (2004)	None	Outer coat assembly	Coat
Cotβ	*ba2104*	None	Mallozzi et al. (2008)	None	Coat surface morphology	Coat
CotA	*ba1902*	CotA (PigA[c])	Donovan et al. (1987)	None	(Oxidase, spore pigmentation)	(Coat)
CotB1[e]	Nucleotides 366229-366678	CotB	Lai et al. (2003)	*cotB2*	Unknown	(Coat)
CotB2[e]	Nucleotides 366208-365687	CotB	Lai et al., (2003)	*cotB1*	Unknown	(Coat)
CotD	*ba1581*	CotD	Donovan et al. (1987)	None	Unknown	Coat
CotE	*ba3906*	CotE	Zheng et al. (1988)	None	Morphogenetic protein	Coat
CotF	*ba3121*	CotF	Cutting et al. (1991)	None	Unknown	(Coat)
CotH	*ba2046*	CotH, YisJ	Naclerio et al. (1996)	None	Morphogenetic protein	(Coat)
CotJA	*ba0805*	CotJA	Henriques et al. (1995)	None	Unknown	(Coat)
CotJB	*ba0804*	CotJB	Henriques et al. (1995)	None	Unknown	(Coat)
CotJC	*ba0803*	CotJC	Henriques et al. (1995)	*ba3134*	Similar to known catalases	(Coat)
CotM	*ba3681*	CotM	Henriques et al. (1997)	*ba2250*	Similar to known chaperones	(Coat)
CotN	*ba4507*	CotN (YqfT[c])	Kim et al. (2006)	None	(Morphogenetic protein)	(Coat)

Table 2.1 *Continued*

Spore protein name	Open reading frame (accession)	*Bacillus subtilis* protein ortholog(s)	First Found in[a]	*Bacillus anthracis* paralog(s)	Known or (putative) function	Known or (putative)[b] subcellular location
CotO	*ba1233*	CotO (YjbX[c])	McPherson et al. (2005)	None	(Morphogenetic protein)	(Coat)
CotS	*ba5188*	YutH, CotS, CotI	Abe et al. (1995)	None	Unknown	(Coat)
CotSA	*ba5012*	CotSA	Takamatsu et al. (1999b)	*ba1558, ba0280, ba5518*	Similar to glycosyl transferases	(Coat)
CotY (CotZ2[c])	*ba1238*	CotY	Zhang et al. (1993)	*exsY*	Morphogenetic protein	Exosporium
CwlJ2[f]	*ba5640*	CwlJ	Heffron et al. (2009)	*cwlJ1, sleB, sleL*	Cortex lytic enzyme	Coat
ExsA	*ba4659*	SafA	Bailey-Smith et al. (2005)	None	Morphogenetic protein	Coat and/or Exosporium/ Interspace
ExsB	*ba2045*	None	Todd et al. (2003)	None	Unknown	Exosporium/ Interspace
ExsC	*ba2894*	None	Todd et al. (2003)	None	Unknown	Exosporium/ Interspace
ExsD	*ba2617*	None	Todd et al. (2003)	*ba2429*	Unknown	Exosporium/ Interspace
ExsE	*ba1786*	None	Todd et al. (2003)	None	Unknown	Exosporium/ Interspace
ExsFA (BxpB)	*ba1237*	None	Steichen et al. (2005); Sylvestre et al. (2005); Todd et al. (2003)	*exsFB*	Morphogenetic protein	Exosporium
ExsFB	*ba2477*	None	Steichen et al. (2005); Sylvestre et al. (2005); Todd et al. (2003)	*ba1237*	Morphogenetic protein	Exosporium/ Interspace
ExsG	*ba2150*	None	Todd et al. (2003)	None	Unknown	Exosporium/ Interspace
ExsJ	*ba1477*	None	Todd et al. (2003)	*ba3841*	Unknown	Exosporium/ Interspace
ExsK	*ba2554*	None	Redmond et al. (2004)	None	Unknown	Exosporium/ Interspace
ExsY (CotZ1[c])	*ba1234*	CotZ	Boydston et al. (2006)	*cotY*	Morphogenetic protein	Exosporium

(*Continued*)

Table 2.1 *Continued*

Spore protein name	Open reading frame (accession)	*Bacillus subtilis* protein ortholog(s)	First Found in[a]	*Bacillus anthracis* paralog(s)	Known or (putative) function	Known or (putative)[b] subcellular location
GerQ	*ba5641*	GerQ (YwdL[c])	Ragkousi et al. (2003)	None	Unknown	Coat
IunH1	*ba2888*	None	Lai et al. (2003)	*ba5338, ba3606, ba2400*	Similar to nucleotide hydrolases	Exosporium/ Interspace
IunH2	*ba3606*	None	Lai et al. (2003)	*ba2888, ba2400, ba5338*	Similar to nucleotide hydrolases	Coat and/or Exosporium/ Interspace
OxdD	*ba1742*	OxdD (YoaN[c])	Costa et al. (2004)	None	Unknown	Coat
RocA	*ba0309*	RocA, YcgN	Charlton et al. (1999)	*dhaS, aldA, mmsA-2, gabD, mmsA-1, gapN, ba2289, ywdH*	Similar to delta-1-pyrroline-5-carboxylate dehydrogenase	Exosporium/ Interspace
SoaA	*ba5269*	NA	Cote et al. (2008a)	None	Facilitates spore opsonization	Coat
Sod15	*ba1489*	SodF, SodA	Passalacqua et al. (2006)	*sodA1, sodC, sodA2*	Superoxide dismutase	Exosporium/ Interspace
SodA1	*ba5139*	SodF, SodA	Passalacqua et al. (2006)	*sod15, sodC, sodA2*	Superoxide dismutase	Exosporium/ Interspace
SpoIVA	*ba1530*	SpoIVA	Giorno et al. (2007)	None	Morphogenetic protein	Coat
SpoVID	*ba4692*	SpoVID	Beall et al. (1993)	None	Morphogenetic protein	Coat
SpoVM[e]	Nucleotides 3674683-3674760	SpoVM	Levin et al. (1993)	None	Morphogenetic protein	Coat
Tgl	*ba0546*	Tgl	Zilhao et al. (2005)	None	Transglutaminase	Coat
YabG	*ba0040*	YabG	Takamatsu et al. (2000a,b)	None	Unknown	Coat
YckK	*ba0855*	YckK	Lai et al. (2003)	*ba4376, ba0640*	Similar to amino acid ABC transporters	Coat
YdhD	*ba3668*	YdhD	Kodama et al. (2000)	*ba3480* (YjdH)	Similar to glycosyl hydrolases	Coat

Table 2.1 *Continued*

Spore protein name	Open reading frame (accession)	*Bacillus subtilis* protein ortholog(s)	First Found in[a]	*Bacillus anthracis* paralog(s)	Known or (putative) function	Known or (putative)[b] subcellular location
YhaX	*ba0870*	YhaX	Eichenberger et al. (2003)	*ba1174, ba5646, ba4188, ba2426, ba2634, ba2077, ba4285, ba4271*	Similar to haloacid dehalogenases	Coat
YhbA	*ba0545*	YhbA	Lai et al. (2003)	None	Similar to iron-sulfur cluster-binding proteins	Coat
YhbB	*ba0546*	YhbB	Lai et al. (2003)	None	Unkonwn	Coat
YheC	*ba0861*	YheC	Eichenberger et al. (2003)	*ba0860*	Unknown	Coat
YheD	*ba0860*	YheD	van Ooij et al. (2004)	*ba0861*	Dynamically localizing protein	Coat
YhjR	*ba3113*	YhjR	Kim et al. (2006)	None	Unknown	Coat
YirY	*ba2360*	YirY	Kim et al. (2006); Lai et al. (2003)	*smc, ba0477, ba3254, ba2149, ba1011, pxo1-90, ba0557, ba4940, pxo2-17, ba0684, ba2967, ba1978, ba3366*	Similar to S-layer proteins	Coat
YisY	*ba5030*	YisY	Lai et al. (2003); Zilhao et al. (2005)	*ba3165*	Similar to chloride peroxidases	Coat
YjdH	*ba3480*	YjdH	Lai et al. (2003)	*ba3668* (YdhD)	Similar to glycosyl hydrolases	Coat
YknT	*pxo1-90*	YknT	Kim et al. (2006)	*ba1663, ba3986* (*smc*)	Similar to S-layer proteins	Coat

(*Continued*)

Table 2.1 *Continued*

Spore protein name	Open reading frame (accession)	*Bacillus subtilis* protein ortholog(s)	First Found in[a]	*Bacillus anthracis* paralog(s)	Known or (putative) function	Known or (putative)[b] subcellular location
YkuD	*ba4372*	YkuD	Kodama et al. (2000)	*ba5274*	Unknown	Coat
YlbD	*ba4146*	YlbD	Lai et al. (2003)	None	Unknown	Coat
YobN	*ba1924*	YobN	Lai et al. (2003)	*ba2018*	Unknown	Coat
YodI	*ba3640*	YodI	Lai et al. (2003)	None	Unknown	Coat
YpeP	*ba1626*	YpeP	Lai et al. (2003)	None	Unknown	Coat
YppG	*ba1580*	YppG	Kim et al. (2006)	*ba2162*	Unknown	Coat
YsxE	*ba4691*	YsxE	Kim et al. (2006)	None	Unknown	Coat
YtaB	*ba3179*	YtaB	Kim et al. (2006)	None	Unknown function, similar to TspO/MBR family proteins	Coat
YusA	*ba5219*	YusA	Lai et al. (2003)	*ba5220, ba0175, ba0314*	Similar to ABC transporters	Coat
YxeE	*ba3589*	YxeE	Kuwana et al. (2002); Takamatsu et al. (2000a)	None	Unknown	Coat

[a] References refer to the first paper to identify the gene/protein in *B. anthracis, B. cereus,* or *B. subtilis.*

[b] Presumed location from studies conducted on the *B. subtilis* ortholog.

[c] Other/older protein designations.

[d] ExsH is the nearest *B. cereus* ortholog of BclB in *B. anthracis.*

[e] Not annotated in the Ames strain of *B.* anthracis.

[f] CwlJ1 and SleB (and not CwlJ2) have been shown be involved in spore cortex hydrolysis in *B. anthracis* during germination (Heffron et al., 2009). However, CwlJ2 is the homolog with the highest identity to the *B. subtilis* protein.

FUNCTIONS OF THE EXOSPORIUM

The exosporium could plausibly be involved in any of the functions ascribed to the coat, including resistance and germination. The exosporium is also almost certainly involved in adhesion. Of these possible functions, germination has received significant attention. As will be discussed below, in most cases where an exosporium protein has been shown

to impact germination, the molecular basis of the effect is unknown. An exception is the enzyme alanine racemase (Alr), an exosporium protein that is present throughout the exosporium except for the cap, the first exosporium structure to be assembled (see below) (Steichen et al., 2007). Alr catalyzes the interconversion of L-alanine and D-alanine, and D-alanine has long been known to inhibit germination (while L-alanine is a spore germinant) (Anmuth et al., 1956; Chesnokova et al., 2009; Fey et al., 1964; Weiner et al., 2003). In *B. anthracis*, racemase-mediated production of D-alanine has been shown to inhibit germination of a subset of spores in a population, a phenomenon known as autoinhibition (McKevitt et al., 2007). Possibly, it is adaptive for spore populations to limit the degree of germination when the spore density is high or when the nutrient level is relatively low (and, therefore, too low to saturate Alr). Furthermore, even when nutrient is plentiful, it may be useful for a subpopulation of spores to remain dormant but capable of germination since conditions unrelated to nutrient (such as temperature or pH) may be unsuitable for continued cell growth. In that event, the spores that did not germinate will survive, and could attempt germination at some later time. Although this explanation is speculative, the presence of ungerminated spore subpopulations (so-called superdormant spores) is well documented (Ghosh and Setlow, 2009; Turnbull et al., 2007). Certainly, other factors are likely to contribute to superdormant spore populations. Recently, roles for low levels of germinant receptor proteins and the need for heat activation in superdormancy have been established (Ghosh and Setlow, 2009). It should be noted, however, that spores with very low levels of receptors will presumably germinate inefficiently (if at all) in response to nutrient and, therefore, are not poised to efficiently germinate at a future date. In contrast, spores inhibited by Alr would be able to germinate whenever nutrient reappears.

Other exosporium proteins have roles in germination that are much less well understood. Spores lacking the exosporium basal layer protein ExsFA/BxpB (discussed in more detail below), which is required for the formation of the nap and for the deposition of most but not all BclA (Sylvestre et al., 2002) have decreased germination efficiency (Giorno et al., 2009). In contast, *bclA* mutant spores (missing BclA and, therefore, the hair-like projections or nap) were found to germinate more efficiently than wild-type spores in one study (Brahmbhatt et al., 2007), and to have no change in germination efficiency in another (Bozue et al., 2007a). In contrast, these data suggest that the germination defect observed in *exsFA*/*bxpB* mutant spores is not due to the loss of BclA. However, the effect of an *exsFA*/*bxpB* mutation on germination was not seen when fresh germinant was supplied constantly via a flow cell, suggesting that this phenotype may be sensitive to the germination conditions (Steichen et al., 2003). It is interesting that, at least under some conditions, the removal of exosporium structures inhibits rather than facilitates germination, as might be expected if the exosporium is largely a passive structure. This suggests that the exosporium has an active role in germination.

The role of the exosporium in the disease anthrax is an important question. It is reasonable to conjecture that the exosporium could affect disease by mediating important interactions with immune cells as well as because of its role in germination. Consistent with this, the autoinhibition phenomenon described above enhances *B. anthracis* spore survival in interactions with macrophages and quantitatively lowers the infectious dose needed for the appearance of disease in an animal model (McKevitt et al., 2007). The inhibition of germination by Alr is important to the spore in at least one other way: in the absence of Alr, germination occurs prematurely in the sporulating cell cytoplasm, before the spore is released (Chesnokova et al., 2009). Therefore, Alr helps the immature spore stay dormant in spite of the nutrient-rich mother cell interior. This finding raises an

interesting question: how do spores from species lacking an exosporium stay dormant prior to release from the mother cell? In the case of *B. subtilis*, it is possible that a poorly characterized spore-associated Alr activity (Kanda-Nambu et al., 2000; Reusch et al., 1982; Yasuda et al., 1993) might have a role in inhibiting germination. Nucleoside hydrolase, which is associated with the exosporium in *B. anthracis* and *B. cereus* (Lai et al., 2003; Todd et al., 2003) might suppress germination in a manner similar to Alr. This protein is present in *B. thuringiensis* and, when overproduced in that organism, suppresses germination (Liang et al., 2008; Todd et al., 2003).

Although the exosporium clearly has an important role in mediating interactions between spores and host cells, so far, experiments in which animal models are inoculated with significant doses of virulent spores suggest that the exosporium (and, in particular, BclA) is dispensable for the disease (Bozue et al., 2007a; Giorno et al., 2007; Sylvestre et al., 2002). However, a modest effect for BclA on increasing the mean time to death was shown using the highly attenuated Sterne strain and complement-defective A/J mice, in which the Sterne strain can cause disease (Brahmbhatt et al., 2007). These results raise the possibility that the exosporium has a role in infection in nature, where the number of bacteria in the inoculum may be much lower than in laboratory infections and, most likely, infection will occur by the cutaneous or gastrointestinal routes. A number of studies, in fact, show functions or activities for the exosporium that are consistent with a role in the host. For example, the exosporium appears to have an enzymatic activity due, most likely, to the protein RocA, that neutralizes the nitric oxide response produced by infected macrophages (Weaver et al., 2007). Not only can the spore neutralize this oxidative burst, but it appears also to be able to utilize it to facilitate germination (Baillie et al., 2005). At first glance, this seems counterintuitive, since germinated spores can be killed much more easily than can dormant spores by macrophages (Hu et al., 2007; Welkos et al., 2002). However, as discussed at the beginning of this section, the expression of virulence factors soon after germination may render the macrophage incapable of eliminating the infecting bacteria.

Although not required for disease in several animal models (Bozue et al., 2007a; Giorno et al., 2007, 2009; Sylvestre et al., 2002), BclA could still impact the natural infection, by an ability to bind specific host cell-surface receptors. For example, in *B. anthracis*, BclA facilitates spore binding to macrophages and to the cell surface integrin CD11b/CD18 (Bozue et al., 2007b; Oliva et al., 2008). Intriguingly, the C terminal domain of BclA (which is exposed at the spore surface) has high structural similarity to the portion of the complement protein C1q that binds antibody (Rety et al., 2005). Possible clues to the significance of the structural similarity of BclA and C1q come from studies suggesting that *B. cereus* spores utilize the C1q receptor on lung and colon epithelial cells to facilitate attachment and entry of spores into the host (Ghebrehiwet et al., 2007). Perhaps, in the case of anthrax, interactions between BclA and C1q receptors on lung epithelial cells somehow facilitate phagocytosis by resident dendritic cells or alveolar macrophages. Supporting the possibility that spore proteins mediate the uptake of spores by phagocytic cells is the finding that the spore protein SoaA facilitates spore opsonization by anti-spore antibodies and, thus, phagocytosis by macrophages (Cote et al., 2008a). Interestingly, in the mature spore, SoaA is detected by immunoelectron microscopy at the interface between the coat and cortex, and not at the coat surface or in the exosporium. Most likely, SoaA becomes exposed on the spore surface during germination.

In addition to engaging certain host cell receptors, it is likely that the exosporium helps ensure that other surface receptors, that might activate host cell defenses, are not triggered. Consistent with this view, the exosporium appears to mask proinflammatory

antigens within the spore (Basu et al., 2007). The degree to which this is relevant to disease is unclear since, at least in some animal models, the exosporium is not needed for virulence (Giorno et al., 2007). Nonetheless, as already stated, this mechanism for avoiding the host innate immune response might provide protection in natural infections.

ASSEMBLY OF THE SPORE OUTER STRUCTURES

Early electron microscopic studies strongly argue that the coat and exosporium are assembled in a stepwise manner that is coordinated with the overall program of sporulation. Studies in *B. subtilis* have revealed the major steps in coat assembly in that organism. Since *B. subtilis* and *B. anthracis* share many coat protein orthologs, it is reasonable to use these prior analyses in *B. subtilis* as a starting point for elucidating outer layer assembly in *B. anthracis* (see Table 2.1). All bacilli form a spore by dividing the cell into two compartments of unequal size. The smaller of these will become a protoplast that develops into the mature spore (Piggot and Losick, 2002). As in *B. subtilis*, it appears that *B. anthracis* coat and exosporium formation involves the stepwise deposition of proteins synthesized in the surrounding cytoplasm to the developing spore.

Early Events in Assembly

An early event in the assembly of the *B. anthracis* outer spore layers is very likely to be the deposition of the coat protein SpoIVA to the forespore outer membrane, at about the time that the asymmetrically positioned sporulation septum appears (Figure 2.2a). The view that SpoIVA has an important role in outer layer deposition is based on the phenotype of *spoIVA* mutant cells, in which the immature spore is neither dehydrated (needed for spore dormancy, see Chapter 3) nor encircled by the coat and exosporium (Giorno et al., 2007). Instead, the coat and exosporium accumulate as swirls in the cytoplasm that surround the spore. This is extremely similar to the *spoIVA* mutant phenotype in *B. subtilis*, where SpoIVA has been found to localize to the forespore outer membrane (along with and under the control of SpoVM) (Catalano et al., 2001; Driks et al., 1994; Pogliano et al., 1995; Ramamurthi et al., 2006). Therefore, the deposition of SpoIVA at the forespore surface likely marks that surface as the site of subsequent coat and exosporium protein deposition. In this model, SpoIVA binds to the forespore at about 90 min into sporulation, the time at which it is synthesized (Giorno et al., 2007). The sequences of SpoIVA from *B. subtilis* and *B. anthracis* are 85% identical and 92% similar. SpoIVA is extremely well conserved in the Bacilli and is present in all Clostridial genomes examined so far. Taken together with the functional similarities in *B. subtilis* and *B. anthracis*, it is likely that SpoIVA has similar roles in coat deposition in all bacterial spores (Driks, 2002a).

The next readily detected event in coat or exosporium assembly in *B. anthracis* is the appearance of a thin layer called the cap, which forms very close to the forespore membrane, on the side of the spore farthest from the sporulating cell envelope (Figure 2.2c). It should be noted that this takes place well before coat assembly is complete (see below). This thin sheet will subsequently extend to engulf the spore, forming the exosporium basal layer (Figure 2.2d) (Giorno et al., 2007; Ohye and Murrell, 1973; Steichen et al., 2007). Attachment of the cap to the forespore is under the control of the coat protein CotE; *cotE* mutant cells fail to form a cap on the forespore, and the exosporium appears to form swirls in the mother cell cytoplasm that are disconnected from the forespore, presumably because of the lack of some connection that requires CotE (Giorno et al.,

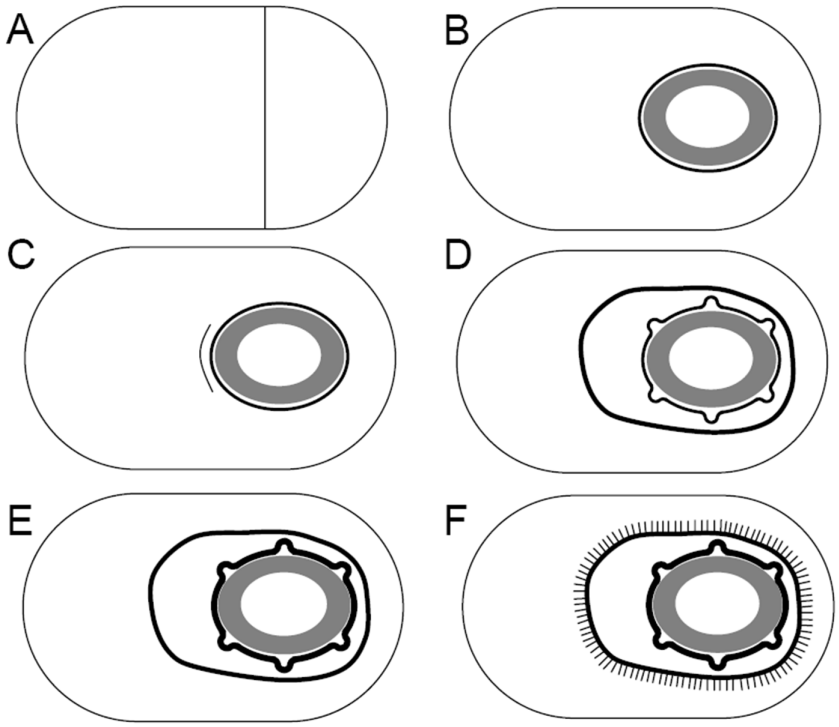

Figure 2.2 Stages of assembly of the *Bacillus anthracis* spore. Each panel shows a successive stage in spore formation and, in particular the assembly of the outer spore structures. (a) A hallmark event indicating the commitment to spore formation is the appearance of an asymmetrically positioned septum that divides the cell into a larger compartment (where the coat and exosporium proteins will be synthesized) and a smaller compartment (which will become the spore). (b) In an endocytic-like event, the septum engulfs the smaller compartment, generating a protoplast possessing an outer membrane (thin line) and, subsequently, a specialized peptidoglycan (thick grey band). (c) The first readily observed event in outer structure assembly is the appearance of the cap (thin line adjacent to the forespore) which sits close to the outer membrane. In some cells, the cap appears even before engulfment is complete. (d) The cap extends around the developing spore, becoming the basal layer. During this process, the distance between the spore and the basal layer increases. By the time the basal layer (thick dark line around the forespore in the figure) has surrounded the forespore, the coat (dark line with ridges) can usually be seen as a thin dark layer. The forespore outer membrane is usually not visible at this stage. (e) Coat assembly continues even after encasement of the spore by the basal layer (indicated by the thicker dark line with ridges). (f) The hair-like projections or nap appears after completion of the basal layer. In the final stage of spore formation, the sporulating cell envelope lyses, releasing the spore into the milieu.

2007). The polymerization of the cap around the forespore depends on the exosporium protein ExsY (Steichen et al., 2005). Without ExsY, released spores possess a small arc of exosporium that appears to have largely fully formed (it harbors the nap) but possesses only the patch of basal layer corresponding to the cap (Steichen et al., 2007).

The protein composition of the cap differs from that of the rest of the exosporium. As already noted, the exosporium protein Alr is excluded from the cap (Steichen et al., 2007). BclA, on the other hand, might initially deposit specifically at the cap; although BclA is present around the entire exosporium surface, in *exsFA*/*bxpB* mutant spores, BclA is more abundant in the cap region of the exosporium (Giorno et al., 2009).

Intermediate and Late Events in Assembly

We define the intermediate and late stages in spore outer layer assembly as the events after cap formation. This is when the majority of coat and exosporium formation occurs, since it is only during or after completion of a contiguous basal layer shell that an electron-dense coat becomes visible (Figure 2.2d) (Giorno et al., 2007; Ohye and Murrell, 1973; Steichen et al., 2007). From TEM, it is clear that once the cap appears, coat and exosporium assembly proceeds concurrently (Giorno et al., 2007; Ohye and Murrell, 1973; Steichen et al., 2007). This observation raises the question of the degree, if any, of coordination between coat and exosporium formation. Presumably, the coat acts as a basement structure upon which the exosporium forms. Beyond that, however, it is possible that exosporium formation follows a distinct assembly program that coincides with coat formation but is not dependent on it. TEM data show that coat assembly continues even after the basal layer is a contiguous shell (Giorno et al., 2007; Ohye and Murrell, 1973; Steichen et al., 2007). Therefore, unassembled coat proteins must be able to reach the coat surface in spite of the basal layer which probably serves as a barrier to protein diffusion (Figure 2.2e) (Ball et al., 2008). Most likely, those coat proteins that assemble after completion of the basal layer have either already accumulated in the interspace (the region between the coat and exosporium) or are synthesized by ribosomes trapped at that location.

In spite of the importance of the intermediate and late stages to spore outer-layer assembly, we know little about coat formation at this time. As in *B. subtilis*, coat assembly in *B. anthracis* is probably a complex process, since there are likely to be as many as 80 *B. anthracis* coat protein species (Driks, 2002a; Giorno et al., 2007; Kim et al., 2006; Lai et al., 2003) (Table 2.1). In *B. subtilis*, assembly of this large number of coat proteins is orchestrated by a relatively small subset of coat proteins, referred to as the morphogenetic proteins (Driks, 1999; Henriques et al., 2004). Given that so many *B. subtilis* morphogenetic coat proteins have *B. anthracis* orthologs, it is plausible that *B. anthracis* coat assembly is also directed by relatively few proteins. Interestingly, neither of the two *B. anthracis* morphogenetic coat protein orthologs that have been studied so far in *B. anthracis*, CotE and CotH, have roles in coat assembly that are as dramatic as their *B. subtilis* orthologs (in *B. subtilis*, mutations in *cotE* and *cotH* result in coats lacking significant numbers of coat proteins; Naclerio et al., 1996; Zheng et al., 1988) (Giorno et al., 2007). TEM reveals that *B. anthracis cotE* mutant spores lack exosporia and that the coat is detached from the underlying cortex in some places, while *cotH* mutant spores appear normal (Giorno et al., 2007). Interestingly, both *cotE* and *cotH* mutant spores have an altered spore surface topography as measured by AFM; spores of both mutants lack the characteristic ridges extending from pole to pole along the long axis of the spore. To the degree that can be determined by gel electrophoresis, *cotE* and *cotH* mutant spores lack only a small number of the usual collection of coat proteins; a *cotE* mutant lacks just one, and a *cotH* mutant about five, of the usual coat protein complement. Consistent with these minor defects in protein composition, *cotE* and *cotH* mutant spore coats resist penetration by lysozyme as well as wild-type spore coats. Therefore, the impact of CotE and CotH on assembly is much less severe than in *B. subtilis* (with the exception of the role of CotE in exosporium assembly, noted above). As described above, both CotE and CotH affect germination in *B. anthracis*.

Coat proteins specific to *B. anthracis* (and its relatives, comprising the *B. cereus* group) also have important roles in coat assembly. Examples of this are Cotα and Cotβ. Cotα controls the assembly of the *B. anthracis* coat outer layer (possibly corresponding

to the *B. subtilis* outer coat) (Kim et al., 2004). *cotα* mutant spores germinate normally and exclude lysozyme but have decreased resistance to phenol, chloroform, and hypochlorite. Therefore, the coat outer layer participates in resistance but is not required for germination or for the coat to act as a barrier to large molecules. Consistent with a role in formation of the outer coat layer, Cotα is synthesized late in sporulation. Cotβ is a small (8.4 kDa) protein on the coat surface that is also specific to the *B. anthracis* and its relatives (Mallozzi et al., 2008). It does not have a readily detected effect on coat protein composition or structure as determined by TEM, but it does control coat surface topography since, in most cases, the surfaces of *cotβ* mutant spores lack clear ridges and have what appears to be a cleft extending along the long axis. It is unknown why the loss of Cotβ results in this morphological feature. Analysis of the timing of Cotβ synthesis (using fluorescence microscopic analysis of cells bearing a Cotβ-GFP fusion protein) suggests that Cotβ is synthesized at or soon after the spore protoplast appears, but does not actually deposit at the spore surface until about an hour later. The hour gap between synthesis and deposition suggests the involvement of an unknown factor that directs Cotβ assembly.

Another spore protein that might impact coat assembly in *B. anthracis* is ExsA, since it has high sequence identity to the *B. subtilis* protein SafA, a coat component playing a major role in coat assembly (Bailey-Smith et al., 2005; Ozin et al., 2000, 2001; Takamatsu et al., 1999a). In *B. cereus*, *exsA* mutant cells produce spores that have highly disorganized coats and exosporia, and in some cases, appear to lack both structures (Bailey-Smith et al., 2005). These phenotypes are consistent with the possibility that ExsA is a coat protein that directs coat formation as well as exosporium deposition.

In contrast to coat assembly, which is poorly understood, some of the critical events in exosporium assembly during the intermediate and late stages are coming into focus. As already discussed, a major intermediate step in exosporium assembly is the formation of the basal layer. The full set of basal layer proteins is still unknown. One of its components is ExsFA/BxpB (Giorno et al., 2009; Redmond et al., 2004; Steichen et al., 2005). However, since *exsFA*/*bxpB* mutant spores possess an intact basal layer, ExsFA/BxpB is unlikely to be a major basal layer structural element. ExsY is a strong candidate for a basal layer protein since it forms a complex with ExsFA/BxpB (Redmond et al., 2004). Since *exsY* mutants assemble the exosporium cap but progress no farther in basal layer formation (Steichen et al., 2007), it is reasonable to propose that either ExsY is a principal structural component of most of the basal layer or that it governs polymerization of other basal layer proteins. Either way, it is a major factor in intermediate exosporium assembly.

The manner in which the cap or the basal layer is connected to the forespore surface is unknown. Presumably, the reason the polymerizing basal layer stays associated with the forespore, ultimately encasing it, is because of as yet unidentified molecules connecting the exosporium to the coat surface. Candidates for these molecules include the large set of proteins that fractionate with the exosporium but have not yet been shown to be localized solely to that layer. Three especially good candidates are CotY, ExsFB, and IunH. In *B. cereus,* scanning electron microscopy suggests that the exosporia of *cotY* mutant spores are in closer contact with the coat than in wild type (Johnson et al., 2006). A simple explanation for this observation is that CotY is an interspace protein that determines interspace width. Possibly, CotY has a similar role in *B. anthracis*. IunH and ExsFB are interspace protein candidates because, in cells bearing either ExsFB-GFP or IunH-GFP, fluorescence localizes to the spore, as expected, but with an unusual pattern (Giorno et al.,

2009). Rather than forming a ring, as do coat and exosporium proteins, the fluorescence from either fusion protein forms a disk that covers the spore. One interpretation of this fluorescence pattern is that ExsFB-GFP and IunH-GFP are in the interspace and, due to the thickness of the interspace (as compared to the exosporium or coat), fluorescence appears as a disc (Giorno et al., 2009). If so, then these two proteins may have some role in connecting the exosporium to the spore.

The nap (or hair-like projections) is the last structure to appear in spore formation (Figure 2.2f). As already discussed, the best characterized protein component of this structure is BclA. ExsFA/BxpB has an important role in BclA deposition. *exsFA*/*bxpB* mutant spores lack the hair-like projections but, interestingly, still retain a small amount of BclA, concentrated at one pole of the spore, as mentioned above (Giorno et al., 2009). Spore-associated BclA is modified by the addition of the sugar anthrose (2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-D-glucose) which is linked to the collagen-like region of BclA (Daubenspeck et al., 2004). Anthrose biosynthesis is most likely directed by a cluster of four genes transcribed during sporulation (Dong et al., 2008). Interestingly, two genes in the cluster are homologs of genes (*spsA-L* and *cgeA-E*) possibly involved in the biosynthesis of spore-associated polysaccharides in *B. subtilis* (Charnock and Davies, 1999). At least one additional cluster of genes, the rhamnose biosynthesis operon (harboring orthologs *spsI-L*), is also involved in spore-surface glycosylation (Bozue et al., 2005; Charnock and Davies, 1999).

The assembly of the exosporium-surface is more complex than just the deposition of BclA since the glycoprotein BclB is also present on the exosporium surface (Thompson et al., 2007; Waller et al., 2005). BclB has high sequence similarity to BclA including, in particular, a collagen-like region. Another intriguing similarity is an N-terminal motif that is shared between BclA and BclB that directs these proteins to the exosporium basal layer during spore assembly (Thompson and Stewart, 2008). Apparently, there are other mechanisms that target spore proteins to the basal layer, as this motif is not present in any other known spore proteins (Mallozzi and Driks, unpublished observations).

SUMMARY AND FUTURE DIRECTIONS

Our understanding of *B. anthracis* spore composition, assembly, and function has significantly improved in recent years. No doubt, these new insights will facilitate better understanding of anthrax pathophysiology and the development of improved therapeutics that target the spore. In particular, from the renewed focus on the composition and structure of the exosporium, it should be possible to elucidate the molecular interactions between spores and host cells as well as inert surfaces, likely to be key factors in survival and pathogenesis. Importantly, these recent findings have also had major impact on basic studies seeking a deeper understanding of how spores remain dormant, defend against severe conditions, and assemble complex multi-protein structures. By analyzing the spore in *B. subtilis* and *B. anthracis* in parallel, we can more reliably identify conserved features of spore function and assembly, and gain better insights into how diverse species solved the problem of surviving in and adapting to a range of harsh environments. Perhaps, from the analysis of these two species, we can begin to understand the adaptive evolutionary processes themselves that led to the fascinating diversity in spore outer structures among the Bacilli.

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Chapter 3

Anthrax Spore Germination

Nathan Fisher, Katherine A. Carr, Jonathan D. Giebel,
and Philip C. Hanna

INTRODUCTION

Anthrax is a zoonosis that primarily inflicts wild and domesticated ruminants, although most mammals are considered susceptible to some extent (Dixon et al., 1999). In the mid-eighteenth century, Casimir-Joseph Davaine first noted that the blood of sheep suffering from anthrax contained rod-shaped bacteria and that their transfer to an otherwise healthy animal would initiate anthrax in the experimental animal (Wilson and Miles, 1975). Robert Koch (1877) later expanded that observation and decidedly demonstrated that *Bacillus anthracis* was the etiological agent of anthrax.

Like many members of the *Bacillus*, *Clostridium*, and other closely related genera, *B. anthracis* is capable of differentiation between two distinct cellular morphologies, the vegetative cell and the spore. The spore is a unique cell type notable for a complete lack of detectable metabolic activity, extreme longevity, and an ability to withstand environmental conditions disadvantageous for vegetative life. Spores are relatively resistant to heat, noxious chemicals, desiccation, starvation, and a number of additional stresses compared to cells of the vegetative morphology (Moir et al., 2002; Setlow, 2003). Enhanced resistance properties are linked directly to their unique cellular structures, chemistry, and morphology. These include, in part, a thick modified cell wall (cortex), protective protein coats, a partially dehydrated core, high concentrations of core Ca^{++}-dipicolinic acid (DPA), a high proportion of protease-resistant proteins, and other specialized attributes (Bergman et al., 2006; Liu et al., 2004; Moir, 2006; Moir et al., 2002; Setlow, 2003). The intentional release of *B. anthracis* spores as an act of biological terrorism remains a threat to target populations (both humans and livestock), and many of the longevity, unit size, and resistance properties inherent in the spore contribute to these concerns (Dixon et al., 1999).

ANTHRAX AND THE SPORE

In his early work, Koch (1881a, b) also showed that *B. anthracis* spores are more pathogenic to experimental animals than are cells of the vegetative morphology. He noted that

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

vegetative cells were only infectious if inoculated directly into the blood of an experimental animal while spores were highly infectious regardless of the route of inoculation. In nature, there are four distinct presentations of anthrax, each depending on the original site of spore inoculation into the host animal. Inoculation into an abrasion of the epidermis results in cutaneous anthrax, the most common form seen in the human population. Gastrointestinal anthrax is caused by ingestion of spores (Dixon et al., 1999; Sirisanthana et al., 1984; Sirsanthana and Brown, 2002; Watts et al., 2008, 2009). For ruminant hosts, by far the most commonly affected species during natural outbreaks, the most common route of entry for *B. anthracis* spores is believed to be through abrasions in the oral cavity or pharynx, which causes oropharyngeal anthrax (Glomski et al., 2007; Minett, 1952; Navacharoen et al., 1985; Quinn and Turnbull, 1998). Gastrointestinal and oropharyngeal anthrax are often confused with each other in the literature, and some sources fail to distinguish the two. Finally, the highest rates of mortality are seen in cases of inhalation anthrax, which occurs upon inoculation of *B. anthracis* spores into the lung airways of a susceptible animal (Dixon et al., 1999). Except for the case of direct inoculation into the blood of a host animal, the infectious dose of vegetative cells is considerably higher than that of spores (Fisher, 2006; Koch, 1881b).

Thus, in addition to mediating environmental survival of *B. anthracis*, the spore morphology provides an initial advantage to the bacterium during the establishment phase of anthrax. Perhaps a successful infection is initiated after the spore morphology provides resistance to certain innate immune mediators as has been observed for defensins and macrophages (Fisher et al., 2006). However, the spore must convert to a metabolically active vegetative cell, through the processes of germination and outgrowth, in order to multiply and maintain the infection. Although spores are metabolically dormant, they passively monitor their surroundings and are able to appropriately trigger germination after sensing a change in their local conditions indicative of inoculation into a host animal. Thus, spore germination is considered the first step in the pathogenesis of anthrax.

SPORE GERMINATION *IN VIVO*

Due to high rates of human morbidity and mortality, inhalational anthrax has received far more research attention than have the cutaneous, gastrointestinal, or oropharyngeal forms. In 1957, Ross published a landmark study on inhalation anthrax in guinea pigs (Ross, 1957). These studies were consistent with clinical observations of no initial bacterial growth in the lungs (e.g., no pneumonia). Germination of spores was not observed in air spaces proper; however, a percentage of spores were observed associated with regional phagocytes, and were efficiently and rapidly phagocytosed by these immune cells. It was concluded that spore germination did not occur in the air spaces of the lungs. Instead, Ross went on to document that a significant number of spore-containing phagocytes (she referred to them as macrophages at that time) detached from the lung and migrated toward the regional lymph nodes in the chest. These nodes seemed to serve as sites of initial bacterial growth ultimately leading to intensive localized necrosis that preceded widespread dispersal of bacilli throughout the body via the lymphatic and circulatory systems. In a series of elegant and thorough studies using staining methods that distinguished ungerminated spores from recently germinated spores from bacilli, Ross observed that some spores likely germinated within these cells while en route from lung to lymph node. Her initial observations provided firm footing for host-induced spore germination studies that were re-initiated decades later.

In 1999, Guidi-Rontani et al. (1999b) used confocal scanning laser microscopy and image cytometry analysis to provide high-resolution images of *B. anthracis* (Sterne strain) spores associated with host immune cells during a murine model of inhalational anthrax. Using immunohistochemistry and a *lacZ*-expressing strain of *B. anthracis*, they showed both dormant spores and spores that had recently germinated within phagolysosomal compartments of alveolar macrophages harvested by bronchial alveolar lavage. In more recent work, Sanz et al. (2008) constructed genetically modified *B. anthracis* (Sterne strain) spores that become luminescent upon germination. Using an *in vivo* imaging system and *in situ* analysis, they showed that these spores rapidly associated with alveolar macrophages after intranasal inoculation, and that cell-associated germination was evident in the lung within 30 min. Notably, they were unable to detect bacterially derived luminescence within lung-associated lymph nodes, indicating that if germinated spores were trafficked there, the luminescence decayed prior to arrival and/or only a small percentage of spores reached the lymph nodes. The results of Sanz et al. (2008) contrast sharply with those of Cote et al. (2006), which showed that the vast majority of Ames strain spores present in the lung remained ungerminated for up to 96 h post-inoculation. This difference could be attributed to the different *B. anthracis* strains used in the two studies. Or, alternatively, the luminescence detected in the lung by Sanz et al. (2008) could have originated from a small percentage of the bacterial population, most of which could have remained dormant and, therefore, non-luminescent. Using spores containing the green fluorescent protein and a variety of immunohistochemical and fluorescent activated cell sorting analyses of tissues and cells harvested from infected animals, Cleret et al. (2007) also observed that alveolar macrophage rapidly phagocytose *B. anthracis* (Sterne strain) spores while generally remaining within the alveolar spaces. However, they were able to extend their analysis and convincingly demonstrate that lung dendritic cells capture spores within 30 min and traffic them to regional lymph nodes—matching very closely the findings of Ross—but with increased resolution between various types of lung phagocytes. It is important to note that the studies by Ross (1957), Guidi-Rontani et al. (1999b), Welkos et al. (2002), Lyons et al. (2004), Cote et al. (2006), and Cleret et al. (2007) provide only snapshots of events during the establishment phase of anthrax and thus cannot conclusively determine if spore germination occurred before or after phagocytosis (both have been shown possible *in vitro*). Furthermore, all of the above studies were unable to determine if the observed processes are part of the actual productive establishment pathway, or simply analysis of the bulk flow of spores, some or most of which may not contribute to fulminating disease given the typically high lethal dose for *B. anthracis* exposure. We believe *in vivo* imaging technologies, like those employed by Sanz et al. (2008), are poised to address both of these complications pending technological improvements beyond the current state of both sensitivity and multi-signal analysis.

Given their apparent importance to the infection process, several groups have attempted to characterize interactions between *B. anthracis* spores and phagocytic immune cells *in vitro* (Basu et al., 2007; Dixon et al., 2000; Hu et al., 2007; Ireland and Hanna, (2002b); Kang et al., 2005; Ribot et al., 2006; Weiner et al., 2003). Both primary macrophages and macrophage-like cell lines readily associate with, and phagocytose, bacterial spores of all genera tested. While spores of those non-pathogenic *Bacillus* species tested show no increase in germination in the presence of cultured macrophages, many groups have observed *B. anthracis* spores to respond both rapidly and efficiently to this stimulation. In our studies, germination begins within minutes of associating with host cells, reaching efficiencies of >85% in 10 min (Dixon et al., 2000; Ireland and Hanna, (2002b); Weiner and Hanna, 2003). It was, however, noted in this work that while phagocytes are adept

and efficient at binding to and phagocytosing spores, neither property was absolutely required for germination. It was found that filtered, cell-free macrophage-conditioned media provided all necessary components for robust germination (Weiner and Hanna, 2003). Control experiments strongly suggested that macrophage conditioning of the medium involved the host cells contributing small molecule germinant(s) to those already present in the medium as opposed to their removing potential germination inhibitors from the medium. Conditioning of Hank's Balanced Salts solution, which does not support *B. anthracis* spore germination without the addition of nutrient germinants, was found to depend on both the number of macrophages in the culture and the amount of time allowed for conditioning.

Hu et al. (2007) extended this finding and showed that spore germination within a macrophage culture system depends on both the phagocytic cells and the choice of media supplements. When all germinants were carefully removed from the media, very little germination took place either before or after phagocytosis, and most spores remained viable within phagocytic compartments, in contrast to those that germinated, which were mostly inactivated by the antibacterial functions of the macrophage. In the course of a natural infection, extracellular nutrients present in interstitial fluids are expected to contact spores and contribute to germination, but perhaps the findings of Hu et al. (2007) are relevant to understanding differences in virulence toward various host species. If the lung interstitial fluids of some hosts are especially poor at triggering spore germination, then a higher percentage of spores could survive phagocytosis and be trafficked to regional lymph nodes where they are able to establish a foci of infection.

Experimental animal models have recently been developed for the study of cutaneous and gastrointestinal anthrax (Bischof et al., 2007; Glomski et al., 2007; Hahn et al., 2005, 2008). Bischof et al. (2007) evaluated *B. anthracis* spore germination after inoculation onto either the intact or abraded skin of experimental mice. They found that significant spore germination occurred only after abrasion of the skin. Using a filter system, they also showed that host-induced germination during cutaneous anthrax did not require contact between the spores and host immune cells, a finding that is consistent with macrophage germination studies outlined above. *B. anthracis* spores germinate readily in whole blood or sera from a number of mammals (Fisher, 2006), and presumably the abraded skin allowed contact between spores and these fluids. Similarly, Glomski et al. (2007) observed that abrasion to the mucosal surface of the intestines enhanced infection by the gastrointestinal route, although it did not seem to be absolutely required. The roles of spore germination or of phagocytic cells were not directly examined in this study, but the authors did find apparent foci of infection at the Peyer's patch, a location dense with phagocytic immune cells. On the whole, and perhaps not surprisingly, these studies indicate that *B. anthracis* spores are exquisitely sensitive to signals present within the host, and there seems to be a variety of situations in which host-initiated germination can take place and lead to fulminating disease.

SPORE GERMINATION *IN VITRO*

The mechanistic components of germination are highly similar among all spore-forming species studied to date, while the regulation of germination and outgrowth appears specific to the particular environmental niche of each species (Fisher and Hanna, 2005). Germination can be initiated by the regulated sensing of environmental signals, termed nutrient-triggered germination, or by the nonregulated degradation of protective spore structures including

the coats, cortex, or inner membrane, which is termed non-nutrient-triggered germination (Paidhungat and Setlow, 1999; Setlow, 2003; Setlow et al., 2001). The latter is generally considered a default mechanism that allows for an attempt at vegetative growth when a spore is damaged by its environment to a degree that will soon affect its viability. In contrast, nutrient-triggered germination is considered the means by which spores sense the niche appropriate for vegetative growth (Paidhungat and Setlow, 2000). Since *B. anthracis* spore germination within a host is a highly regulated process, it is most likely that this occurs via nutrient-triggered pathways.

Nutrient-triggered germination has been well studied in a variety of spore-forming species, and much is known regarding the specific signals that initiate the process (Moir et al., 2002). The signaling molecules vary widely among spore-forming bacterial species, but in general, small molecule nutrients are recognized by receptors located within the inner spore membrane (Clements and Moir, 1998; Fisher and Hanna, 2005; Foster and Johnstone, 1986; Hornstra et al., 2005; Hudson et al., 2001; Paidhungat and Setlow, 2001; Preston and Douthit, 1988; Racine et al., 1979; Rossignol and Vary, 1979). For *B. anthracis* spores, in contrast to non-pathogenic spores, no single chemical species alone at physiological concentrations was found to be sufficient to initiate the process. Instead, combinations of two or more different chemical species are required, with the presence of additional germinants reducing the combined concentrations of total germinants in the solution required for breaking of dormancy. The discovery of the specific chemical combinations capable of germinating *B. anthracis* spores was accomplished via studies involving chemically defined solutions in combinations with phenotypic analyses of mutant strains harboring defined deletions in germinant receptor genes (*gerA* family) believed responsible for recognition of those chemicals. The receptor genes are discussed below in detail. To summarize a number of studies, the major germinants for *B. anthracis* spores are alanine *or* purine ribonucleosides, with a variety of amino acids capable of serving as co-germinants in the presence of alanine or purine ribonucleosides (Fisher and Hanna, 2005).

In *B. anthracis*, five distinct germination pathways are now recognized (Fisher and Hanna, 2005; Ireland and Hanna, 2002a; Weiner and Hanna, 2003). The alanine germination pathway (Ala) requires only the presence of L-alanine in concentrations considerably greater than what is believed available in the host. At physiologically relevant concentrations, L-alanine can cooperate with L-proline, comprising the alanine and proline (AP) response, or with L-histidine, L-tyrosine, or L-tryptophan to make up the aromatic amino acid-enhanced alanine (AEA) pathway. Purine ribonucleosides have been noted to be of particular importance to germination of some *Bacillus* spores (Barlass et al., 2002; Clements and Moir, 1998; Preston and Douthit, 1988). In *B. anthracis*, these nutrients must be in combination with a second co-germinant in order to trigger endospore germination (Fisher and Hanna, 2005; Ireland and Hanna, 2002a). Inosine is the most potent purine co-germinant and is able to combine with several amino acids to comprise the amino acid and inosine-dependent (AAID) responses. AAID-1 includes the binary combination of inosine and L-alanine, L-serine, L-valine, L-methionine, or L-proline, while in AAID-2 inosine pairs with L-histidine, L-tyrosine, L-tryptophan, or L-phenylalanine (Ireland and Hanna, 2002a).

Exposure to these specific combinations of nutrients (or recognition of a favorable environment through as yet undiscovered pathways) rapidly initiates a series of complex biophysical and biochemical changes within the cell, ultimately leading to restoration of the vegetative growth cycle. Two distinct components are recognized within this differentiation process: germination and outgrowth (Figure 3.1). Germination begins with a

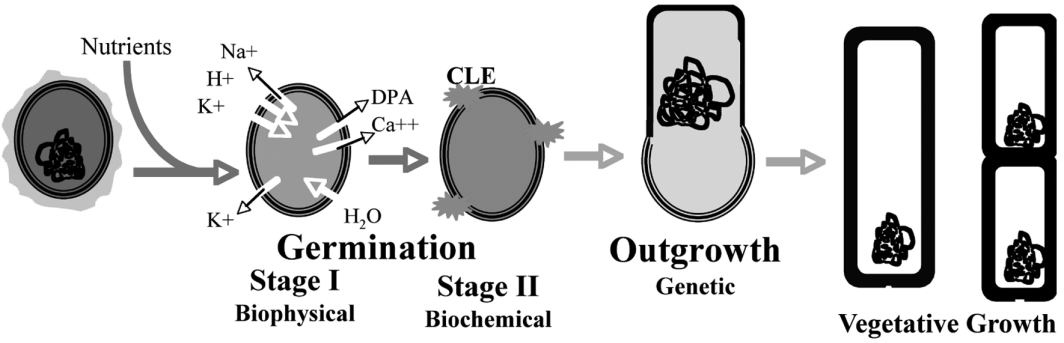

Figure 3.1 Germination of *Bacillus anthracis*. Upon recognition of specific chemical signals that are indicative of conditions desirable for vegetative growth, the cell undergoes a differentiation program to return to the vegetative morphology. The first step in this process is germination, defined as the biophysical (Stage I) and biochemical (Stage II) breaking of dormancy. The second step is outgrowth, defined as the novel synthesis of vegetative cell components. Upon the completion of outgrowth, the cell returns to the vegetative morphology where replication is possible (Fisher, 2006, PhD dissertation).

trigger reaction that is unidirectional. The germination trigger reaction is regulated by germinant receptors located within the inner spore membrane. Through unknown mechanisms, which presumably involves ligand–receptor interactions, receptor "activation" initiates Stage I of germination. Stage I consists of a series of ion and solute fluxes across the inner membrane, most notably hydration of the core and expulsion of large depots of calcium and DPA. In turn, Stage I events initiate Stage II germination which involves activation of preformed spore enzymes that function to degrade spore-specific protective structures such as the small acid soluble proteins in the core that served to preserve the chromosome as well as the modified cortex peptidoglycan responsible for cellular rigidity during dormancy. Novel synthesis of cellular components of the vegetative cell (of all macromolecular varieties) is considered the onset of outgrowth, which continues until the first round of binary fission when cells are defined as having resumed vegetative growth (Setlow, 2003).

GERMINATION MACHINERY

The known proteins residing in the *B. anthracis* spore that are believed either to regulate or to contribute to germination include: (a) enzymes that may inhibit inappropriate, or premature, germination; (b) systems for facilitating permeation of outer spore layers by host-specific germinants; (c) membrane receptors that specifically recognize germinants and act to signal membrane fluxes of water, ions, and DPA into and out of the core; (d) a newly discovered sensory protein capable of initiating germination in response to rapidly growing cells in the vicinity of the spore; and (e) spore cortex lytic enzymes acting to hydrolyze the protective spore wall.

Germinant Modification by Spore Enzymes

As mentioned earlier, the fact that spores are considerably more infectious than vegetative *B. anthracis* cells indicates a specific function for the spore morphology during infection.

Moreover, recent studies show that premature initiation of germination, either by *in vitro* germination of spores immediately prior to inoculation or by adding a germination-stimulating compound to the inoculation mix, results in an increased infectious dose. Furthermore, addition of germination-inhibiting compounds can have the opposite effect of decreasing the infectious dose (Cote et al., 2008; Fisher, 2006; McKevitt et al., 2007). Interestingly, *B. anthracis* spores contain proteins that appear to function in order to prevent premature activation of germinant pathways. As germinant molecules diffuse through the outer structural layers of the *B. anthracis* spore, they are subject to modification by spore-associated enzymes present in these layers. Titball and Manchee (1987) were the first to report that *B. anthracis* spores germinated poorly when inoculated into a germination medium at high cell densities. However, the addition of an alanine racemase inhibitor, O-carbamyl-D-serine, removed the cell density-dependent inhibition of germination (Titball and Manchee, 1987). The D-enantiomer of alanine serves as a competitive inhibitor of L-alanine-induced germination in a number of *Bacillus* spp, and work by Chesnokova et al. (2009) clearly established the presence of alanine racemase in the outer layers of the *B. anthracis* spore. McKevitt et al. (2007) showed a strict, nonlinear relationship between spore density, D-alanine production, alanine racemase activity, and spore germination profiles both *in vitro* and in association with the RAW 264.7 murine macrophage-like cell line. Interestingly, higher multiplicities of infection (MOI) progressively inhibited spore germination in an alanine racemase-dependent fashion. Ribot et al. (2006) reported a strong MOI-dependent correlation between macrophage-mediated bacterial killing in a co-culture model of infection wherein macrophages that phagocytosed small numbers of spores readily eliminated the bacterial cells, but those that phagocytosed larger numbers of spores achieved incomplete killing and eventually succumbed to the ensuing bacterial infection. Since dormant spores are relatively resistant to macrophage-mediated bacterial killing, an MOI-dependent delay in spore germination could prove an important factor in determining the outcome of interactions between *B. anthracis* and these important cells of the host immune system.

Early studies on *Bacillus* spores revealed the presence of purine hydrolase activity in species closely related to *B. anthracis* (Lawrence, 1955). Subsequent proteomic analysis of either whole *B. anthracis* spores or purified exosporia indicated the presence of putative purine hydrolase enzymes in the outer layers of the spore (Bergman et al., 2006; Liu et al., 2004; Redmond et al., 2004; Steichen et al., 2003; Todd et al., 2003). Homologous enzymes have been characterized in the closely related species *B. thuringiensis* where they function to inhibit purine-mediated spore germination (Liang et al., 2008). While it has not yet been directly tested, it is likely that these enzymes also serve to inhibit purine-dependent germination pathways in *B. anthracis* spores. Since it is likely that purine nutrients are at least one signal recognized by *B. anthracis* spores upon exposure to macrophages (Weiner and Hanna, 2003), the purine hydrolase activity of the spore may function in a manner similar to that described above for alanine racemase in delaying spore germination and enhancing survival when phagocytosed by host immune cells.

Germinant Permeation of the Outer Layers of the Spore

Initiation of germination requires that small molecule germinants are able to make their ways through the spore's outer layers and find their appropriate receptors at the inner membrane. As spores germinate within minutes upon contact with appropriate small mol-

ecules, germinant entry may involve some manner of selectivity through outer protective barriers. First identified in *Bacillus cereus*, a six gene operon termed $gerP_{ABCDEF}$ (*gerP*) may be involved in this process (Behravan et al., 2000). The *gerP* operon, with the "P" denoting "permeation," is under control of a σ^K promoter, characteristic of spore coat-associated genes transcribed in the mother cell during sporulation (Behravan et al., 2000; Kroos and Losick, 1989). A similar operon has been identified in *B. anthracis*, which shares 99% sequence similarity, and 97% protein identity to the *gerP* operon in *B. cereus* (Read et al., 2003).

The genes of the *gerP* operon are not predicted to encode any known enzymes, yet its disruption results in a kinetic defect in germination *in vitro* for all *Bacillus* species tested, including *B. anthracis* (Behravan et al., 2000; Carr et al., 2010; Fisher, 2006). Additionally, *B. cereus* spores lacking the full *gerP* operon do not exhibit any morphological defect, suggesting that GerP proteins do not play a major role in structural integrity or stability of the spore itself (Behravan et al., 2000). As GerP proteins are predicted to reside in the metabolically dormant spore coat and disruption of the operon results in a germination defect, it is possible that GerP proteins localize to the outermost layers of the spore, facilitating interactions between germinants and receptors. It is hypothesized that this can be accomplished through the formation of a pore-like complex, or a chaperone-like system, shuttling germinants across the spore's outer layers to the inner membrane. It is possible that GerP proteins might also function as an outward-flowing channel, as previous work has demonstrated that Ca^{++}-DPA released from the core during germination can activate cortex hydrolysis by the coat-localized enzyme CwlJ (Paidhungat et al., 2001). Therefore, the possibility exists that the GerP proteins may function in facilitating the translocation of Ca^{++}-DPA out through various spore layers to target enzymes (Carr et al., 2010). Although much remains to be learned about GerP proteins, they are the most popular candidates for facilitating germinant entry into the spore. Further studies will provide better insight into mechanisms of germinant selectivity and transport and, perhaps, the stability of *B. anthracis* spores.

Germinant Recognition

Guidi-Rontani et al. (1999a) were the first to describe a genetic basis of *B. anthracis* spore germination. Upon examination of the DNA sequence of a pathogenicity island on the pXO1 virulence plasmid, they identified a tricistronic operon, which they named *gerX*, with similarities to germinant receptor loci identified in other spore forming species. Using a transcriptional fusion to the *lacZ* reporter, they showed that *gerX* is expressed solely within the developing spore. Mutation of the *gerX* locus through allelic exchange prevented normal germination of spores after exposure to murine alveolar macrophages and slightly attenuated virulence in a murine model of anthrax. While the *gerX*-null strain was still capable of initiating disease, appearance of vegetative cells, and their subsequent spread in the host was significantly delayed. Thus, the authors hypothesized that the rate of spore germination may play a significant role in the establishment of anthrax—a possibility that contemporary studies have largely corroborated (Fisher, 2006; McKevitt et al., 2007). Interestingly, mutation in *gerX* does not affect *in vitro* germination in response to any known germinant molecules, and the cognate ligand of this receptor remains unknown (Fisher and Hanna, 2005).

As the whole genome sequence of *B. anthracis* became available, the remaining repertoire of germinant receptor loci was identified and characterized (Read et al., 2003).

The *gerX* locus proved to be the only germinant receptor loci on either of the two *B. anthracis* virulence plasmids. However, an additional six homologous operons were found on the chromosome. Two of these, *gerA* and *gerY*, contain pseudogenes and were experimentally determined not to contribute to virulence or known germination responses (Fisher, 2006; Fisher and Hanna, 2005; Read et al., 2003). Analysis of transcriptional fusions with the *lacZ* reporter gene and whole transcriptome analysis of *B. anthracis* cells grown in nutrient-limiting conditions showed that the remaining four chromosomally encoded operons, *gerS*, *gerH*, *gerL*, and *gerK*, were all expressed in developing *B. anthracis* spores (Bergman et al., 2006; Fisher and Hanna, 2005; Liu et al., 2004). Although germinant receptors are believed to be present in very low abundance, with only a few copies of each protein per cell (Bergman et al., 2006; Setlow, 2003), two of the three GerH moieties were detected in the *B. anthracis* spore proteome (Bergman et al., 2006; Liu et al., 2004).

B. anthracis spore germination can be readily achieved in the laboratory following exposure of spores to specific combinations of small molecule germinants. Therefore, a genetic approach was used to find cognate ligands for the GerS, GerH, GerL, and GerK receptors. Multiple, large-scale surveys of potential host-associated germinant molecules (including all common amino acids and nucleosides as well as most commercially available hormones and vitamins) revealed several nutrient combinations that trigger efficient germination of *B. anthracis* spores *in vitro*. L-alanine is the only known germinant that can trigger *B. anthracis* spore germination without the presence of a second co-germinant molecule. Concentrations of L-alanine greater than 30 mM efficiently triggers spore germination, but due to the high concentration requirements, this response is not believed to be physiologically relevant. Carr et al. (2010) showed that the presence of only one germinant receptor was sufficient to stimulate germination, as long as the appropriate nutrient signals were present.

Various binary combinations of micromolar concentrations of L-alanine, L-proline, L-serine, L-valine, L-methionine, L-histidine, aromatic amino acids, and purine ribonucleosides serve as potent germinants for *B. anthracis* spores *in vitro*. [It is worth noting that the ionic conditions and pH of the aqueous buffer used can have a significant effect on spore germination profiles. In the majority of our work, we have used commercially obtained phosphate-buffered saline (PBS) at a pH of 7.0 in order to provide what we believe to be a consistent and physiologically relevant ionic environment. The mechanism(s) of ion contribution to the germination process is currently unknown. Although the *in vitro* surveys of potential germinant molecules were not exhaustive and additional small molecule germinants likely exist, the studies conducted to date allow us to present a hypothesis on the contributions of germinants and receptors to anthrax pathogenesis. Interestingly, *B. anthracis* germinant receptors appear to function as molecular pattern receptors, whereas each receptor recognizes several nutrients from a broad class of compounds. For example, germination profiles of spores lacking GerS indicate that this receptor functions to recognize aromatic amino acids and L-histidine. Similarly, the GerH receptor recognizes multiple purine ribonucleosides. The GerK receptor recognizes L-alanine, L-proline, and L-methionine, while GerL recognizes L-alanine, L-serine, and L-valine. The most likely mechanism for such promiscuity is the existence of flexible ligand binding sites within this class of receptors, although this possibility has not yet been studied directly. Whatever the mechanism, promiscuous germinant recognition may contribute to the extremely broad host range and/or effective routes of inoculation of *B. anthracis*. Indeed, Carr et al. (2010) showed that strains expressing only one germinant receptor exhibited a wide range of abilities to stimulate germination in a murine model of infection. For example, one host

species or physiological site of inoculation may expose invading spores to increased concentrations of inosine, while another host or site may expose it to L-alanine, but in either case, the GerS receptor recognizes the change in local conditions and contributes to an appropriate germination response.

Alternative Means of Germination Initiation

Recently, Shah et al. (2008) described a newly identified germination pathway that functions in *B. anthracis* and *Bacillus subtilis* in which the dormant spore senses and germinates in response to peptidoglycan fragments released by other bacteria growing in the local vicinity. This germination pathway functions independently from the nutrient pathways described above in that it does not require germinant receptors, but instead requires a membrane-associated Ser/Thr kinase, PrkC, which was shown to directly bind peptidoglycan. Interestingly, pharmacological agonists and antagonists of similar Ser/Thr kinases were used to demonstrate an apparently direct role for the kinase activity of PrkC in this germination pathway. While the results were very compelling, it remains unclear how such activity exists within the metabolically dormant endospore and how the signal is transduced to the rest of the germination machinery. Furthermore, it is immediately obvious that environmental bacteria could use proximal growth of similar species as a reliable signal of a supportive local environment, but the significance of this pathway to anthrax pathogenesis is unclear. In addition to this newly found role in germination, the PrkC protein is important for the vegetative growth cycle as well; thus, it is possible that this finding represents an ancestral germination pathway that has been maintained in *B. anthracis* due to selection for PrkC function during vegetative growth and not germination. Alternatively, the PrkC germination pathway could function to synchronize spore germination within a host, where a particular spore that germinates and begins to grow within the host will trigger the germination of nearby spores, thus increasing the number of active bacterial cells capable of contributing toward disease establishment.

Degradation of the Spore Cortex

The spore cortex is a thick layer of modified peptidoglycan that contributes much to the spore's environmental resistance properties (Popham, 2002). Upon germination, removal of the protective cortex is believed to be required for outgrowth and the subsequent resumption of the vegetative growth cycle. The cortex barrier is broken down by enzymes believed to reside in the spore, dormant until activated during germination. These enzymes are named germination-specific lytic enzymes (GSLEs) and allow full core rehydration and cell outgrowth (Setlow, 2003). Typical bacterial cell wall peptidoglycan consists of polysaccharide chains of repeating *N*-acetyl glucosamine and *N*-acetyl muramic acid, joined by β(1,4) glycosidic bond; this structure is modified in the spore. In one major modification, 50% of muramic acid residues (alternating every other) are converted to muramic-δ-lactam residues (Lambert and Popham, 2008; Popham, 2002). This modification is essential for the specificity observed with the GSLEs and prevents degradation of the bacterium's vegetative cell wall during cortex hydrolysis (Moir et al., 2002).

For *B. anthracis,* the enzymes SleB and CwlJ serve partially redundant roles and are necessary for full cortex hydrolysis and spore germination (Heffron et al., 2009). SleB is a lytic transglycosylase that when activated, by an unknown mechanism, hydrolyzes the bond between *N*-acetyl muramic acid and *N*-acetyl glucosamine (Boland et al., 2000). In

B. anthracis, and in *B. subtilis*, the *sleB* gene is found in a bicistronic operon with *ypeB*. Although the function of YpeB is not known, deletion of *ypeB* prevents SleB activity during spore germination (Atrih and Foster, 2001; Boland et al., 2000). No specific enzymatic activity, *in vitro*, has been attributed to CwlJ; however, it is required for full germination, and it shares a homologous catalytic domain with SleB (Moir, 2006). It has been shown that CwlJ is localized to the spore coat and that it is necessary for spore germination by exogenous Ca^{++}/DPA treatment (Bagman and Setlow, 2002; Paidhungat et al., 2001).

In *B. subtilis* and *B. cereus cwlJ* is found in an operon with *gerQ*, and in *B. subtilis gerQ* was shown to be required for CwlJ activity (Ragkousi et al., 2003). The *B. anthracis* genome contains two homologs of *cwlJ* (termed *cwlJ1* and *cwlJ2*; Heffron et al., 2009; Read et al., 2003) compared to the single copy found in *B. subtilis* and *B. cereus. B. anthracis cwlJ1* is found in an operon with *gerQ,* but *cwlJ2* is in a different locus, not in an operon with a *gerQ*-like homolog (Read et al., 2003). GSLE activation represents a critical step in germination, and there is renewed interest in understanding the mechanisms underlying the regulation of these enzymes in the context of spore germination. A recent study indicates that SleB or CwlJ1 are required for full virulence in a murine model of inhalation anthrax. Although in this study loss of CwlJ2 results in further attenuation in the absence of SleB and CwlJ1, loss of the CwlJ2 alone does not significantly impact virulence (Giebel et al., 2010). The relatively small number of genes involved, the presence of their proteins prepackaged in the spore, and the apparent essential nature of their activity make them attractive targets for new therapeutics as well as environmental decontamination compounds.

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Chapter 4

Genetic Manipulation Methods in *Bacillus anthracis*

Brian K. Janes and Scott Stibitz

INTRODUCTION

"Reverse genetics" whereby specific genes of interest are mutated and the resulting effects are assessed, is a well-established strategy for investigating all aspects of bacterial life, but has had particular success in the study of bacterial pathogenesis. For example, the application and fulfillment of "molecular Koch's postulates" generally involves the mutation of a specific gene of interest, and if a decrease in virulence is measured, the restoration of that virulence by genetic complementation (Falkow, 2004). The number of attractive candidates for study has increased dramatically due to recent advances in genomic methods, such as whole genome sequencing, microarray technology, and proteomics methodology. The premier biological warfare agent *Bacillus anthracis* is no exception, and is currently the target of a great deal of research of all types. Methods for genetic manipulation in *B. anthracis* have been available for some time, and this chapter summarizes both these established protocols as well as several recently developed techniques. These advances have broadened the array of genetic techniques available for studying *B. anthracis* and have significantly reduced the time and effort involved in mutant creation compared to the classic approaches.

DELIVERY OF FOREIGN DNA

Generalized Transduction

Transduction is a method by which genetic material from one strain of bacteria (the donor strain) is transferred via a bacteriophage to another strain (the recipient strain). Generalized transduction is often the most useful example of this in that any donor sequence can be transferred, and typically occurs as follows. The bacteriophage, propagated on the donor strain, will, at low frequency, package a portion of bacterial DNA in place of the normal phage genome. Subsequent infection with this population of phage will transfer the donor strain DNA to the recipient strain, where the recipient can incorporate the foreign DNA

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into its chromosome through homologous recombination. As this is a rare event, a strong selection, such as antibiotic resistance, is required.

The phage CP-51, originally isolated as a transducing phage for *Bacillus cereus* (Thorne, 1968), has been shown to mediate transduction between strains of *B. anthracis* (Green et al., 1985; Ruhfel et al., 1984). This method is most frequently used to move alleles that include an antibiotic resistance cassette (isolated via other methods) to new background strains. Several protocols have been described (Green et al. 1985; Hoffmaster and Koehler, 1997; Welkos and Marrero, 1996). CP-51 is also capable of transducing the large virulence-associated plasmid pXO2 into strains lacking the plasmid (Bourgogne et al., 2003; Green et al., 1985). Transductants are selected for in the presence of the phage CP-54, which lyses cells lacking capsule. Capsule production is dependent on the presence of genes located on pXO2, so only those recipient cells that receive the plasmid will survive in the presence of CP-54. As most protocols used for making mutants by allelic exchange (see below) have been described for non-pathogenic strains that lack pXO2, this procedure presents an attractive method for testing the virulence of these mutants. UT500, a $pXO2^+$ strain constructed by this method (Bourgogne et al., 2003), has been shown to have greatly enhanced virulence as compared to its $pXO2^-$ parent and is nearly as virulent as a clinical isolate of *B. anthracis* (Drysdale et al., 2005).

Electroporation

Electroporation, the method of transformation that occurs when a mixture of cells and DNA are subjected to a short electrical pulse, is the method of choice for the direct transfer of plasmid DNA purified from *Escherichia coli* into *B. anthracis*. Many electroporation protocols for *B. anthracis* have been described (including, but not limited to, Bartkus and Leppla, 1989; Dunny et al., 1991; Kim et al., 2004; Koehler et al., 1994; Quinn and Dancer, 1990; Schurter et al., 1989). Briefly, overnight cultures of vegetative cells are diluted and grown in rich media for a length of time that depends on the particular protocol. Some protocols allow the cells to grow for only an hour, while others allow the cells to grow until mid-, to late-, log phase. The cells are then washed with and concentrated in electroporation buffer (for composition of the various electroporation buffers used, see the previous citations). Outgrowth conditions post-shock also vary, but antibiotic resistant transformants (resistance conferred by markers on the plasmid) are readily obtained.

One aspect common to all the protocols is the source from which the DNA is isolated. *B. anthracis* is resistant to transformation with DNA isolated from *E. coli* strains capable of methylating the DNA (Marrero and Welkos, 1995). Therefore, any plasmid DNA to be electroporated into *B. anthracis* should be isolated from a $dam^- dcm^-$ strain of *E. coli*. Several different *E. coli* host strains have been used successfully (descriptions for many of these can be found in Palmer and Marinus, 1994), some of which are commercially available (e.g., SCS110 (Stratagene), GM2163 (New England Biolabs), and C600 (Sigma-Aldrich)).

Conjugation

Bacterial conjugation is another method by which DNA can be transferred from cell to cell. Many naturally occurring plasmids contain genes whose products confer the ability of the host cell to produce machinery to facilitate the transfer. This genetic transfer is not limited to the conjugative plasmid itself. Compatible plasmids present in the cell contain-

ing the conjugative plasmid, so long as they contain the origin of replication for the transfer (*oriT*), can be mobilized from the donor strain into the recipient. This phenomenon has not only been exploited to transfer DNA from cell to cell of the same species of bacteria, but from one bacterial species to another. Indeed, using a protocol of conjugation established for the transfer of plasmids from *E. coli* to gram-positive bacteria (Trieu-Cuot et al., 1987), Cataldi et al. (1990) demonstrated that interspecies bacterial conjugation was a viable means of introducing DNA into *B. anthracis.* Transconjugants can be selected via antibiotic resistance conferred by markers present on the plasmid, while the donor *E. coli* strain is killed by the presence of an antibiotic such as trimethoprim or polymixin B to which many gram-positive bacteria, including *B. anthracis*, are naturally resistant.

As stated above, the plasmid to be mobilized must contain the *oriT* necessary for the conjugal transfer. A "helper plasmid" that contains the conjugal transfer genes is also necessary; Cataldi et al. (1990) used donor strains that contain both the helper plasmid and the plasmid to be transferred, but it has also been established that "three-way," or "triparental" crosses, in which the recipient *B. anthracis* strain is mixed with two *E. coli* strains, one with the plasmid to be transferred and a separate strain containing the helper plasmid, can efficiently produce transconjugants (Janes and Stibitz, 2006). In this case, transfer of the helper plasmid into the strain with the plasmid to be mobilized creates a transient situation where both plasmids are present in one strain. Although transfer of the helper plasmid into the *B. anthracis* recipient presumably also occurs, since it cannot replicate and is not selected for post-conjugation, this is irrelevant.

ISOLATION OF MUTANTS

Transposons

Random insertion of mobile DNA elements (transposons) to generate libraries of bacterial mutants has long been a powerful tool in bacterial genetics. Classically, transposon mutagenesis was preferred because it allowed for easier identification of the gene in which the transposon was inserted. While the availability of whole-genome sequences and allelic exchange mutagenesis have diminished the appeal of traditional transposon mutagenesis protocols, transposons remain an important component of several current genetic technologies, including signature-tagged mutagenesis and transposon site hybridization (TraSH) assays (for a review, see Hayes, 2003).

Until recently, the use of transposons in *B. anthracis* has been limited due to the fact that the transposons available inserted preferentially at specific hot spots, eliminating the possibility of good gene coverage in a random mutagenesis. Tn*916*, for example, was used to generate auxotrophs, but the results suggested the insertions were not random (Ivins et al., 1988). Tn*917* showed an even greater bias, selectively targeting the virulence plasmids pXO1 and pXO2 over the chromosome (Hoffmaster and Koehler, 1997; Welkos, 1991). In order to circumvent this issue, mutagenesis can be performed in a strain that lacks these plasmids, but even then, Tn*917* appeared to insert in a nonrandom fashion (Samant et al., 2009).

Recently, several groups have developed transposon systems that appear to have much more random insertion patterns compared to Tn*917*. These are summarized in Table 4.1, and share several general characteristics: they are delivered to the cells via a plasmid, and this plasmid is unstable and easily cured after selection for insertion of the transposon into the chromosome or virulence plasmids (the antibiotic resistance provided by each

Table 4.1 Transposon Systems for Use in *Bacillus anthracis*

Transposon type	Characteristics[a]	Reference
Bursa aurealis[b]	Em^R; dual plasmid delivery system	Tam et al. (2006)
Mini-*Himar-1*[b]	Km^R	Barua et al. (2009)[c]
Mariner	Sp^R; single plasmid delivery system	Wilson et al. (2007)
Mini-Tn*10*	Sp^R; single plasmid delivery system	Wilson et al. (2007)
Mini-Tn*10*	Sp^R; single plasmid delivery system	Day et al. (2007)

[a] Em^R, erythromycin resistant; Km^R, kanamycin resistant; Sp^R, spectinomycin resistant.

[b] Mariner-based transposons.

[c] Construction of this transposon system cited as "unpublished data."

transposable element can be found in Table 4.1). The transposase responsible for mobilizing the DNA is also outside the transposon itself, so after transposition and plasmid curing, the mutation should be stable. These transposons, both Mariner and mini-Tn*10*-based systems, do not appear to preferentially insert into the virulence plasmids, and transposon insertions have been isolated in a wide variety of chromosomal locations (see the references cited in Table 4.1). Indeed, the mini-Tn*10* system described by Day et al. (2007) associated with greater than 80% of the genes in the *B. anthracis* strain tested. This improved chromosome coverage allowed for TraSH analysis of genes important in growth, sporulation, and germination in laboratory growth media. All of these studies were performed with non-pathogenic strains of *B. anthracis*, but Cote et al. (2008) used the mini-Tn*10* system of Day et al. (2007) to identify a protein recognized by anti-spore antibodies from a fully virulent strain.

Mutants Derived from Simple (Campbell-Type) Plasmid Insertion

Where classical genetic analysis most often relies on the generation of random mutants followed by their identification and characterization (transposon mutagenesis belongs to this class), so-called reverse genetics begins with a gene of interest which is targeted for mutation and subsequent analysis. The simplest form of reverse genetics involves the insertion of a plasmid into the wild-type gene on the chromosome by homologous recombination. This requires a plasmid containing a conditional origin of replication (temperature sensitivity being the most prevalent), a selectable marker (antibiotic resistance), and an internal portion of the gene lacking the 5′ and 3′ termini. This plasmid construct can be introduced into the host under permissive conditions, and selection can be maintained when the cells are shifted to nonpermissive conditions. This allows for the isolation of strains in which the plasmid has been inserted into the gene of interest via a single crossover event, resulting in a merodiploid condition where an allele lacking the 3′ terminus of the gene and an allele lacking the 5′ terminus are separated by the DNA sequence from the plasmid. It should be noted that mutants constructed via this method can be unstable, as the high degree of homologous DNA can allow for excision of the plasmid if both levels of selection (e.g., nonpermissive temperature and presence of antibiotic) are not rigorously maintained. For this reason, generation of stable mutants by allelic exchange is the preferred method of isolating mutants in *B. anthracis*.

This method of isolating single crossover insertion of plasmids has been used to examine whether particular genes of interest are essential (Paige et al., 2008); the inability to isolate viable cells when both levels of selection are administered is consistent with the conclusion that the genes in question are essential for growth. These protocols have also been adapted for regulatory studies of genes in *B. anthracis*. For example, the regulatory region of the gene of interest can be fused to a reporter gene on an appropriate plasmid and mutants that have inserted the plasmid can be isolated. This approach allows for reporter constructs that maintain a wild-type copy number. Both β-galactosidase (encoded by *lacZ*) and catechol 2,3-dioxygenase (encoded by *xylE*) have been used for such studies (Sirard et al., 1994, 1995). Fisher and Hanna (2005) further modified a plasmid insertion mutagenesis system developed by Vagner et al. (1998) for use in *B. anthracis*. Approximately 500 bp of DNA of the gene of interest, including the translational start site and some upstream sequence, is used as the target for integration of the plasmid. Integration places the gene or operon under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, thus additionally providing a null mutation in the absence of IPTG while fusing the promoter of the gene to the reporter construct, *lacZ*.

Allelic Exchange

More stable mutations can be isolated using the technique of allelic exchange. In this process, the mutant allele of choice is constructed using standard molecular biology techniques and is introduced into the target strain, where the mutant allele then replaces the wild-type allele. This process is illustrated in Figure 4.1. Mutants isolated by this method are desirable due to their genetic stability; unlike the mutants derived from a single crossover event described above, there is no simple method of reversion back to wild type.

Theoretically, any type of mutation can be localized to the chromosome of *B. anthracis* using allelic exchange; however, two classes are most frequently isolated: disruption/replacement of the target gene with an antibiotic resistance cassette, or in-frame deletions that remove the gene in its entirety. Introduction of an antibiotic resistance cassette is advantageous in that the mutants are easily selected or screened for, given that the mutation confers antibiotic resistance, and this also allows for movement into other genetic backgrounds using generalized transduction (see above). The disadvantage of inserting an antibiotic resistance cassette is the potential for inducing polar effects on downstream genes, although cassettes designed to be nonpolar can be produced (Mesnage et al., 2000). The limited number of resistance cassettes available also limits the number of mutant alleles that can be introduced into one strain. In-frame deletions do not have either of these limitations, but can be more difficult to isolate due to the lack of selection for the mutant allele.

The first step in allelic exchange is similar to the single crossover event described in the previous section. The mutant allele, flanked by upstream and downstream regions of homology (usually 500 bp or more for good results in *B. anthracis*), is cloned into an allelic exchange vector (see Figure 4.1 for a generic example), and the resulting plasmid is introduced into the target strain under conditions permissive to plasmid propagation. Conditions are shifted to nonpermissive for plasmid replication, and insertions of the entire plasmid into the chromosome are isolated by selection for the antibiotic resistance carried on the plasmid. The single crossover insertion can occur on either side of the wild-type allele.

The second step is to isolate a second crossover event that results in the removal of the inserted plasmid. If this excision event occurs in the same area (upstream or

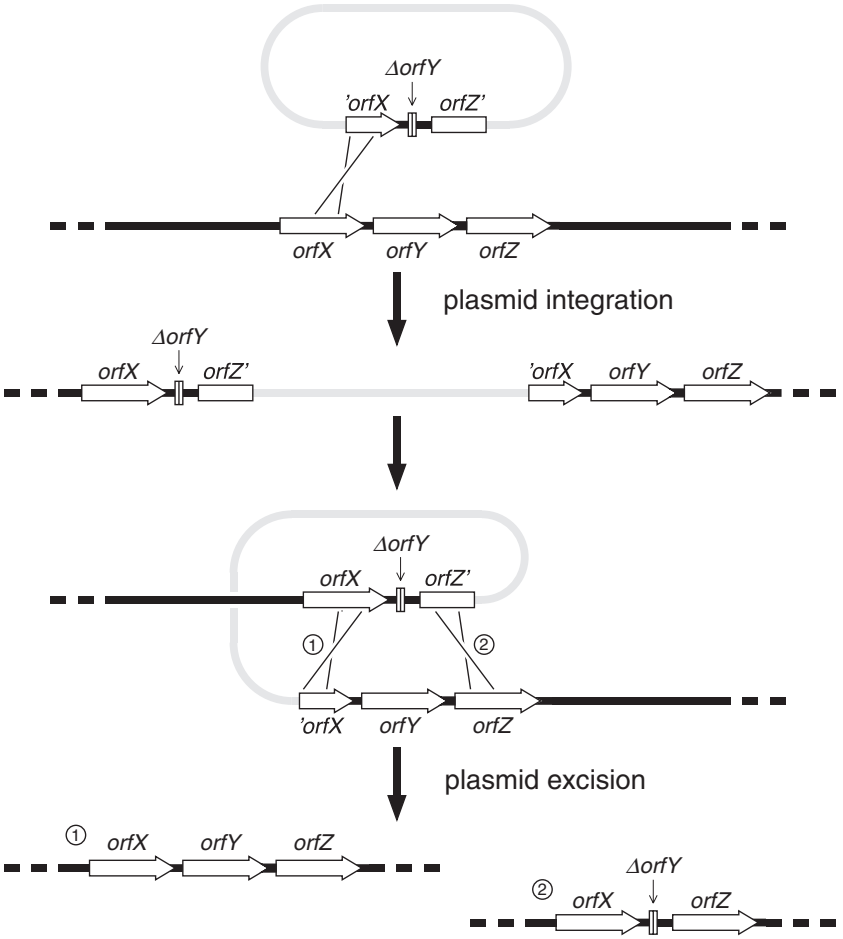

Figure 4.1 Allelic exchange. Allelic exchange is performed via a two-step process involving an initial crossover event resulting in plasmid integration, followed by a second crossover event resulting in plasmid excision. In this example, the mutation of choice (Δ*orfY*), and flanking regions both upstream and downstream are produced by standard methods. This construct is then moved into an allelic exchange plasmid (for examples, refer to the text and Table 4.2; plasmid sequence represented by the gray bar). For this example, the plasmid integrates by homologous recombination into *orfX*, although integration into *orfZ* should be equally probable. The second crossover event, in which the plasmid is excised, can proceed through one of two routes. In route 1, the crossover occurs in *orfX*, and since this was the site of integration, the wild-type *orfY* is regenerated upon plasmid excision. In route 2, the crossover occurs in *orfZ*, leaving the mutant allele on the chromosome resulting in the desired allelic exchange.

downstream) where insertion occurred, the wild-type allele is regenerated (Figure 4.1, route 1 of plasmid excision). However, if the crossover event occurs in the region opposite of where the original insertion occurred, the plasmid is excised along with the wild-type allele, leaving the desired mutant allele on the chromosome (Figure 4.1, route 2). Plasmid excision can be achieved by passaging the strain isolated in the first step for many generations in the absence of antibiotic selection. The second crossover event occurs naturally, and individual bacterial colonies can be screened for the loss of the antibiotic resistance. As this second crossover is a rare event, many passages in nonselective media may be

necessary. If the mutant allele in question possesses a selectable marker, one can screen for the mutant simultaneously by identifying isolates that retain the resistance provided by the mutation but lack the resistance conferred by the plasmid. For markerless mutations, antibiotic-sensitive candidates for allelic exchange must be screened by another method to determine which of the two possibilities presented in Figure 4.1 has occurred. In the absence of an easily detectable phenotype, PCR using diagnostic primers that anneal to regions close to but outside the regions of homology originally cloned into the allelic exchange vector is performed on candidates. The DNA product isolated from a successful exchange will be different (usually significantly smaller if the gene has been deleted) from the wild type. It should be noted that essentially, any sort of genetic modification, including single base-pair changes, can be introduced by this approach (Janes and Stibitz, 2006).

A list of plasmid vectors that have been used successfully to perform allelic exchange in *B. anthracis* is presented in Table 4.2. The earliest reports of allelic exchange experiments in *B. anthracis* involved screening for crossover events where the mutant allele was present on a plasmid with a temperature-stable replicon (Cataldi et al., 1990; Dai et al., 1995; other examples are included in Table 4.2). These are labeled as "temperature stable" in Table 4.2. After isolation, transformants or transconjugants containing the allelic exchange construct were passaged without selection for the plasmid for many generations. The mutant alleles in question contained antibiotic markers themselves, so selection for the mutant allele could be maintained for the entirety of the process.

The most reported method for isolating allelic exchange mutants involves vectors that contain temperature-sensitive origins of replication. These vectors are labeled as temperature sensitive (ori^{TS}) in Table 4.2. Transformants or transconjugants were isolated at the permissive temperature, and then shifted to the higher, nonpermissive temperature in order to isolate the primary insertion. Selection was removed, and after multiple passages, bacteria that had lost the plasmid were identified as those sensitive to the appropriate antibiotic; mutants were verified by phenotype or PCR methods. One limiting step of this procedure is the ease at which the plasmid, once excised via the second recombination event, is lost from the growing cell. Thus, maintenance at the nonpermissive temperature is critical during the passage of the cells in the absence of the antibiotic selection conferred by the plasmid. One strategy that expedites the loss of the plasmid at this stage uses a plasmid for allelic exchange that does not have the ability to replicate in gram-positive bacteria at all. Allelic exchange constructs cloned into these so-called suicide vectors (labeled as such in Table 4.2) were integrated into their designated site after conjugation. Upon excision, the plasmid is readily lost as it fails to replicate. Kedar et al. (2007) further modified this approach by adapting a method used for allelic exchange in *Staphylococcus*

Table 4.2 Plasmids Used for Allelic Exchange in *Bacillus anthracis*

Plasmid	Characteristics[a]	Reference[b]	Source[b]
	Temperature stable replicons		
pAT18	Em^R; conjugation proficient (RP2)	Pezard et al. (1991)	Trieu-Cuot et al. (1991a)
pAT187	Km^R; conjugation proficient (RP2)	Cataldi et al. (1990)	Trieu-Cuot et al. (1987)
pLC1	Km^R		Ahn et al. (2006)
pMR1	Tc^R, $(Km^R)^c$		Giorno et al. (2007)
pUTE29	Tc^R	Dai et al. (1995)	Koehler et al. (1994)
pUTE583	Em^R		Chen et al. (2004)

(*Continued*)

Table 4.2 *Continued*

Plasmid	Characteristics[a]	Reference[b]	Source[b]
	Temperature-sensitive origin of replication (ori^{TS})[d] or suicide vector		
pASD2	Km^R; Sp^R; ori^{TS} (pE194)		Day et al. (2007)
pATΔS28	Sp^R; conjugation proficient (RP2); no origin of replication in *B. anthracis* (suicide vector)		Namy et al. (1999)
pAT113	Em^R, Km^R; conjugation proficient (RP2); no origin of replication in *B. anthracis* (suicide vector)		Trieu-Cuot et al. (1991b)
pBAK	Km^R; conjugation proficient (RP2); no origin of replication in *B. anthracis* (suicide vector)		Mignot et al. (2003)
pCLT1376	Em^R; ori^{TS} (pE194)		Dong et al. (2008)
pEO-3	Em^R; ori^{TS} (pE194)		Mendelson et al. (2004)
pGhost5	Em^R; ori^{TS} (pWVO1)	Vetter and Schlievert (2007)	Maguin et al. (1996)
pHY304	Em^R; ori^{TS} (pWVO1)	Pomerantsev et al. (2006)	Pritzlaff et al. (2001)
pKS1	Em^R, (Km^R)[c]; ori^{TS} (pWVO1)		Shatalin and Neyfakh (2005)
pKSV7	Km^R; ori^{TS} (pE194)	Weiner et al. (2003)	Smith and Youngman (1992)
pLM4	Km^R; ori^{TS} (pTV1*ts*)		Marraffini and Schneewind (2006)
pLTV1ΔXba	Tc^R; ori^{TS} (pE194)		Uchida et al. (1997)
pORI-Cm	Cm^R; ori^{TS} (pWVO1)		Brunsing et al. (2005)
	Plasmids with counterselection		
pBKJ236	Em^R; conjugation proficient (RP4); ori^{TS} (pWVO1); I-SceI recognition site[e]		Janes and Stibitz (2006)
pBKJ258	Em^R; conjugation proficient (RP4); ori^{TS} (pWVO1); I-SceI recognition site[e]		Lee et al. (2007)
pORI-I-SceI	Cm^R; ori^{TS} (pWVO1); I-SceI recognition site[e]		Bongiorni et al. (2007)
pSABA-3	Em^R, (Km^R)[c]; *repC* counterselection		Kedar et al. (2007)

[a] ori^{TS}, temperature-sensitive origin of replication (see each reference for permissive and nonpermissive temperatures); Em^R, erythromycin resistant; Km^R, kanamycin resistant; Tc^R, tetracycline resistant; Sp^R, spectinomycin resistant; Cm^R, chloramphenicol resistant; note that antibiotic resistance is noted for selection in *B. anthracis*, and may or may not work as well in *Escherichia coli* (refer to each reference for details and concentrations). Additional antibiotic resistance genes nonfunctional in *B. anthracis* (but functional in *E. coli*) may be present.

[b] The column labeled "Reference" cites an example (others may exist) in which the plasmid was used in *B. anthracis*. The column labeled "Source" cites the original reference for the plasmid. In cases where the original reference demonstrated the use of the plasmid for allelic exchange in *B. anthracis*, the Reference column is left blank.

[c] Antibiotic resistance noted in parentheses is designed to be part of the allelic exchange, so while the plasmid contains this marker, it is ultimately part of the mutant allele and is not lost after allelic exchange (see each specific reference for details).

[d] The source of the temperature-sensitive origin of replication (pWV01, pTV1, or pE194) is noted parenthetically.

[e] The presence of the I-SceI restriction endonuclease recognition site allows for enrichment of strains that have cleared the plasmids by *trans*-expression of I-SceI. See text and Janes and Stibitz (2006) for details.

aureus (Xia et al., 1999) for use in *B. anthracis*. This method takes advantage of the fact that overexpression of the RepC protein is inhibitory to DNA replication for plasmids bearing the origin of replication from plasmid pT181 (Iordanescu, 1995). Mutant alleles were constructed that contained antibiotic resistance on the plasmid pSABA-3, which also contains this RepC-sensitive origin of replication. After the isolation of the primary insertion, a second plasmid was introduced that overexpressed *repC*, and the original plasmid was more readily cleared, expediting the ability to isolate the appropriate mutant.

As described above, replacing the wild-type allele with an antibiotic resistance marker greatly simplifies mutant isolation, as selection can be maintained to ensure that the desired mutation remains following the second recombination event. The presence of the antibiotic marker itself can, however, restrict the number of mutations that can be sequentially generated in a single strain. Pomerantsev et al. (2006) devised a strategy to circumvent this issue, adapting a method that uses Cre recombinase to remove DNA sequences flanked by *loxP* sites (see Palmeros et al., 2000 for details). Mutant alleles were designed such that a spectinomycin cassette, flanked by the *loxP* sites, were introduced into the target strains on a plasmid with a temperature-sensitive origin of replication. Selection for spectinomycin was maintained during the second recombination event to ensure allelic exchange and then the Cre recombinase was expressed in trans to remove the antibiotic cassette. It should be noted that while this technique did allow for sequential mutagenesis of *B. anthracis*, the small sequences of DNA that are left after Cre-specific excision can mediate unintended recombination events. For example, a double mutant generated by this method rearranged such that the 30 kb span between the two mutation sites was deleted due to the *loxP* sites that remained.

Recently, another method has been reported that significantly enhances the frequency of the second crossover event during allelic exchange, allowing for more efficient generation of the mutants after isolating primary insertions of the allelic exchange construct. Janes and Stibitz (2006) adapted the strategy Pósfai et al. (1999) developed for allelic exchange in *E. coli*. In this method, the second recombination event is both accelerated and selected for by introducing double-stranded breaks near the site of allelic exchange with restriction endonuclease I-SceI. This enzyme has an 18 bp recognition site that does not exist naturally in the entire *B. anthracis* genome. The I-SceI recognition site was engineered onto the allelic exchange plasmid (pBKJ236), and after isolation of the primary insertion, a second plasmid (pBKJ223) constitutively expressing the I-SceI gene was introduced. Candidates that had lost the resistance provided by pBKJ236 were quickly isolated and the desired mutants were identified by PCR. This method can be adapted to any available allelic exchange plasmid system by the addition of an I-SceI site, as was reported by Bongiorni et al., 2007 with the creation of pORI-Cm-SceI from pORI-Cm. The strategy has been further refined by the creation of pSS4332 (Cybulski et al., 2009), a functional equivalent of the I-SceI expressing plasmid pBKJ223 that contains a different selectable marker (kanamycin vs. tetracycline) and can be introduced into *B. anthracis* via conjugation. Both pBKJ223 and pSS4332 were derived from pUTE29, which is known to have unstable replication properties and is easily cleared by growth in the absence of selection (Saile and Koehler, 2002).

Complementation and other Uses of Plasmids

Many plasmids that can stably replicate in *B. anthracis* have been described (see Table 4.3 for details). These replicative plasmids are most commonly used to provide a platform

Table 4.3 Plasmids with Stable Replicons in *Bacillus anthracis*

Plasmid	Characteristics[a]	Reference[b]	Source[b]
pAT18	Em^R; conjugation proficient (RK2)	Candela et al. (2005)	Trieu-Cuot et al. (1991a)
pAT187	Km^R	Guignot et al. (1997)	Trieu-Cuot et al. (1987)
pAT28	Sp^R; conjugation proficient (RK2)	Candela et al. (2005)	Trieu-Cuot et al. (1990)
pBKJ236[c]	Em^R; conjugation proficient (RP4); ori^{TS}	Lambert and Popham (2008)	Janes and Stibitz (2006)
pBKJ258[c]	Em^R; conjugation proficient (RP4); ori^{TS}	Passalacqua et al. (2007)	Lee et al. (2007)
pCE104	Em^R	Vetter and Schlievert (2007)	Murray et al. (1996)
pCN55	Sp^R	Samant et al. (2009)	Charpentier et al. (2004)
pHP13	Cm^R, Em^R	Cendrowski et al. (2004)	Haima et al. (1987)
pHT304	Em^R	Samant et al. (2008)	Arantes and Lereclus (1991)
pHT315	Em^R	Wilson et al. (2008)	Arantes and Lereclus (1991)
pMK4	Cm^R	Thompson and Stewart (2008)	Sullivan et al. (1984)
pOS1	Cm^R	Gaspar et al. (2005)	Schneewind et al. (1993)
pUTE29	Tc^R		Koehler et al. (1994)
pUTE568	Cm^R, Em^R		Saile and Koehler (2006)
	Plasmids with promoter-less reporter genes		
pHT304-18z	Em^R; promoter-less *lacZ* cassette	Hadjifrangiskou and Koehler (2008)	Agaisse and Lereclus (1994)
$pGuv_{MCS-5}$	Km^R; promoter-less *gfpuv* cassette		Gat et al. (2003)
pPL703	Km^R, promoter-less *cat* cassette	Bartkus and Leppla (1989)	Mongkolsuk et al. (1985)
pTCV-*lac*	Em^R, Km^R; promoter-less *lacZ* cassette	Bongiorni et al. (2007)	Poyart and Trieu-Cuot (1997)

[a] Em^R, erythromycin resistant; Km^R, kanamycin resistant; Tc^R, tetracycline resistant; Sp^R, spectinomycin resistant; Cm^R, chloramphenicol resistant; note that antibiotic resistance is noted for selection in *B. anthracis*, and may or may not work as well in *Escherichia coli* (refer to each reference for details and concentrations). Additional antibiotic resistance genes nonfunctional in *B. anthracis* (but functional in *E. coli*) may be present.

[b] The column labeled "Reference" cites an example (others may exist) in which the plasmid was used in *B. anthracis*. The column labeled "Source" cites the original reference for the plasmid. In cases where the original reference demonstrated the use of the plasmid for complementation in *B. anthracis*, the Reference column is left blank.

[c] These plasmids have temperature-sensitive origins of replication, but have been used for complementation studies at the permissive temperatures.

for wild-type gene expression to trans-complement mutant alleles. Plasmids created using these vectors can be constructed by traditional methods, passaged through a methylation-deficient strain, and introduced into the *B. anthracis* strain of choice as described above. Trans-complementation is an important element of genetic studies, particularly in establishing the nonpolar nature of mutations introduced by the methods described above.

Promoter expression studies can be performed on plasmids, as vectors containing promoter-less reporter genes have been reported (see Table 4.3). Examples include the genes encoding chloramphenicol acetyltransferase (Bartkus and Leppla, 1989), β-galactosidase (Cataldi et al., 1992), or green fluorescent protein (Gat et al., 2003). In addition, the plasmid pXX_{MCS-5} can be used to insert the promoter-less reporter gene of choice (Gat et al., 2003). While plasmids have their uses, recently developed quantitative PCR techniques that measure the transcript levels of the gene more directly may be preferable to these plasmid-based type of studies.

Kedar et al. (2007) constructed a xylose-inducible expression vector that was used to express anti-sense libraries in an attempt to define target genes that were essential for growth. Expression of anti-sense fragments constructed from the genes *metS1* and *murB2* led to a xylose-dependent growth inhibition. This system seems a good approach toward defining genes essential for growth, and the plasmid pBAX-2 (the xylose-responsive expression vector) could have further utility for expressing genes under controllable conditions.

CONCLUDING REMARKS

There have been many advances in genetic manipulation of *B. anthracis* that have improved both the speed and efficiency with which mutants may be generated. Although not as robust as those developed for model organisms such as *E. coli* and *B. subtilis*, many of the systems developed in these and other genetically tractable systems have been successfully adapted for use in *B. anthracis*. Certainly, the generation and characterization of mutants is no longer a hurdle in the study of anthrax. In addition, some of these genetic approaches have been further adapted for other applications, including the production of chromosomally localized protein-tagged fusions. Fusions of proteins to fluorescent markers have allowed for cellular localization studies (e.g., a protein–*cherry* fusion to BasI; see Marraffini and Schneewind, 2007). Indeed, various fluorescent proteins have been optimized for expression in *B. anthracis* (Sastalla et al., 2009) that should allow for a variety of visualization studies to be performed. In addition, localization of a constitutively highly expressed *lux* operon to the chromosome has allowed for the monitoring of cells during infection in a mouse model using bioluminescence (Loving et al., 2009).

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Chapter 5

The *Bacillus anthracis* Genome

Timothy D. Read

INTRODUCTION

Genome sequences tell fascinating stories. In the case of *Bacillus anthracis*, a string of more than 5.2 million A, C, G, and T bases describes the entire protein coding and genetic regulatory machinery of the pathogen that causes anthrax, one of the most lethal bacterial infections. The genome cryptically outlines the secrets of anthrax infection and points to how the bacterium evades destruction by the immune system of its host. In trying to gain an understanding of how *B. anthracis* pulls off this feat, researchers use the sequence to identify weaknesses that can be translated into medical countermeasures. The genome sequence is also a historical record that can, by comparison to the swathe of closely related but mostly anonymous bacteria of the *Bacillus cereus* species, be used to reconstruct the evolutionary path that led to *B. anthracis* impinging on the human world.

This chapter will briefly overview the history of the first *B. anthracis* genome projects and try to serve as a guide to the sometimes confusing array of the sequences now in the public domain. The focus will then turn to a discussion of the gross features of the genome and some of the discoveries that have come from early sequence-based investigations. The final section covers how the genome sequence has spurred the understanding of *B. anthracis* population biology, evolution, and biogeography. Unfortunately, there are a ream of interesting topics that cannot be covered in just one chapter. Most of the experiments in pathogenesis, cell biology, and proteomics that have used the sequence cannot be described here, although several recent reviews (including Fouet and Mock, 2006; Kolsto et al., 2009; Mock and Fouet, 2001; Passalacqua and Bergman, 2006; Perego and Hoch, 2008; Tournier et al., 2009) and other chapters in this book cover the ground well. The virulence plasmids pXO1 and pXO2 are discussed in detail in their own portion in this book. It is also just too big a subject to provide a comprehensive overview of the genomic comparisons between the *B. anthracis* and the ever-growing collection of non-pathogenic *B. cereus* group species; instead some interesting vignettes will be presented. For the same reason, the role played by genomics in the Amerithrax investigation can only be touched on lightly in this chapter.

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

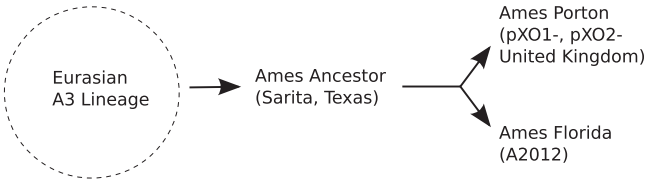

Figure 5.1 Recent pedigree of sequenced Ames strains.

B. ANTHRACIS SEQUENCING PROJECTS

For about 20 years after the development of technology for sequencing DNA by extension in 1977 (Sanger et al., 1977), short regions of the *B. anthracis* genome were laboriously characterized, gene by gene. On the eve of the first *B. anthracis* genome project in 1998, there were about 100 entries in the National Center for Biotechnology Information (NCBI) Nucleotide database. These included the genes for the lethal toxin cluster and poly-D-glutamic acid clusters that had been discovered in the preceding decade (Bragg and Robertson, 1989; Escuyer et al., 1988; Makino et al., 1989; Welkos et al., 1988). From the 1970s until the first Gulf War (1990–1991), *B. anthracis* had been a relatively quiet field of study. However, hints of extensive biowarfare programs in hostile countries and disturbing incidents such as the activities of the Aum Shinrikyo cult (Keim et al., 2001) reawoke the U.S. government to the possibility of deliberate anthrax release. At about this time, the *B. anthracis* Sterne pXO1 (Okinaka et al., 1999a) and Pasteur pXO2 (Okinaka et al., 1999b) virulence plasmid sequences were completed. The Institute for Genomic Research (TIGR) in Rockville, Maryland has been pioneer of complete bacterial genome sequencing using the whole genome shotgun approach (Fleischmann et al., 1995). In 1998, a group of U.S. government agencies initiated a project at TIGR to sequence the first *B. anthracis* genome.

The *B. anthracis* strain was of the Ames lineage (Figure 5.1) from Porton Down in the United Kingdom. The strain had been cured of pXO1 and pXO2 plasmids by heat treatment and chemical mutagenesis. Ames was selected because it was considered to be an unusually virulent isolate that was used for vaccine challenge studies in several defense laboratories. In 1998, when the cost and time of sequencing was very much greater than the present day, it was an advantage to not waste resources resequencing the large plasmids of the Ames strain. The whole genome shotgun method used for sequencing bacterial genomes, pioneered by Sanger (Sanger et al., 1978) and modeled by Lander and Waterman (1988), was still a somewhat controversial strategy in 1998 (Green, 1997), but today it has become the basis of almost every sequencing project. Genomic DNA was sheared randomly into small fragments and cloned into a plasmid vector. Primers annealing to both ends of the insert generated "mate pair" sequencing reads. Enough sequence was generated this way to give an average of more than 8 reads per base (8× coverage in genomics parlance), a sweet spot on the perfect Lander–Waterman model curve, where the rate of filling of gaps in the sequence inflected toward an asymptote. These DNA sequences were then assembled by specialized software that attempted to efficiently pile up overlapping sequences. The resulting "contigs" or "assemblies" of individual reads could be linked to one another by mate pairs (sequencing gaps) or in the cases when there was no linking information, physical gaps. The final, drawn-out phase of the project was the completion of the genome mostly by manual inspection and editing of the sequence, and sequencing

Table 5.1 Properties of the *Bacillus anthracis* 0581 Ames Ancestor Genome

	Chromosome	pXO1	pXO2
Database[a] accession	AE017334/ NC_007350	AE017336/ NC_007322	AE017335/ NC_007323
Size (bp)[b]	5,227,419	181,677	94,830
G+C%[b]	35	32	33
Number of protein-encoding genes[b]	5,309	177	98
Gene density %[b]	80	62	63
Structural RNA genes[b]	128	0	0
Noncoding RNA genes[c]	106	0	0

[a] NCBI GenBank/RefSeq.

[b] Source: NCBI RefSeq website July 2009 (http://www.ncbi.nlm.nih.gov/).

[c] Source: Department of Energy Integrated Microbial Genomes website July 2009 (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

of PCR amplicons bridging gaps. Physical gaps often had to be linked by combinatorial methods (Tettelin et al., 1999), involving hundreds, or even thousands of reactions, and several person-years of effort for large bacterial projects.

The *B. anthracis* Ames Porton genome was constructed from four libraries, two small (2–3 kb insert) and two large (4–8 kb insert). In total, 77,338 small insert library reads and 32,430 large insert reads were sequenced with average lengths of ~600 nt for more than 13× coverage of the 5.2 Mbase genome. The random shotgun phase of the project took about 18 months, while the finishing of the genome took a little over 2 years. Every base in the genome assembly was manually assessed for quality. From the outset of the project, TIGR made regularly updated preliminary data from the project available to the researchers through its own and public NCBI websites, accelerating the pace of a number of exciting discoveries and assays. The genome sequence was published in 2003 (Read et al., 2003) (Table 5.1).

The deliberate release of a *B. anthracis* Ames strain via the U.S. Mail in October 2001 and the subsequent killing of five people (CDC, 2001) occurred as the Porton Ames genome was being finished. A project sequencing an isolate of Ames from an anthrax victim in Florida for comparison began at TIGR in November 2001. The Ames Florida genome DNA was sequenced to an average 6× coverage of the chromosome, 20× coverage of pXO1, and 14× coverage of pXO2. The 3:2:1 ratio of chromosome : pXO1 : pXO2, reflecting the elevated plasmid copy numbers, was a consistent feature of *B. anthracis* genome projects (Ravel et al., 2009; Read et al., 2002). Despite the relatively low coverage, and relatively high number of chimeric reads (where random inserts are ligated together during library construction), high-quality regions of the whole genome shotgun data were parsed bioinformatically to identify four differences between the chromosomes of Ames Porton and Florida that were subsequently validated by independent testing (Read et al., 2002). A number of single nucleotide polymorphisms (SNPs), indels, and inversions were also detected between the Florida plasmids and the Sterne pXO1 and Pasteur pXO2 plasmids. This study was a proof of concept of the use of *de novo* genome sequencing to discover rare variants in very closely related strains (Cummings and Relman, 2002) and laid the groundwork for extensive use of variant detection using whole genome shotgun sequencing in the Amerithrax investigation.

As the comparison of the Ames Porton and Florida genomes progressed, it became apparent that little was actually known about the provenance of the strains used for biodefense studies and genomic characterization (Figure 5.1). Ames turned out to have been isolated in 1981 from a cow that died of anthrax in Sarita, Texas. The U.S. Army Research Institute (USAMRIID) acquired the strain from the veterinary laboratory at Texas A&M University. Because the strain was shipped in a box with an old return label from Ames, Iowa, it was misnamed when it arrived at USAMRIID (Ravel et al., 2009). The Ames strain was later shared with the Porton Down laboratory, and genomic DNA of two independently cured variants were sent to TIGR. All the four differences between Porton and Florida isolates were unique to Porton, probably an indirect result of the curing experiments (Read et al., 2002). Furthermore, a number of genetic differences were found between the two batches of genomic DNA from Porton sequenced at TIGR. Casual use of names, untracked exchanges, and unawareness of the true provenance of strains has been a general feature of the culture of microbiology for more than a century. Cheap whole genome sequencing in the future will doubtless reveal many more surprising features of the workhorse laboratory bacteria that have been used for genetic studies for many decades (Hobman et al., 2007).

A number of other *B. anthracis* genomes have now been sequenced. By June 2009, there were 5 complete *B. anthracis* genomes and 14 genomes at the whole shotgun sequence stage submitted to the NCBI database (Table 5.2). The incomplete genomes include the phylogenetically diverse strains Kruger B and Vollum. One of the completed genomes is the chromosome of common laboratory strain Sterne (Rasko et al., 2005). Recently, the Ames Ancestor strain was completed at an average chromosomal coverage

Table 5.2 *Bacillus anthracis* Genome Projects, July 2009

Strain	Accession numbers[a]	NCBI Taxon ID	Source notes	Complete genome	Reference
A2012 (Ames Florida)	AAAC00000000, AE011190-91/ NZ_AAAC00000000, NC_003980-81	191218	Patient; Florida, USA	No	Read et al. (2002)
Ames (Porton)	AE016879/ NC_003997	198094	Porton Down, UK (plasmid cured)	Yes	Read et al. (2003)
Sterne	AE017225/ NC_005945	260799	Originally isolated in South Africa	Yes	Rasko et al. (2005)
Ames Ancestor Ames 0581	AE017334-36/ NC_007530, NC_007322-23	261594	Cow; Sarita, Texas, USA	Yes	Ravel et al. (2009)
CDC 684	CP001214-16/ NC_012577, NC_012581, NC_012579	568206	Originally classified as *B. megaterium*	Yes	Ezzell et al. (1990)
A0248	CP001597-99/ NC_012655-56, 59	592021	Human isolate from Ohio, USA	Yes	
Kruger B	AAEQ00000000/ NZ_AAEQ00000000	205919	Kruger National Park, South Africa	No	Rasko et al. (2005)

Table 5.2 *Continued*

Strain	Accession numbers[a]	NCBI Taxon ID	Source notes	Complete genome	Reference
Western North America USA6153	AAER00000000/ NZ_AAER00000000	212045	Bison; Canada	No	Rasko et al. (2005)
Vollum	AAEP00000000/ NZ_AAEP00000000	261591	Variant/close relative of Vollum	No	Rasko et al. (2005)
Australia 94	AAES00000000/ NZ_AAES00000000	261592	Victoria, Australia	No	Rasko et al. (2005)
CNEVA-9066	AAEN00000000/ NZ_AAEN00000000	280354	Human isolate, Southern France	No	Rasko et al. (2005)
A0155	AAEO00000000/ NZ_AAEO00000000	280355	Louisiana, USA	No	Rasko et al. (2005)
Tsiankovskii-I	ABDN00000000/ NZ_ABDN00000000	405536	Attenuated Russian vaccine strain containing both pXO1 and pXO2	No	
A0193	ABKF00000000/ NZ_ABKF00000000	486619	Bovine isolate obtained in South Dakota, USA	No	
A0465	ABLH00000000/ NZ_ABLH00000000	486620	Bovine isolate obtained from France	No	
A0442	ABKG00000000/ NZ_ABKG00000000	486621	Kudu (antelope) in the Kruger National Park, South Africa	No	
A0174	ABLT00000000/ NZ_ABLT00000000	486622	Canada	No	
A0389	ABLB00000000/ NZ_ABLB00000000	486623	Isolated in Bekasi, Indonesia	No	
A0488	ABJC00000000/ NZ_ABJC00000000	486624	Vollum-type strain isolated from infected cattle in the United Kingdom in 1935	No	

[a] NCBI GenBank/RefSeq.

of 11× (Ravel et al., 2009). Because the Ames Ancestor genome has the best source information and is from a strain that has been cultured in laboratory probably for the fewest number of generations before sequencing, and the data have been carefully closed, edited, and reannotated, it should be now considered the reference genome for comparison purposes.

Since the introduction of massively parallel bead-based pyrosequencing in 2005 (Margulies et al., 2005), the annual rate of increase of sequence production has overtaken Moore's law, the name given in the electronics industry to the long-term trend of

exponential growth in the ratio of transistors per integrated circuit (Shendure and Ji, 2008). The production stage of whole shotgun sequencing *B. anthracis Ames* Porton (generation of 40 Mbase of sequence in 500 bp reads) took a team of technicians operating multiple instruments several months to achieve in 1998. Today, one technician can sequence 10 times this amount in 2 days (and other instruments that produce shorter reads yield far greater amounts of raw sequence). Soon, there will be a very small cost barrier to sequence generation, and genomic data analysis will be so tightly integrated into microbiology that the practice of labeling "genomics" as a separate discipline may effectively disappear.

FEATURES OF THE *B. ANTHRACIS* GENOME

The Ames Ancestor genome consists of a 5,227,419 bp chromosome with an 181,677 bp pXO1 plasmid and a 94,830 bp pXO2 plasmid. Of the bases,35.4% are G or C, the rest of course, are A and T (Table 5.1). How can these data be translated into insight about the biological activities of *B. anthracis*? In considering some of the features of the genome, *B. anthracis* can be viewed both against a global backdrop of all other sequenced bacteria and also on how it compares to its much closer relatives, the polyphyletic *B. cereus sensu lato* (s.l.) "species" (which includes *B. anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, and *Bacillus weihenstephanensis*) (Helgason et al., 2000).

The size of the *B. anthracis* chromosome itself informs about the biology of the bacterium. Bacterial genomes vary over two orders of magnitude, from 180 kb to 13 Mb with a bimodal distribution around peaks at ~2 and ~5 Mb (Koonin and Wolf, 2008). *B. anthracis* therefore belongs very much in the center of the second band, which consists exclusively of free-living bacteria (not relying on sustenance from a host organism). Large genome size may be an adaptation to environments where resources are scarce, but there is a penalty for slow growth (Bohlin et al., 2008)—a description of a typical soil. Within the *B. cereus* s.l. species, there seems to be uniformity of chromosome size around 5.2 Mb (Rasko et al., 2005). While there is much evidence of the uptake of new genes into the genome of *B. anthracis* and *B. cereus* by horizontal gene transfer, there must also be a counterbalancing genome reduction process in these organisms that settles at a dynamic equilibrium at about 5.2 Mbase. What the selection pressures are that prevent the streamlining of the genome size of *B. anthracis* on the one hand and that constrain the acquisition of novel genes on the other, are not understood.

The nucleotide composition across the genome is the product of several interacting processes. The correlation between G+C nucleotide richness and increasing genome size was noticed after the first genomes were sequenced (Rocha and Danchin, 2002). The reasons for the association have been debated vigorously; some authors have argued that low G+C content is a by-product of energy limitation in nucleoside synthesis in the pathogenic or endosymbiotic lifestyle (Rocha and Danchin, 2002). *B. anthracis* lies at the extreme low range for a 5.2 Mbase genome (Bohlin et al., 2008) (Figure 5.2). The figure of ~35% G+C is consistent across *B. cereus* s.l. genomes (Rasko et al., 2005), suggesting it might be a conserved function of the DNA metabolism of the species. There is a trend for G+C content to decline slightly across the *B. anthracis* genomes, with the replication terminus lower than the origin of replication region (Figure 5.3). The replication region tends to contain more conserved genes, while there is a higher concentration of ORFans and horizontally acquired genes at the terminus (Read et al., 2003). The lower G+C may be a feature of horizontally transferred DNA (Rocha and Danchin, 2002): pXO1 and pXO2

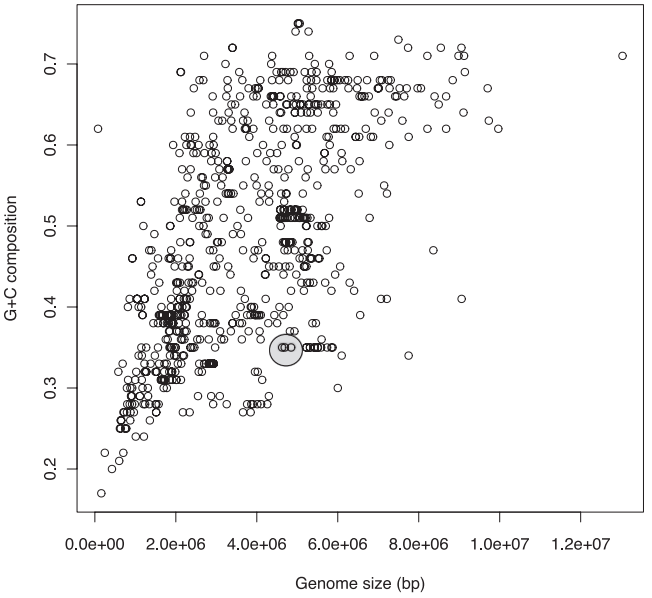

Figure 5.2 Bacterial chromosome size and percent G+C nucleotide content. Data from 782 finished eubacterial projects is included. The points corresponding to *Bacillus anthracis* and *Bacillus cereus* s.l. genomes are indicated by the gray circle.

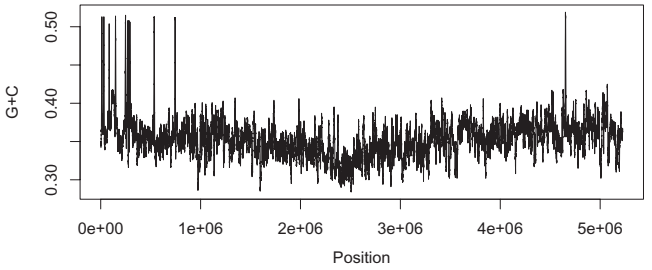

Figure 5.3 G+C variation across the *Bacillus anthracis* chromosome. G+C fraction is plotted against position in 10,000 bp windows. The graph shows the gradual decline in G+C bases from the replication origin (coordinate 0) to the termination region. Strong peaks in G+C correspond to rRNA operons. Putative islands in the genome (see Table 5.3) do not have a notable bias in G+C content.

have slightly lower G+C content (32.5% and 33.0%) and a distinctively different dinucleotide signature (Suzuki et al., 2008; van Passel et al., 2006). However, a more sensitive nucleotide composition test suggests that the pXO1 plasmid may have evolved in a similar host background to *B. anthracis* (Suzuki et al., 2008). Another marked composition bias in the genome is the relative strand abundance of G over C nucleotides, commonly referred to as GC skew (Lobry, 1996) (Figure 5.4). The switch in strand bias clearly demarks the origins of replication and termination. More than 75% of the *B. anthracis* genes are oriented to be transcribed in the same direction as the replication fork, presumably to maximize mRNA production (Rocha and Danchin, 2003). There is an even stronger bias for "essential," highly conserved genes in gram-positive bacteria to be aligned with the

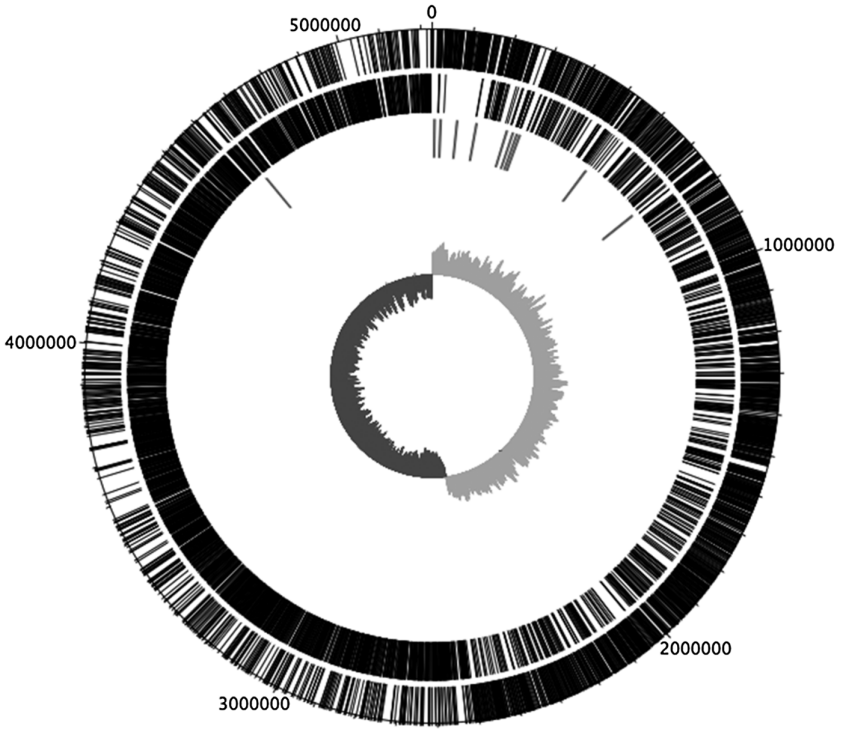

Figure 5.4 Circular representation of the *B. anthracis* genome showing GC skew bias. The outer two rings of the circle are the starting positions of genes on the forward and reverse strands. There is a greater density of genes with transcription oriented in the same direction as the direction of replication. The third circle shows the location on the 11 rRNA operons, clustered near the origin of replication. The innermost circle shows the GC skew (Lobry, 1996) with values below the average shown below the line of the plot and the reverse for positive values. This figure was produced using DNAPlotter (Carver et al., 2009).

replication fork (Rocha and Danchin, 2003). Interestingly, all four prophages are orientated in the same direction as the replication fork.

The *B. anthracis* chromosome is closely packed with genes (84.3% coding [Read et al., 2003]), typical for bacteria that are not undergoing reductive evolution. Highly conserved genes encoding functions such as spore formation and DNA metabolism that cluster near the replication origin may benefit from transiently higher copy number due to their adjacency to the replication fork initiation. Conversely, the replication terminus has a lower total mRNA coverage depth when measured by whole-genome transcriptional profiling (Passalacqua et al., 2009). There are predicted to be at least 1,120 multigene operons in *B. anthracis* (Bergman et al., 2007). Thirty-seven pseudogenes were originally identified in the genome by manual annotation, although the number has been estimated to be as high as 166 using comparisons to *B. cereus* genomes and intergenic distance models to detect potentially anomalous open reading frames (Price et al., 2005). Even though their numbers are small, some of these defective genes lead to striking changes in *B. anthracis*. Perhaps the most sweeping phenotypic variant is produced by a null mutation of the *plcR* (phospholipase C regulation) gene. PlcR is a pleiotrophic global regulator of extracellular functions such as phospholipases, endonucleases, and hemolysins (Agaisse et al., 1999). The canonical binding site TATGNAN$_4$TNCATA is present 56 times on

Table 5.3 Chromosomal DNA Segments Unique to *Bacillus anthracis* Identified by Read et al. (2003)

Coordinates[a]	Size (bp)	G+C(%)	Genes	Description	Unique to *B. anthracis*?[b]
447,283-484,667	37,385	0.35	BA0427-BA0846	PHAGE01	Yes
2,037,962-2,068,884	30,923	0.34	BA2184-2215	IS110 family IS element and several small, hypothetical proteins	No[c]
3,456,508-3,507,049	50,482	0.34	BA3759-BA3941	PHAGE04	No[d]
3,745,626-3,789,668	44,043	0.35	BA4134 BA4064-	PHAGE03	No[e]
4,298,186-4,310,327	12,140	0.36	BA4726-BA4738	Deaminase/ oligonucleotide binding proteins	No[f]
4,841,735-4,858,493	16,759	0.35	BA5363-BA5339	PHAGE02	Yes

[a] AE016879 Porton Ames Chromosome.

[b] Region aligned against the NCBI RefSeq Genome Database, July 2009 Using BlastN (megablast). Matches >60% of region length and >90% maximum identity considered significant.

[c] Match to *B. weihenstephanensis* KBAB4, *B. cereus* E33L, AH820, Al Hakam, O3BB102 (>60% coverage, >95% identity).

[d] Match to *B. weihenstephanensis* KBAB4 (64% coverage, 97% identity), *B. cereus* ET33L (60% coverage, 100% identity).

[e] Match to *B. cereus* AH157—63% coverage , 98% maximum identity.

[f] Match to *B. cereus* E33L, AH820, *B. thuringiensis konkukian* (>99% coverage, >99% identity).

either strand of the *B. anthracis* chromosome and 2 times in pXO2 with a putative regulon of over 45 genes. The lack of production of phospholipase C is a classical *B. anthracis* phenotypic marker (Turnbull, 1999). Another important feature for clinical typing of *B. anthracis*, its lack of motility, appears to be primarily due to a mutation in the flagellar motor switch gene, *fliM*, and the deletion of flagellin structural subunits. Resistance of *B. anthracis* to gamma phage is caused by mutation in the gene encoding a sortase anchored surface protein, GamR (Davison et al., 2005).

In the Ames Porton analysis, Read et al. (2003) identified only six "genome islands" in *B. anthracis*: blocks of DNA which, by sequence and microarray-based hybridization, were not present in any of a range of *B. cereus* s.l. close relatives (Table 5.3). The G+C content of these regions was not significantly different from the rest of the genome, in contrast to what is often reported for classic islands within the Enterobacteriaceae (Hacker et al., 1997). Four of these islands were prophages that were conserved in more than 100 diverse *B. anthracis* isolates tested (Sozhamannan et al., 2006). Although all four prophages were able to excise and form circular replication intermediates, as shown by real time PCR, they were all found to be defective in production of an intact virion. As more and more sequencing of close relative genomes is completed, the extent of unique sequence in *B. anthracis* will continue to dwindle. Alignment of the six identified islands against the 2009 NCBI nucleotide database revealed that only two phage regions still lack significant homology to another *B. cereus* s.l. strain (Table 5.3).

Repeat sequences play a highly significant role in genome evolution, serving as templates for gene duplications, deletions, and rearrangements. Treangen et al. recently

published a survey of the prevalence of repeats in 720 bacterial chromosomes, using their *ab initio* repeat detection tool, Repeatoire (Treangen et al., 2009). Based on this scan, 6.3% of the *B. anthracis* chromosome is covered by large repeat sequences (329,028 bp). Within 720 genomes tested, this percentage is toward the median figure, between the high of 42% for the degrading *Ricketssia* relative *Orientia tsutsuamushi* and lows of 0% for some endosymbionts such as *Buchnera aphidicola* (Treangen et al., 2009). The values for *B. cereus* ATCC 10987 and ATCC 14579 were 5.5% and 6.1%, respectively. The 11 ribosomal RNA operons in *B. anthracis* make up 15% of the repetitive DNA >400 nt long (the *B. cereus* genomes similarly have 12 and 13 operons, respectively). The number of ribosomal genes generally scales with bacterial genome size. The necessity for the large number of operons in *B. cereus* has not been adequately explained, although there is a somewhat controversial association of total rRNA DNA with growth rate (Treangen et al., 2009). Transposons constitute another portion of the repeat DNA although number of annotated genes falling into this category (18) is quite small for a genome of 5.2 Mbase (Read et al., 2003). Three of the known transposons (some may have been missed through lack of similarity to known sequences or degradation) are members of the IS231 family of composite transposons (Jensen et al., 2003) that are widely distributed throughout the *B. cereus* species.

The bcr1 repeats are an important family of short dispersed repeats of ~155 bp (therefore falling below the cutoff used for the Repeatoire analysis mentioned above) that are found only in the *B. cereus* s.l. strains (Okstad et al., 2004). The bcr1 repeats appear to be highly mobile, existing at different locations in different *B. cereus* genomes. The number of bcr1 repeats is quite variable between genomes. *B. anthracis* has only 12 bcr1 elements, whereas *B. cereus* ATCC 10987 has 79 and *B. cereus* ATCC 14579 has 54. These repeats appear to target TTTAT motifs, causing direct repeats on either side of the insertion. This suggests that bcr1 may be mobilized by a transposon common to *B. cereus* species genomes. The repeats show strong bias for insertion in a particular orientation with respect to the origin of replication (Okstad et al., 2004). The bcr1 repeats have been shown experimentally to be part of RNA transcripts when inserted into genes (Klevan et al., 2007). Full-length bcr1 are predicted to form hairpin structures with low free energy; hence, they may have a role in modulating transcription or mRNA stability. The bcr1 is merely the best studied of at least 18 families of 100–400 bp repeats in the *B. cereus* s.l. species (Klevan et al., 2007).

Another distinctive repeat class putatively present in the *B. anthracis* genomes are CRISPRs (clustered regularly interspaced short palindromic repeats) (Jansen et al., 2002). This heterogeneous group of sequences, found in many bacterial lineages, is composed of short direct repeats intermingled with copies of *cas* (CRISPR-associated recombinase) genes and invariant sequences. Recently, CRISPRs have been shown to function as an immune-like phage defense, recruiting direct repeats from the genome of infecting viruses to provide protection through an RNA interference mechanism (Brouns et al., 2008). A recent search of the *B. anthracis* genome using an online CRISPR database tool (Grissa et al., 2007) located two likely elements, at coordinates 2720036-2720233 and 5014881-5015014 on the Ames Ancestor chromosome. Whether these functionally offer *B. anthracis* protection against phage infection (Kunin et al., 2007) is a question inviting exploration.

One of the most surprising findings from the initial *B. anthracis* sequencing study was the insertion of a type I intron in the *recA* gene, the product of which is essential for homologous recombination in bacteria. Ko et al. (2002) showed that the intron is excised during transcription, leaving the RecA protein functional in *B. anthracis*. The

recA intron insertion is not unique to *B. anthracis*—recent genome sequencing studies have turned up the same insertion in other *B. cereus* genomes. In a recent thorough analysis, Tourasse and Kolsto uncovered two other type I and 2 type II introns in the Ames Ancestor sequence (Tourasse and Kolsto, 2008) genome. These and other related introns may be spread horizontally through the *B. cereus* group, with bacteriophage the likely carriers.

GENES ENCODED BY *B. ANTHRACIS*

Arguably the most important reason for sequencing bacterial genomes is to discover the function of new genes and understand better the variations in known genes. Nevertheless, there must be some caution used in interpreting annotated genomes. Most annotation pipelines start with *in silico* prediction of open reading frames likely to encode true genes. The genes are then subjected to searches against databases, usually using BLAST (Altschul et al., 1997) or Hidden Markov Model-based alignment of gene models. In some cases ("manual annotation") experts adjust the gene calls and carefully name the genes based on their own extended investigation. In other systems, gene naming is achieved purely computationally. Therefore, annotation is a somewhat subjective art that can produce quite different results for even very similar organisms depending not only on *how* the analysis was performed but also on *when* it was performed. An example of how annotation changes over time is the discovery in the *B. anthracis* genome of numerous predicted transcribed small RNA molecules, including riboswitches (Griffiths-Jones et al., 2005) that has been made possible by experimental work completed after publication of many of the early bacterial genomes. In the original Porton Ames, small noncoding RNAs were annotated simply as blank intergenic regions (Read et al., 2003). Transcription of many of these regions was confirmed by whole-genome sequencing of the *B. anthracis* transcriptome (Passalacqua et al., 2009).

One of the most popular and robust methods for global gene analysis is the Clusters of Orthologous groups (COGs) database maintained by the NCBI (Tatusov et al., 2000). Predicted proteins in a genome can be assigned to a curated COG based on BLAST alignment, and a reasonable inference about function can be drawn. In a recent analysis of the Ames Ancestor on the NCBI website 3,719 proteins could be assigned to known groups (Table 5.4). The overall composition of *B. anthracis* COG functional classes very much reflects general trends across bacteria. *B. anthracis* has a larger than average genome size for bacteria in general. As genome size increases, the relative proportion of ORFans (genes of unknown function—probably horizontally transferred plasmids and phage determinants) and informational genes involved in transcription increases while the number of genes involved in the basic translational machinery remains level and hence decreases as a fraction (Koonin and Wolf, 2008). Early genome-based analysis of the metabolism led to suggestions that *B. anthracis*/*B. cereus* was unusually rich in proteases and ABC protein transporters but deficient in complex polysaccharide metabolism compared to *Bacillus subtilis*, which was not presumed to be an adapted pathogen. However, it is harder to discern this trend as more genome data and more whole-genome analysis tools have become available. The MEROPS database (Rawlings et al., 2008), an exhaustive, manually curated resource for protease information, lists approximately similar ranges of matches across all *Bacillus* genomes (200–270 putative proteases), and these numbers are not unusually high for soil-dwelling bacterial genera. Likewise, comparative analysis of *B. subtilis* and *B. anthracis* using the SEED subsystems-based annotation website (Overbeek

Table 5.4 COG Functional Category Breakdown for *Bacillus anthracis* Ames Ancestor

Code	Number of COGs	COG category description	*B. anthracis* (%)	Average *Bacillus* (%)	Average all bacteria (%)
J	201	Translation	3.3	5.0	4.3
A	0	RNA processing and modification	0.0	0.0	0.0
K	448	Transcription	7.4	7.0	6.0
L	190	Replication, recombination, and repair	3.1	5.1	4.8
B	2	Chromatin structure and dynamics	0.0	0.0	0.0
D	43	Cell cycle control, mitosis, and meiosis	0.7	0.8	0.8
Y	0	Nuclear structure	0.0	0.0	0.0
V	110	Defense mechanisms	1.8	1.9	1.3
T	208	Signal transduction mechanisms	3.4	3.3	4.0
M	209	Cell wall/membrane biogenesis	3.4	3.9	4.4
N	49	Cell motility	0.8	0.7	1.5
Z	1	Cytoskeleton	0.0	0.0	0.0
W	0	Extracellular structures	0.0	0.0	0.0
U	45	Intracellular trafficking and secretion	0.7	1.1	1.8
O	114	Posttranslational modification, protein turnover, chaperones	1.9	2.3	2.9
C	210	Energy production and conversion	3.5	3.4	4.8
G	273	Carbohydrate transport and metabolism	4.5	6.2	5.0
E	458	Amino acid transport and metabolism	7.5	7.6	7.3
F	106	Nucleotide transport and metabolism	1.7	2.4	1.8
H	149	Coenzyme transport and metabolism	2.4	2.6	3.0
I	130	Lipid transport and metabolism	2.1	2.2	2.9
P	287	Inorganic ion transport and metabolism	4.7	4.7	4.6
Q	111	Secondary metabolites biosynthesis, transport, and catabolism	1.8	1.5	2.3
R	727	General function prediction only	12.0	10.9	10.5
S	419	Function unknown	6.9	7.2	6.1
—	1592	Not in COGs	26.2	20.0	20.2

Based on data from the NCBI COG website: http://www.ncbi.nlm.nih.gov/sutils/.

et al., 2005) failed to yield an obvious gross genome-wide difference in the numbers of genes involved in protein transport and polysaccharide degradation.

The annotated genome sequence has greatly accelerated the study of the structure of the *B. anthracis* surface (Anderson et al., 2005; Fouet, 2009). Seven hundred seventy *B. anthracis* proteins contain a type II signal sequence for transmembrane export, and 95 contain predicted transmembrane helices (using the prediction software SignalP [Nielsen et al., 1997] and TMHMM [Krogh et al., 2001], respectively). There are at least two gram-positive specific systems for attaching proteins on the vegetative cells surface. Three sortase genes (*srtA*, *srtB*, and *srtC*) are responsible for cleaving proteins at an LPXTG-like (or LPTNA, in the case of *srtC* [Marraffini and Schneewind, 2006]) C-terminal amino acid motif and attaching them to peptidoglycan (Read et al., 2003). At least nine proteins in the *B. anthracis* genome are potential substrates for sortases (Read et al., 2003). The products of the *csaAB* genes are responsible for attaching proteins with S-layer homology (SLH) domains to peptidoglycan, including the major surface proteins Eag and Sap

(Mesnage et al., 1999). There are up to 32 other proteins with SLH domains in the genome (Read et al., 2003). BslA, a pXO1-located SLH domain protein, was shown to be incorporated into the S-layer and necessary for attachment of vegetative *B. anthracis* to human cells (Kern and Schneewind, 2008). The genome sequence has been used to further examine the structure of the *B. anthracis* spore (Anderson et al., 2005; Fouet, 2009): among other discoveries was the identification of an eight gene cluster encoding exospore component (Todd et al., 2003).

Genome-based analysis of *B. anthracis* yielded clues that the organism may have evolved from an insect-infecting ancestor. *B. anthracis* has genes encoding chitanases, chitosan cleavage enzymes, and *N*-acetyl glucosamine utilization functions (although these are not expressed, owing to the *plcR* mutation). There are two orthologs of the immune inhibitor metalloprotease (IhnA) first described in *B. thuringiensis* for counteracting insect defenses against infection. IhnA and another secreted neutral protease, Npr599, appeared to be virulence factors in the systemic phase of mouse infection (Chung et al., 2006). Genome sequencing also led to the discovery that *B. anthracis* has a homolog of the enhancin protease that aids baculoviruses infection of gypsy moth guts (Lepore et al., 1996; Read et al., 2003). Since research on the determinants for insect virulence is still in the early phase, more genes may later be assigned to this role. *B. thuringiensis*, which produces diverse crystal toxins, is an insect pathogen. *B. cereus* strains also have been found to be associated with insect guts, including a filamentous "Arthromitus" stage (Jensen et al., 2003; Margulis et al., 1998). Although little is known about *B. anthracis* and *B. cereus* ecology outside direct host interactions, some have speculated that the *B. cereus* s.l. group in general may have extensive endosymbiotic relationships which only occasionally are directly pathogenic (in the case of toxigenic *B. thuringiensis*). Mahillon et al. and others have suggested that in Africa, *B. anthracis* may be disseminated via ingestion and excretion by Tabanid flies (Jensen et al., 2003).

Pathogenenesis of anthrax in mammals is believed to follow in a discrete series of stages, from engulfment of the endospore, survival in the harsh intracellular environment, through to escape from the phagocytotic vacuole and outgrowth of the vegetative cell (Dixon et al., 1999; Passalacqua and Bergman, 2006). While knowledge of the plasmid-encoded virulence factors such as the tripartite anthrax toxin cluster and *atxA* virulence regulator (pXO1) and poly-D-glutamate capsule cluster predated the genome sequence, much new data on genes involved in interaction with the mammalian host directly have been generated as a result of the project. Several *B. anthracis* genetic knockout strains have defects infecting cultivated macrophages. A mutant in the putative spore protein gene *soaA* showed reduced uptake by RAW264.7 macrophage cells (Cote et al., 2008). Anthrolysin O (ALO), a cholesterol-dependent cytolysin (Shannon et al., 2003) can complement the phagocytotic vacuole escape function of its *Listeria* counterpart (Wei et al., 2005). In combination with three phospholipase mutants, ALO knockouts are attenuated in mouse infection (Heffernan et al., 2007), suggesting that *B. anthracis* has a battery of toxins for phagosome disruption. *B. anthracis* also encodes an array of five catalases, and four superoxide dismutases, which may serve to protect the endospore from oxidative damage in the hostile macrophage environment after engulfment (Baillie et al., 2005; Passalacqua et al., 2006). Mutant studies suggest that the product of the *dltABCD* locus, a D-alanine esterification system, protects germinated cells from cationic antibacterial peptides (Fisher et al., 2006).

Following the establishment of systemic infection, *B. anthracis* growth is limited by micronutrients, most importantly, iron. The *B. anthracis* genome sequence revealed biosynthetic genes for two iron-binding catecholate siderophores, bacillibactin (BB) and petrobactin (PB), secreted via membrane-associated substrate-binding proteins (SBPs) (Read et al.,

2003). Mutant analysis (Cendrowski et al., 2004; Wilson et al., 2006) showed that PB, which is also found in some closely related *B. cereus* strains, is necessary for maximal *B. anthracis* Sterne virulence in mice. The SBPs for PB have now been identified (Zawadzka et al., 2009). Other micronutrient scavenging systems are coming to light. A surface protein, IsdC, anchored to peptidoglycan by SrtB, is required for heme-iron scavenging (Maresso et al., 2006). Mutants of the *mntA* gene, part of a manganese ATP transport system, show impaired growth and delayed release from macrophage release (Gat et al., 2005).

What was discussed previously was by necessity a very brief overview of some of the recent post-genomic development in characterization of the *B. anthracis* proteome. A common feature of almost all the genes detailed is their conservation across many *B. anthracis* close relatives: there are few, if any, unique *B. anthracis* chromosomal virulence factors. There is also increasing interest in the role of gene deletions and disruptions in the evolution of pathogenesis, the so-called pathoadaptive mutations (Maurelli et al., 1998). As comparative genome sequencing of near neighbors gathers momentum, it will be possible to identify more potentially important recent loss-of-function mutations. One example is the deletion of the arginine deaminase gene cluster in *B. anthracis* (Ivanova et al., 2003).

POPULATION BIOLOGY

The genome sequence has revolutionized our understanding of the population genetics of *B. anthracis*. The bacterium has been described as a highly monomorphic species, with few to zero biochemical or morphological features available to subtype strains (Turnbull, 1999). Early sequence-based studies revealed very low levels of nucleotide diversity, making subtyping inefficient (Patra et al., 2002; Price et al., 1999). At least four multilocus typing schemes (MLTS) have been developed for the *B. cereus* group over the past 10 years (Helgason et al., 2004; Ko et al., 2004; Priest et al., 2004; Sorokin et al., 2006; Tourasse and Kolstø, 2008). Each MLTS scheme samples 2,500–3,500 bp nucleotide sequence spread over six to eight chromosomal loci. For each scheme, variant *B. anthracis* strains show only two to four different sequence types, making the discrimination at the subspecies level practically impossible. Therefore, genetic tests with a much greater level of resolution were needed. The publication of the genome sequence dramatically increased the number of markers available to researchers and opened the door to unraveling the history of the pathogen. Since the genome sequence, *B. anthracis* population biology and evolution have been dissected by a variety of molecular methods, including tandem repeat analysis (Keim et al., 2000), tiling and resequencing microarray analysis (Kenefic et al., 2009; Zwick et al., 2005, 2008), SNP analysis (Van Ert et al., 2007b), and whole-genome sequencing (Pearson et al., 2004). The latter technology is likely to be increasingly used in the future of *B. anthracis* population studies.

A major step toward uncovering natural *B. anthracis* diversity was the publication of the *B. anthracis* variable number of tandem repeat (VNTR) typing scheme by Keim et al. in 2000. The authors utilized eight VNTR loci, based on results from previous amplified fragment length polymorphism studies. The rate of change in the number of repeats for the VNTR loci was significantly greater than that seen for SNPs. Based on VNTR profiles, Keim et al. (2000) were able to discriminate a worldwide collection of 426 *B. anthracis* into 89 genotypes and to propose phylogenetic relationships between strains. The broad outline of the results from this study has been supported by every subsequent population analysis. The species split into two main subgroups, A and B (a third, rarer subgroup C was discovered in later analysis [Pearson et al. 2004]). Also, pXO1 and pXO2 VNTR

markers cosegregated with the chromosome (Keim et al., 2000; Price et al., 1999), providing no evidence of horizontal exchange of plasmids between *B. anthracis* strains (a similar result was obtained by Zwick et al., 2005 using resequencing microarrays).

Early comparative sequencing of five genomes using a shotgun Sanger approach gave an overview of the extent of genetic variation within *B. anthracis* (Pearson et al., 2004). There were 1168–1350 high-quality SNPs separating Ames (subgroup A3) from the B subgroup strains and 2075 from the C group representative. Four hundred twenty-eight SNPs separated Ames from Vollum, a representative A1 subgroup strain. A molecular clock estimation (Van Ert et al., 2007a) suggested divergence of the C branch from A and B to have occurred 12–26,000 years ago and the radiation with the A subgroup to be between 3–6,000 years old, although these numbers may be greater if alternative models for the generation time of *B. anthracis* spores are used (Kenefic et al., 2009). As would be expected from genomes with such low level of variation, there were very few homoplasious nucleotide sites (sites with more than one character variant). Van Ert et al. (2007b) introduced the concept of canonical SNPs (canSNPs), a genotyping scheme based on a robust subset of the ~3,500 SNPs discovered from the sequence of multiple diverse *B. anthracis* genomes that efficiently classified strains into the major branches of the VNTR-based phylogeny. In a global survey, 1033 isolates from 42 countries were typed using a combination of 12 canSNPs and VNTR analysis (Van Ert et al., 2007a). There appeared to be a significant geographic population structure, with the A subgroup strains being broadly dispersed around the globe and the B subgroup being much more commonly encountered in Africa. This may be a result of differential fitness of A over B (there is limited evidence of elevated soil survival profiles of some lineages (Smith et al., 2000)) or more likely, a chance consequence of the transport of livestock in the colonial era (Van Ert et al., 2007a). An Affymetrix resequencing microarray based on 2850 SNPs discovered in the early *B. anthracis* genome comparison (Pearson et al., 2004; Van Ert et al., 2007a) was used as a tool to dissect the very few differences between the Western North America (WNA) group of *B. anthracis* strains (Kenefic et al., 2009). The most recent ancestor of this lineage is a Eurasian strain, while newer offshoot braches accumulate in a Southerly gradient. These results lead to the development of a hypothesis that WNA *B. anthracis* strains were introduced to the Western United States and Canada via a Bering Sea land bridge rather than through European livestock import radiating out from the East Coast ports. One place where *B. anthracis* strains of different provenance seem to have encountered each other is Texas, where the Ames strain was originally isolated. Ames itself may have its recent origins in China and may have been shipped to East Texas, rather than pre-Columbian importation from Russia (Simonson et al., 2009).

The ultimate goal of genomics-based population analysis is to use both rare and common genetic variants. One approach toward this is to use microarray-based resequencing (Hacia et al., 1999) of regions of the reference genome strains. Zwick et al. (2005, 2008) designed a microarray, based on the unpublished Ames Porton sequence, to probe 29,500 bp of sequence spread over eight regions of the chromosome and one region each of pXO1 and pXO2. Therefore, the study design resembled traditional MLTS but with approximately 10-fold greater sequence coverage per strain. Fifty-six diverse strains of *B. anthracis* were sequenced, yielding 37 high-quality differences (about 10 times the number found in MLTS studies). Key findings were that the genetic variation in the *B. anthracis* population was in line with the Neutral model of molecular evolution (Kimura, 1983) and that there was no evidence of recombination between loci on any of the eight dispersed regions of the chromosome. The phylogeny of *B. anthracis* based on the resequencing studies also broadly agreed with the VNTR results (Keim et al., 2000).

B. anthracis then, is clearly a clonal population recently emerged from within the greater *B. cereus* species. There is some argument about whether the lack of diversity between isolates is to some degree a function of an unusually low mutation rate. Using a mutation detection system based on the *rpoB* gene resistance to the antibiotic rifampicin, Ziebell et al. (2007) showed that the spontaneous mutation rate per available site per replication in *B. anthracis* (1.1×10^{-10}) was similar to *Escherichia coli* and the radiation resistant bacterium *Deinococcus radiodurans* (both 1.9×10^{-10}). However, a comparative genomic analysis suggested that there may be auxiliary, as yet uncharacterized, DNA repair pathways in *B. anthracis* (Zeibell et al., 2007).

CONCLUSIONS: AN EXTRAORDINARY PATHOGEN WITH AN ORDINARY CHROMOSOME?

The genome of *B. anthracis*, a bacterium that causes lethal infections of mammals when almost all other *Bacillus* strains are non-pathogens, seems to tell two very different stories. The narrative gleaned from the analysis of the chromosome is of a free-living environmental bacterium, typical in the composition of metabolic, transport, and informational genes for a bacterium of 5.2 Mb. It shares its unusually low G+C content with other *B. cereus*, which may be an indication of their common facultative pathogenic ancestry. The many toxins and virulence genes that allow macrophage invasion and iron scavenging may have been carried forward from a previously invertebrate-infecting progenitor. In terms of numbers, most features of the chromosome (repeats, introns, etc.) fall within the normal range for *B. cereus* s.l. strains. The population genetics of *B. anthracis* are consistent with recent clonal expansion from a single bacterium. Worldwide dispersal may have been facilitated by the development of human agriculture and intercontinental trade routes. The acquisition of the virulence plasmids is probably the impetus behind the successful emergence of *B. anthracis*. Under these circumstances, the other features that distinguish the entire clonal group (such as the flagellum and the *plcR* mutations) must have either been present on the chromosome of the *B. anthracis* ancestral cell, occurred soon after the plasmid acquisition event(s) and been fixed by stochastic genetic drift, or must have been the result of selective pressure.

The plasmids, particularly pXO1, are the other story. pXO2-like plasmids carrying poly-D-glutamic capsules are quite common in *Bacillus*, although *B. anthracis* may have specific regulatory adaptations to enhance anthrax pathogenesis (Drysdale et al., 2004; Pannucci et al., 2002; Park et al., 2007). pXO1 shares a common backbone with other replicons in the *B. cereus* s.l. group (Rasko et al., 2004), but it contains the toxin genes necessary for anthrax. Other rare *B. cereus* strains carrying plasmids essentially identical to pXO1 are linked with diseases suspiciously similar to anthrax (Hoffmaster et al., 2004; Klee et al., 2006; Miller et al., 1997). pXO1 profoundly changes the pattern of gene regulation while in the host, the AtxA protein orchestrating plasmid and chromosomal gene transcription (Fouet and Mock, 2006; Perego and Hoch, 2008). Signals from conserved regulatory systems such as the bicarbonate sensing signal transducer (Wilson et al., 2008) and the sporulation phosphorelay system (Brunsing et al., 2005) are intercepted to reprogram the cell for expression of virulence determinants. Other pXO1 regulators of chromosomal genes with possibly important roles in virulence are being discovered (Aronson et al., 2005). It may turn out that the pXO1 plasmid has been a mammalian pathogen for much longer than the chromosome of its current host, the bacterium we call *B. anthracis*, and may have acquired a greater arsenal of specific virulence adaptations.

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Chapter 6

Bacillus anthracis Plasmids: Species Definition or Niche Adaptation?

David A. Rasko

THE *B. CEREUS* GROUP

Bacillus anthracis is a member of the *Bacillus cereus* group that includes *B. cereus*, the namesake member of the group, which causes a wide range of infections in humans; *Bacillus thuringiensis*, the world's most widely used biopoesticide, and *B. anthracis*, the etiological agent of anthrax, as pathogenic members of the group. In addition to these pathogenic members, the group includes *Bacillus mycoides, Bacillus pseudomycoides, Bacillus medusa*, and *Bacillus weihenstephanensis*, which have not been associated with any type of disease. Historically, pathogenic potential and diverse host range has defined the members of the *B. cereus* group (Jensen et al., 2003; Rasko et al., 2005). The small, usually metabolic-based differences found in the chromosomal content cannot account for the spectrum of disease and host range observed in this group (Ivanova et al., 2003; Rasko et al., 2004; Read et al., 2003). However, much of the disease and host specificity in this group can be attributed to plasmid content (Rasko et al., 2005, 2007). The plasmids associated with the *B. cereus* group of organisms have broad size range (5–200 kb) and vary in number. Traditional typing systems are inadequate in defining *B. anthracis* from other closely related species within the *B. cereus* group. There have been multiple studies that have suggested that the members of the *B. cereus* should be considered a single species; however, this has met with resistance from both the biopesticide research groups as well as the biodefense research communities (Helgason et al., 2000a,b).

The most striking example of the plasmid content affecting host range and pathogenesis occurs in *B. anthracis*. *B. anthracis* harbors two plasmids, one that encodes the tripartite lethal toxin complex, pXO1 (Okinaka et al., 1999a), and the other, which contains the biosynthetic genes for the poly-γ-D-glutamic acid capsule (Okinaka et al., 1999b; Pannucci et al., 2002). While each plasmid is a distinct entity, it has been shown that loss of either one results in an attenuated *B. anthracis* isolate (Mock and Fouet, 2001; Turnbull, 1991). The role of the *B. anthracis* plasmids in pathogenesis is exquisitely known; however, there is still much to discover about the role of the plasmid content in the general metabolic state of *B. anthracis* as well as regulatory networks (Bourgogne et al., 2003).

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

Table 6.1 Distinguishing Characteristics of *B. anthracis* and *B. cereus*

	B. anthracis	*B. cereus*
Hemolysis	Nonhemolytic	Hemolytic
Motility	Nonmotile	Motile
Gamma-phage susceptibility	Susceptible	Resistant
Penicillin susceptibility	Susceptible	Resistant
Cell wall DFA	Positive	Variable
Capsule DFA	Positive	Negative
16S rRNA type	Type 6	Variable (not type 6)

DFA, direct fluorescence assay.

PLASMIDS OF *B. ANTHRACIS*

Historically, *B. anthracis* has been defined from other closely related *Bacillus cereus* group members through a number of variable phenotypic features (Table 6.1). These traits are related to chromosomal features that are very minor differences. By way of example, motility separates *B. cereus* from *B. anthracis*; however, when one examines the genomes in detail, there are fewer than five changes in the chromosomal content encoding the flagella machinery in *B. anthracis*, and most of those are point mutations resulting in frameshifts and the introduction of premature stop codon (Rasko, unpublished material). The current absolute definition of the "*B. anthracis*" is related to the carriage of the two virulence-associated plasmids pXO1 and pXO2 that encode the tripartite anthrax toxin and poly-γ-D-glutamic acid capsule, respectively (Okinaka et al., 1999a,b; Pannucci et al., 2002). These plasmids are the basis for the molecular definition of *B. anthracis* and rapid molecular tests that are now utilized to positively identify this species; however, as was discovered by Hoffmaster et al (2004), this is based on the flawed concept that these plasmids only exist in the *B. anthracis* species.

The pXO1 Plasmid

The plasmid known as pXO1 is the largest plasmid in *B. anthracis*, at ~181 kb, and encodes the tripartite lethal toxin that will be discussed in detail elsewhere in this volume. There are multiple versions of the pXO1 plasmid that have been sequenced (Table 6.2); however, three are of critical importance: one from the isolated plasmid preparation from the Sterne vaccine strain (Okinaka et al., 1999a,b) as this isolate is the basis for vaccine studies and this was the first plasmid sequenced; one from a whole-genome shotgun preparation isolated from the spinal fluid of an anthrax attack victim in 2001 (Read et al., 2002; Ames Florida); and one from a complete genome project of *B. anthracis* Ames Ancestor representing the only isolate of *B. anthracis* to have the full genomic completely sequenced (Ravel et al., 2009). The content of the pXO1 plasmid does not vary greatly between these sources. The greatest variation is observed between the two Ames strains and the Sterne strain, most likely a result of the plasmid curing process that removed the pXO2 plasmid from the Sterne isolate. The lack of the pXO1 plasmid results in the formation of the Pasteur vaccine strain that is commonly used in Europe to vaccinate livestock (Mock and Fouet, 2001), and hence it is considered essential for virulence. The genome sequencing of diverse *B. anthracis* isolates identified only one isolate that lacked the pXO1 plasmid or any homologous sequencing data. This strain represented a new lineage of *B. anthracis*

Table 6.2 Genome Sequencing Efforts and Plasmid Status of Projects

Strain	Source	Genome completed	Plasmid genotype	GenBank accession
Bacillus anthracis Ames	Texas, Cow; plasmids cured	Y	pXO1−,pXO2−	AE016879
B. anthracis Ames Ancestor	Texas, Cow; plasmids included	Y	pXO1+,pXO2+	AE017334, AE017336 (pXO1), AE017335 (pXO2)
B. anthracis Ames Florida	Patient, Florida, USA	N	pXO1+,pXO2+	AAAC01000001, AE011190 (pXO1), AE011191 (pXO2)
B. anthracis Kruger B	Kruger National Park, South Africa	N	pXO1+,pXO2+	AAEQ00000000
B. anthracis Western North America	Bison; Canada	N	pXO1+,pXO2+	AAER00000000
B. anthracis CNEVA-9066	Patient, Southern France	N	pXO1+,pXO2+	AAEN00000000
B. anthracis A0155	Louisiana, USA	N	pXO1−,pXO2+	AAEO00000000
B. anthracis Australia 94	Victoria Province, Australia	N	pXO1+,pXO2+	AAES00000000
B. anthracis Vollum	—	N	pXO1+,pXO2+	AAEP0000000
B. anthracis SterneA	pXO2-deficient; the basis for animal vaccines throughout the world	N	pXO1+,pXO2−	AE017225
B. anthracis Tsiankovskii-I	Russian vaccine strain	N	pXO1+,pXO2+	ABDN00000000
B. anthracis str. A0174	Canada	N	pXO1+,pXO2−	ABLT00000000
B. anthracis str. A0193	South Dakota, USA	N	pXO1+,pXO2+	ABKF00000000
B. anthracis str. A0389	Bekasi, Indonesia	N	pXO1+,pXO2+	ABLB00000000
B. anthracis str. A0442	Kudu (antelope) in the Kruger National Park, South Africa	N	pXO1+,pXO2+	ABKG00000000
B. anthracis	Bovine in France	N	pXO1+,pXO2+	ABLH00000000

that has not been observed with any frequency (Pearson et al., 2004). It is thought that the lack of pXO1 plasmid leads to a significant decrease in virulence and results in a decreased dissemination of this clone. It is also possible that pXO1 might have been lost during laboratory passages. All other *B. anthracis* isolates contain a highly similar pXO1 plasmid with little variability.

In the 10 years since the original publication of the pXO1 plasmid sequence 148 of the 204 predicted coding sequences, greater than 70% remain uncharacterized (Okinaka et al., 1999a,b). This is incredible considering that the plasmid is responsible for much of the virulence associated with anthrax. Ongoing detailed transcriptional mapping studies should be able to refine the annotation and in the process identify novel genes involved in the pathogenicity. It is to be hoped that the new technologies will spur the characterization of these unknown genes encoded on pXO1.

Virulence Encoded on the pXO1 Plasmid

The pXO1 plasmid is essential in the virulence of *B. anthracis*. Anthrax toxin has been examined in detail for a number of decades, as it is responsible for many of the adverse effects observed with *B. anthracis* infection (Friedlander, 1990; Lacy and Collier, 2002; Leppla, 1995; Mock and Fouet, 2001). The toxin is composed of three subunits. The three portions of the toxin are produced separately and are assembled on the eukaryotic membrane without the aid of any other microbial factors. The protective antigen (PA) is expressed as a pre-protein and is proteolytically processed while associated with the eukaryotic membrane into its active form, known to elicit a protective immune response against anthrax (Gladstone, 1946). The toxin has two additional subunits—the edema factor (EF) and the lethal factor (LF), which convert intracellular ATP into cAMP and cleave several mitogen-activated protein kinases (MAPKKs), respectively. The complete complement of the subunit activities are not fully characterized but do result in aberrant signaling inside the macrophages and fluid accumulation in the lung (Lacy and Collier, 2002). All three subunits are required for active toxin production and activity (for reviews, see Friedlander, 1990; Leppla, 1995; Mogridge et al., 2001; Pannifer et al., 2001; Singh et al., 2001). The three structural genes (Figure 6.1), *cya* (EF), *lef* (LF), and *pagA* (PA) are under the control of at least two regulatory elements, AtxA and PagR (Bourgogne et al., 2003), and have been shown to be expressed early in the growth of *B. anthracis* (Liu et al., 2004). These genes are encoded within a 44.5 kb pathogenicity island that is transpositionally active (Mock and Fouet, 2001) (Figure 6.1, red). An associated mobile element is thought to be responsible for this inversion event, but the mobility does not alter the virulence of the isolate (Read et al., 2002).

pXO1 Encoded Regulation of Virulence

As one would imagine, virulence regulation is exquisitely controlled, and multiple levels of control have been described involving the three genetic elements within *B. anthracis* (Figure 6.2). The dominant regulator of anthrax toxin production is AtxA, a pXO1 plasmid-encoded regulatory factor that increases the expression of all three toxin subunits, as well as the pXO2 plasmid genes for capsule biosynthesis (Bourgogne et al., 2003; Dai and Koehler, 1997; Koehler, 2002; Uchida et al., 1997). A self-regulating loop is also encoded into the toxin production by the transcription and translation of PagR, which negatively regulates the expression of *pagA*, encoding the PA subunit (Bourgogne et al., 2003). An additional toxin negative regulator of AbrB, a chromosomally encoded regulator, which also plays a key role in the sporulation pathways of other *Bacillus* species. Interestingly, the pXO1 plasmid encodes an additional copy of the *abrB* gene; however, how this impacts this regulatory network is unclear at this time. Of the predicted 204 genes encoded on pXO1, the functional characterization of only a handful has been completed; however, we must assume there is a benefit to maintaining this plasmid and clearly, more work is required on the regulation of the pXO1 features.

Copy Number

The copy number of the pXO1 plasmid has been debated over the last few years when sequencing technologies and direct measures appeared to disagree. The sequencing-based method compares the plasmid sequence coverage to the average coverage for the chromosome. This molecular ratio predicts that the pXO1 copy number is approximately two to three copies per chromosome copy (Ravel et al., 2009; Read et al., 2002). This molecular ratio was also examined in the sequencing projects listed in Table 6.2, and a similar value

Figure 6.1 Identification of the virulence, conserved, and replication regions of the pXO1 and pXO2 plasmids. Multiple plasmid comparisons have indicated that there is a conserved region in pXO1 that is shared with many other *B. cereus* plasmids (indicated by blue). Putative replication regions are highlighted in green. Virulence related factors are highlighted in red. The regions in black are conserved in some other *B. cereus* group plasmids but not all. See color insert.

was obtained for each isolate that contains pXO1. This estimate is based on the pXO1 copy number at the time of isolation and assuming that all manipulations of the genomic DNA are equally applied to the plasmid DNA. Interestingly, this estimate is much lower than indicated by other direct measurements (Coker et al., 2003). The copy number estimate based on sequencing agrees with other measures of pXO1-like plasmids in *B. cereus*, which are thought to have the same replicon as the pXO1 plasmid (Turgeon et al., 2008).

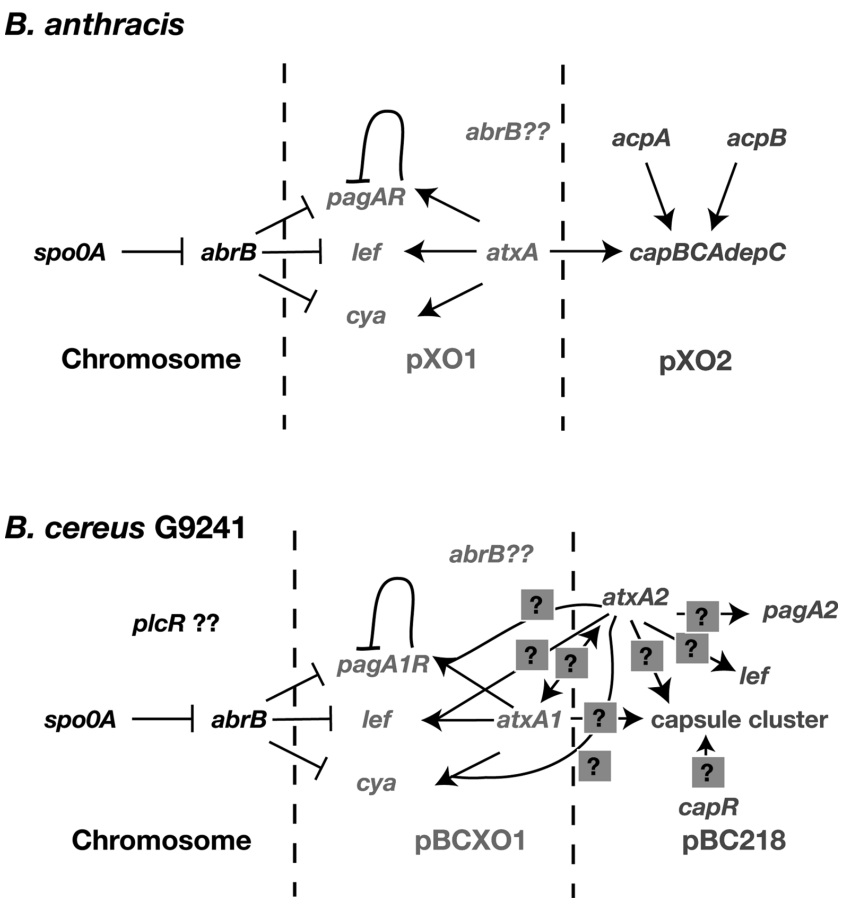

Figure 6.2 Potential cross talk between the chromosome features of *B. anthracis* and in *B. cereus* G9241. The gene features colored in red are from the pXO1 and pXO1-like plasmid; features in blue are encoded on pXO2; and features in purple are encoded on pBC210. In *B. anthracis* the AtxA peptide activates the expression of the pXO1 encoded tripartite lethal toxin as well as the capsule loci on pXO2. Additional features, AcpA and AcpB encoded on pXO2, also positively regulate the expression of the capsule. In *B. cereus* G9241 a pXO1-like plasmid is present; however, the pXO2 plasmid is absent and an alternative plasmid is present, pBC210. pBC210 encodes an additional copy of *atxA*, named *atxA2*, but it is unclear if the gene product regulates the same genes and in the same way as the pXO1 encoded AtxA. The pBC210 plasmid also encodes divergent virulence genes with unknown functional and regulatory activities. See color insert.

Replication of pXO1

The origin of replication or replication machinery of pXO1 has never been conclusively identified (Andrup et al., 1996; Okinaka et al., 1999a). A number of hypotheses have been put forward to account for this lack of clearly identifiable replication machinery, including that these plasmids carry a novel replication mechanism (Rasko et al., 2004). Attempts have been made to identify the regions involved in the replication machinery of pXO1 using subcloning. Kaspar et al. identified an 11-kb region that is thought to play a role in pXO1 replication (Kaspar and Robertson, 1987). However, this region does not encode genes with similarity to any other plasmid replication system, suggesting that pXO1 replication is unique.

Tinsley et al. have described a 5-kb region that, when cloned into a replication-deficient plasmid, could initiate replication (Tinsley and Khan, 2006). This 5-kb region contained one open reading frame encoding a gene known as RepX (aka pXO1-45). RepX is similar to the cell division protein, FtsZ, which also functions as a GTPase (Anand et al., 2008). It is unclear if this region is the only feature required for pXO1 replication or if chromosomal features are also required. A conserved region of plasmids similar to pXO1 identified in other *B. cereus* group members (Figure 6.1 and Table 6.3) all contain a RepX homolog. Alignment of the RepX homolog from each of the pXO1-like plasmids demonstrates that this peptide is highly conserved (>98% identity among the examined isolates), differing at only eight amino acids. All *B. anthracis* pXO1 plasmids have identical RepX sequence. None of the variation observed in the RepX homologs is in the

Table 6.3 Plasmid Content of Close Relatives to *B. anthracis*

Species	Strain	Plasmid name	Size (Bp)	Similar to pXO1 or pXO2	GenBank accession
Bacillus anthracis	Ames Ancestor	pXO1	181677	—	AE017336
B. anthracis	Ames Ancestor	pXO2	94830	—	AE017335
Bacillus cereus	ATCC10987	pBc1098	208369	pXO1-like	AE017195
B. cereus	AH187	pCER270	270082	pXO1-like	DQ889676
B. cereus	E33L	pE33L466	466370	—	CP000040
B. cereus	E33L	pE33L5	5108	—	CP000041
B. cereus	E33L	pE33L54	53501	—	CP000042
B. cereus	E33L	pE33L8	8191	—	CP000043
B. cereus	E33L	pE33L9	9150	—	CP000044
B. cereus	G9241	pBC210	209385	pXO1-like	DQ889679
B. cereus	G9241	pBCXO1	190861	pXO1-like	DQ889680
B. cereus	AH818	pPER272	272145	pXO1-like	DQ889678
B. cereus	NVH 391-98	pBC9801	7135	—	CP000765
B. cereus	03BB108	p03BB108_10	9797	—	ABDM02000066
B. cereus	03BB108	p03BB108_239	238933	pXO1-like	ABDM02000063
B. cereus	03BB108	p03BB108_282	282009	pXO1-like	ABDM02000062
B. cereus	03BB108	p03BB108_42	42470	—	ABDM02000065
B. cereus	03BB108	p03BB108_86	85879	—	ABDM02000064
B. cereus	AH187; F4810/72	pAH187_12	12481	—	CP001178
B. cereus	AH187; F4810/72	pAH187_3	3091	—	CP001181
B. cereus	AH187; F4810/72	pAH187_45	45173	—	CP001180
B. cereus	AH187; F4810/72	pAH187_272/ pCER270	270082	pXO1-like	CP001179
B. cereus	AH820	pAH820_272/ pPER272	272145	pXO1-like	CP001285
B. cereus	AH820	pAH820_10	10915	—	CP001286
B. cereus	AH820	pAH820_3	3091	—	CP001284
B. cereus	G9842	pG9842_140_ pseudo	140807	—	CP001188
B. cereus	G9842	pG9842_209_ pseudo	209991	pXO1-like	CP001187

(*Continued*)

Table 6.3 *Continued*

Species	Strain	Plasmid name	Size (Bp)	Similar to pXO1 or pXO2	GenBank accession
B. cereus	H3081.97	pH308197_10	10077	—	CP001168
B. cereus	H3081.97	pH308197_11	11567	—	CP001167
B. cereus	H3081.97	pH308197_258	258484	pXO1-like	CP001166
B. cereus	H3081.97	pH308197_29	29189	—	CP001169
B. cereus	H3081.97	pH308197_3	3424	—	CP001171
B. cereus	H3081.97	pH308197_73	72792	—	CP001170
B. cereus	NVH0597-99	pNVH0597_6	60944	—	ABDK02000071
B. cereus	W	pW_3	3750	—	ABCZ02000102
B. cereus	W	pW_7	7688	—	ABCZ02000101
B. cereus	W	pW_87	85757	—	ABCZ02000100
Bacillus thuringiensis	Al Hakam	pALH1	55939	—	CP000486
B. thuringiensis	HD73	pAW63	71777	pXO2-like	DQ025752
B. thuringiensis	—	pBMB175	14841	—	DQ364061
B. thuringiensis	YBT-1520	pBMB2062	2062	—	AF050161
B. thuringiensis	—	pBMB67	67159	—	DQ363750
B. thuringiensis	YBT-1520	pBMB9741	6578	—	AF202532
B. thuringiensis	INTA 14-4	pBMBt1	6700	—	AY822042
B. thuringiensis	97–27	pBT9727	77112	pXO2-like	CP000047
B. thuringiensis	—	pBtoxis	127923	pXO2-like	AL731825
B. thuringiensis	INTA-FR7-4	pFR12	12095	—	EU362917
B. thuringiensis	INTA-FR7-4	pFR12.5	12459	—	EU362918
B. thuringiensis	INTA-FR7-4	pFR55	55712	—	EU362919
B. thuringiensis	H1.1	pG11	8254	—	AY138809
B. thuringiensis	—	pG13	11365	—	Y11173
B. thuringiensis	K1	pk1S1	5475	—	EF406356
B. thuringiensis	—	pTX14-1	5415	—	BTU67921
B. thuringiensis	4Q2	pTX14-2	6829	—	AY138808
B. thuringiensis	BOE406	pTX14-3	7649	—	X56204
B. thuringiensis	LBIT-113	pUIBI-1	4671	—	AF516904

homologous or catalytic domains identified by Tinsley et al. (Tinsley and Khan, 2006). Further work is required to fully understand how RepX controls and maintains the replication functions of pXO1.

It is possible that pXO1 is a degenerate chromosome and replication is completed by the chromosomal replication machinery; therefore, it does not require additional plasmid-encoded replication machinery. Many genes found on pXO1 have significant similarity with chromosomally encoded genes from other members of the *B. cereus* group, *B. subtilis*, *Bacillus halodurans*, and other low G+C gram-positive species such as *Listeria* and *Staphylococcus* (Okinaka et al., 1999a,b; Rasko et al., 2007). Additionally, the GC skew, gene organization, and gene orientation bias of these large plasmids appear more similar to that of chromosomes (Rasko, unpublished material). In line with the chromosomal reduction theory, one can speculate that the replication machinery for these plasmids is actually chromosomally encoded, and only the actual origin of replication is present on

the plasmids themselves. In the work by Tinsley et al. the RepX protein, acting as a GTPase, provides the energy required for plasmid replication in addition to the chromosomal replication apparatus (Tinsley and Khan, 2006). A chromosomally encoded helicase described by Anand et al. in *B. anthracis* and other member of the *B. cereus* group is similar to that of *Staphylococcus aureus* plasmid pT181 (Anand and Khan, 2004; Anand et al., 2004; Naqvi et al., 2003). This helicase, PcrA, functions as a nickase and can initiate replication of pT181. The *B. anthracis* PcrA has been shown to function as a helicase and initiate replication of pT181. It is thought that the *B. anthracis* homolog performs a similar function for pXO1. The combination of the RepX and PcrA may provide the nicked pXO1 substrate required for replication of these plasmids.

The pXO2 Plasmid

The second large plasmid, ~96 kb, in *B. anthracis* encodes for the poly-γ-D-glutamic acid capsule (Uchida et al., 1985). *B. anthracis* isolates lacking the pXO2 plasmid are also not fully virulent (Mock and Fouet, 2001; Uchida et al., 1985), and the prototype strain is *B. anthracis* Pasteur that has a plasmid profile of $pXO1^{-}$, $pXO2^{+}$. The first pXO2 sequence was obtained from this isolate (Okinaka et al., 1999b). The plasmid encodes 104 genes for which 78 do not have functional annotation (78/104, 75%). In contrast to pXO1, the pXO2 replication region has been identified. pXO2 is a theta replicating plasmid similar to the prototypical pAMβ1plasmid from *Enterococcus faecalis* (Wilcks et al., 1999). Detailed analysis of the replication machinery is provided below. Several studies have indicated that pXO2 sequences are not widely distributed among closely related *B. cereus* group species based on comparative genomic hybridization (CGH), and PCR experiments (Pannucci et al., 2002; Read et al., 2003). Only two isolates, *B. thuringiensis* AW06 and *B. thuringiensis* plasmid pBT9727, showed any significant sequence similarity to pXO2. This could be an indicator of the origin of the pXO2 plasmid; however, with so few members of this group, it is difficult to make any definitive associations.

Virulence Factors of pXO2

Encapsulation is a common mechanism used by bacteria to evade the immune system. In the case of *B. anthracis*, it is unclear what role the capsule plays in pathogenesis as the spores are taken up by the macrophage at the site of infection and the vegetative cells grow rapidly in the bloodstream. There must be a transient phase where the vegetative cells are protected by the capsule as unencapsulated strains are the basis for vaccines and are shown not to infect and/or proliferate as well as encapsulated strains (Hoffmaster et al., 2004; Makino et al., 2002). Some studies have demonstrated that the capsular material protects the vegetative bacterial cells while they are *within* the macrophage, allowing growth resulting in systemic sepsis when the bacteria lyse the cell and enter the bloodstream (Makino et al., 1988, 1989). The poly-γ-D-glutamic acid capsule is produced by a three-gene biosynthetic operon, *capABC,* which is under the control of multiple regulators including the pXO1-encoded toxin regulator, AtxA (Bourgogne et al., 2003), and the pXO2-encoded regulators AcpA and AcpB (Koehler, 2002; Pannucci et al., 2002) (Figure 6.2). It is known that CO_2 and other environmental factors increase the production of the capsular material (Koehler, 2002; Pannucci et al., 2002). While synthesis of the capsule is required for virulence, it appears as though the degradation is also an important feature as degradation of the capsular material, by CapD (Uchida et al., 1993). It was demonstrated by Makino et al. that high and low molecular weight forms of the capsule appear to be

essential for infection (Makino et al., 2002). The *capD* gene is encoded on pXO2 but is not part of the *capABC* biosynthesis operon, and the regulation of this additional peptide is not well understood. It is unclear if this degradation process leads to recycling of the materials required to assemble the capsule or if the proteinaceous is also degraded by the large number of proteases encoded in the chromosome (Read et al., 2003). Almost nothing is known about the molecular mechanism for the transport of the capsular material to the surface of the bacterial cell.

While the capsule is an important feature in the virulence, it has been demonstrated that the poly-γ-D-glutamic acid capsule can be functionally complemented by other type of capsules resulting in some strains of *B. cereus* harboring pXO1-like plasmids and remaining as infective as *B. anthracis* (Avashia et al., 2007; Cheung et al., 2005; Hoffmaster et al., 2006; Sue et al., 2006; Tatti et al., 2006). Further work will be required to determine if *B. anthracis* requires the poly-γ-D-glutamic acid capsule or if "just any capsule" will be sufficient for virulence.

Replication of pXO2

The replicative machinery of the pXO2 plasmid was identified based on similarity to other replication proteins identified from plasmids in the *B. cereus* group and other gram-positive organisms. The pXO2 replicon is similar to the prototypical theta replicating plasmid, pAMβ1, identified in *E. faecalis* (Wilcks et al., 1999). Additionally, the plasmid replicon is similar to the pAW63 replicon isolated from *B. thuringiensis* (Wilcks et al., 1998). Tinsley et al. (2004) characterized the functional components of the pXO2 replicon independently and demonstrated the functionality of the replicon directly. They demonstrated that the minimal replicative unit is solely composed of the RepS peptide (BXB0039) and the origin of replication (Tinsley et al., 2004). The functional origin of replication was shown to be limited to a 60 bp region encoding the RepS protein. The nick site for the initiation of replication is highly conserved in this 60 bp region and contains a "TACAT" sequence where nicking occurs following the cytosine residue (Tinsley et al., 2004). All theta replication proteins from *B. cereus* group plasmids share a significant level of similarity and cluster together phylogenetically, suggesting a common ancestral origin. A more detailed analysis of the pXO2-like plasmids will be discussed below. As mentioned above, the pXO2-like replicons appear to be restricted to the *B. thuringiensis* members of the *B. cereus* group.

pXO2 Copy Number

As predicted for the pXO1 plasmid above, the pXO2 plasmid copy number can be predicted from the sequencing projects. As with the pXO1 plasmid, pXO2 has a relatively low copy number estimated to be between 1 and 2 copies per chromosome equivalent (Ravel et al., 2009). Interestingly, these copy number figure match more closely the values obtained from the direct measurement of the plasmid (Coker et al., 2003). These plasmids exist at relatively low copy number, but appear to be maintained in *B. anthracis* as there have been no isolates sequenced that lacked the pXO2 plasmid (Table 6.2).

B. anthracis Plasmid Mobility

The *B. anthracis* plasmids have not been directly shown to be self-transmissible; however, previous reports have demonstrated that some of them can be mobilized with the help of

conjugative plasmids (Andrup et al., 1996). *B. thuringiensis* subsp. *israeliensis* pXO16 is an example of such a conjugative "helper" plasmid (Andrup et al., 1996, 1998). No typical transfer or mobilization regions are identified in the sequence of the *B. anthracis* plasmids. In contrast, the mobilization proteins encoded on the smaller *B. cereus* ZK and other *B. thuringiensis* plasmids suggest that they may be self-mobilizable but appear to lack the ability to create pores to transfer themselves to the recipient cells. Van der Auwera et al. published a series of elegant studies demonstrating that the pBT9727 and pAW63 plasmids, both similar to the pXO2 plasmid, can initiate and complete the transfer of these plasmids (Van der Auwera and Mahillon, 2008; Van der Auwera et al., 2005, 2008). Additionally, those studies have demonstrated through *in silico* studies that the pXO2 plasmid lacks the key genetic features responsible for self-transmission. It appears as though pXO2 has lost the genes required for this function as opposed to the other plasmids acquiring the ability to transfer (Van der Auwera and Mahillon, 2008; Van der Auwera et al., 2008); however, the reason for the plasmidic reduction was not apparent.

No such system has been identified in the pXO1-like plasmid; however, candidate pore formation genes, such as the TraD/G conjugation proteins, are harbored in the highly conserved region. Sequencing of mobilizable *B. cereus* group plasmids such as pOX11 (Battisti et al., 1985), pXO12 (Battisti et al., 1985), or pXO16 (Andrup et al., 1996; Jensen et al., 1996) will further advance our understanding of plasmid transfer mechanisms in this group. A number of limited functional studies have been undertaken that demonstrate that *B. cereus* group plasmid transfer is not affected by DNase, involves membrane interaction (Andrup et al., 1998), and in some cases employs an "aggregation substance" (Andrup et al., 1998), all of which suggest a conjugative transfer mechanism.

B. CEREUS GROUP PLASMIDS SIMILAR TO *B. ANTHRACIS* PLASMIDS

B. anthracis is only one member of the *B. cereus* group, and so it would not be surprising that the plasmids would be shared among these closely related species; however, until Hoffmaster et al. (2004) described the presence of a pXO1 plasmid in a *B. cereus* isolate, it was thought that the pXO1 and pXO2 plasmids were only found in *B. anthracis*. Due to the size and difficulty of working with these plasmids and the fact that they do not transfer readily, it was assumed they were *B. anthracis* specific. Recent studies and sequencing programs have discovered that the plasmids of *B. anthracis* represent only one flavor of the *B. cereus* group plasmids (Table 6.3).

In contrast to the conserved plasmid content observed among *B. anthracis* (Table 6.2), other *B. cereus* group isolates contain a diverse range of plasmids—no strains have yet been identified with identical plasmid content (Table 6.3). *B. cereus* group plasmid content can vary from 5 kb to almost 500 kb in size and from one to six plasmids in number; however, only a limited number have been implicated in pathogenesis. A subset of plasmids does not encode for any obvious phenotypes and can be identified as cryptic plasmids. These cryptic plasmids are often small, <50 kb, and are not shared with *B. anthracis*, but are shared among *B. cereus* and *B. thuringiensis* (Table 6.3). *B. cereus* group genome projects have identified a number of large plasmids that share features with the *B. anthracis* plasmids which will be discussed below; however, it must be noted that in both *B. cereus* and *B. thuringiensis*, large plasmids have been identified that are not related to the *B. anthracis* plasmids, suggesting that there may be more variability in these species in terms of plasmid content, most likely related to the more diverse environments they thrive in.

pXO1-Like Plasmids

There are a number of plasmids that have been identified that are similar to the *B. anthracis* pXO1 plasmid; however, these plasmids are exclusively restricted to *B. cereus*. Plasmid similarity is based on a ~50-kb region of the pXO1 plasmid that not only encodes the putative replication genes, but also includes a number of genes that are not related to replication, and many of them do not have any assigned function (Rasko et al., 2007) (Figure 6.2, blue region). This region forms the conserved backbone for the pXO1-like plasmids. Each of the plasmids with significant similarity to pXO1 contains this conserved region; however, none of the other plasmids contains the regions encoding the anthrax toxin. Each of the plasmids with the pXO1-backbone also contains a unique insert where the virulence genes are in pXO1 (Rasko et al., 2007). Since many of the pXO1-like plasmids are obtained from *B. cereus* of clinical origins, it is possible that the genes in these nonconserved regions are responsible for the virulence in these pathogens. An example of this is the *B. cereus* isolates from emetic food poisoning which all contain a pXO1-like plasmid; however, in place of the anthrax toxin and associate genes, these plasmids contain the emetic toxin (Ehling-Schulz et al., 2006; Lapidus et al., 2008; Rasko et al., 2007). Comparison of the RepX protein from each of these pXO1-like plasmids reveals that there are fewer than 10 amino acid differences and none appear to be in the essential amino acids identified previously (Tinsley and Khan, 2006, 2007).

Interestingly, the pXO1 plasmid is the smallest of the pXO1-like plasmids (Table 6.3). It is unclear why this is the case as the replication machinery can obviously undertake the replication of much larger molecules. It is possible that the *B. anthracis* lifestyle prevents the plasmids from acquiring novel genetic material, whereas the other *B. cereus* group members are more amenable to genetic acquisition.

The pXO1-like plasmids are mosaic plasmids that each contain unique regions responsible for the observed phenotypes of the host. Two examples will be discussed below: a *B. cereus* that contains an almost identical copy of the pXO1 plasmid and a *B. cereus* containing a divergent plasmid from an environmental source.

pBCXO1

B. cereus G9241 was isolated from a patient with an illness resembling inhalation anthrax (Hoffmaster et al., 2004). Initial polymerase chain reaction and comparative genomic hybridization studies revealed that *B. cereus* G9241 contained genes with similarity to pXO1, including all three anthrax toxin genes; however, there was no similarity to pXO2. Upon sequencing, a ~181 kb plasmid with a high degree of similarity and synteny to *B. anthracis* pXO1 was identified, in addition to one other plasmid of 210 kb, named pBC210, and a ~15 kb phage-like element (Hoffmaster et al., 2004). The pXO1-like plasmid, named pBCXO1, for the pXO1 plasmid from *B. cereus*, is 99.6% identical to pXO1 from *B. anthracis*. This homology extends to the amino acid identity of the pXO1 virulence genes, PA (99.7%), LF (99%), and EF (96%), as well as the virulence regulatory proteins AtxA (100%) and PagR (98.6%) (Hoffmaster et al., 2004). This combination of these features indicates that a functional toxin could be generated from this plasmid and be regulated in a manner that would be similar to *B. anthracis* (Bourgogne et al., 2003; Koehler, 2002). Closer examination of the toxin using a typing system developed by Price et al. (1999) based on minor variations in the PA protein indicated that the pBCXO1 toxin was most similar to genotype V, often associated with the western North America diversity group of *B. anthracis*.

The vaccine strain of *B. anthracis* lacks the pXO2 plasmid as an attenuating factor; however, *B. cereus* G9241 was isolated from a patient with an anthrax-like disease. This apparent incongruity is clarified by the presence of the other plasmid, pBC210. The plasmid encodes a number of features that are similar to virulence genes on pXO1 but does not contain the conserved region discussed above and thus, it is not considered a pXO1-like plasmid. However, pBC210 does encode a second copy of AtxA, which is only 78% identical to the pXO1 homolog, as well as the PA (~60% amino acid identity) and LF homologs (~36% amino acid identity). No homolog of the EF is found on pBCXO1. The LF gene is truncated by a premature stop codon and is most likely nonfunctional. The pBC210-encoded PA peptide is identical at all 10 dominant negative amino acid residues identified in pXO1 (Mourez et al., 2003). An additional 33 residues were identified by Mourez et al. that resulted in decreased activity when mutated, of which 27 are identical in the pBC210-encoded PA, with the remaining 6 being conservative substitutions (Hoffmaster et al., 2004; Mourez et al., 2003). ELISA experiments demonstrated that PA was present in the supernatant of the *B. cereus* G9241 culture; however, it is unclear which PA subunit, pBCXO1 or pBC210, was recognized by this assay (Hoffmaster et al., 2004).

In addition to the putative virulence factors, the pBC210 plasmid encoded a putative polysaccharide capsule cluster, representing the only capsule biosynthetic cluster identified in the genome of *B. cereus* G9241. This gene cluster is thought to be responsible for the capsule produced by this isolate. India ink staining and microbiological analyses have shown that *B. cereus* G9241 is encapsulated; however, it is not regulated like the *B. antrhacis* capsule (Hoffmaster et al., 2004). Interestingly, it was previously thought that *B. cereus* as a species in the group did not produce capsule (Jensen et al., 2003). The lack of any other capsule suggests that this plasmid-encoded polysaccharide capsule might compensate for the lack of poly-γ-D-glutamic acid capsule and help *B. cereus* G9241 to evade the host immune system. Recent studies by Hoffmaster's group have shown a wide range of capsule types in relation to *B. cereus* from anthrax-like clinical presentations (Avashia et al., 2007; Cheung et al., 2005; Hoffmaster et al., 2006; Sue et al., 2006; Tatti et al., 2006).

Regulation of these various virulence factors becomes convoluted based on what we know of regulation in *B. anthracis* (Bourgogne et al., 2003; Koehler, 2002). Previous regulatory studies had suggested that the *plcR* and *atxA* gene products from the chromosome and pXO1 plasmid could not exist in the same cell at the same time (Mignot et al., 2001; Slamti et al., 2004); however, *B. cereus* G9241 clearly contains both regulators in functional forms and actually contains two copies of AtxA. It is possible that there are other compensatory mutations in G9241 that result in this apparent inconsistency; however, further examination will be required.

The high level of identity between pXO1 and pBCXO1 suggests that both isolates acquired their plasmid from a common ancestor or that transfer occurred into *B. cereus* G9241 from *B. anthracis*. The successful transfer of pXO1 from *B. anthracis* to a close relative with the aid of a mobilizing plasmid (Battisti et al., 1985) supports the latter. The plasmid content of this isolate represents a chimeric *B. anthracis/B. cereus* species. This isolate and others like it raise interesting questions regarding the assembly of toxin and the role of the novel capsule in pathogenesis.

pBc10987

B. cereus ATCC10987 was isolated from cheese in Canada in the 1930s and when sequenced demonstrated to maintain a single 208-kb plasmid, named pBc10987 (Rasko et al., 2004). The pBc10987 plasmid was the first of the pXO1-like plasmids to be identified through

in silico analysis. pBc10987, like other pXO1-like plasmids, contains the conserved backbone region of ~50 kb and lacks all features associated with *B. anthracis* virulence. In lieu of the pXO1 pathogenicity island, pBc10987 contains genes for adaptation to either an environmental or pathogenic lifestyle. Environmental adaptations of pBc10987 include a copper-requiring tyrosinase, arsenite resistance and its associated regulators, as well as an amino acid transport system. These features would be essential to a species living in the soil environment. The pathogenic adaptations comprise of two potential novel toxins that have not been investigated to date (Rasko et al., 2004). When these unique plasmid-borne features are combined with the chromosomal features identified suggest that there is an evolution of the whole genome toward an increased metabolic fitness of the isolate.

While the conservation between pXO1 and pBc10987 is only ~40% overall, there are features of basic plasmid biology that are conserved, one being replication and the other being potential chromosome-plasmid crosstalk. Divergent copies of *abrB*, a pleiotropic transition state regulator, are present on pXO1 and pBc10987, as well as the chromosomes of *B. anthracis* and *B. cereus* ATCC 10987 (Rasko et al., 2004; Read et al., 2003). In *B. anthracis*, AbrB has been shown to negatively regulate toxin production (Koehler, 2002; Saile and Koehler, 2002), whereas in *B. subtilis*, AbrB modulates the switch between biofilm formation and sporulation (Banse et al., 2008; Hamon and Lazazzera, 2001), in addition to regulating competence (Hamoen et al., 2003a,b). The presence of global regulators on the plasmid suggests that plasmids play a significant role in the adaptation to environmental conditions whether that be in the host or the larger environment.

pXO2-Like Plasmids

Far fewer plasmids have been identified that are similar to pXO2 when compared to pXO1 (Table 6.3); however, the similarity between the pXO2-like plasmids covers a much greater proportion of the plasmid. In this case one defining feature is that the replicative machinery is highly conserved (see above). All pXO2-like plasmids encode homologs of the RepS protein and the 60 bp origin of replication region identified by Tinsley et al. (2004). The isolates that contain the pXO2-like plasmids are all from *B. thuringiensis* isolates; however, none of them carry the poly-γ-D-glutamic acid capsule gene cluster (Van der Auwera and Mahillon, 2008; Van der Auwera et al., 2008) (Figure 6.3a). There is very little known about the genes encoded on pXO2 and, by extension, the pXO2-like plasmids. The majority of the pXO2 plasmid research has been focused on the poly-γ-D-glutamic acid capsule and thus, much of the plasmid content remains unexamined.

pBT9727

A pXO2-like plasmid has been identified in *B. thuringiensis* 97-27 (Van der Auwera et al., 2008). The *B. thuringiensis* 97-27 isolates have been shown to produce crystal protein in sporulated culture by direct microscopic examination (Hernandez et al., 1999). While toxin crystal production is most often linked to plasmids in *B. thuringiensis*, no genes with similarity to the Cry toxin could be identified, suggesting that the toxin genes are chromosomally encoded or that the toxin plasmid was lost prior to sequencing. Interestingly, pBT9727 shows similarity to *B. anthracis* pXO2. Comparison of the predicted coding regions of the two plasmids revealed that pBT9727 shares 89% (82/92) of its putative coding sequence with pXO2. The pBT9727 origin of replication (59/60 nucleotides) and RepS are similar (91% amino acid identity) to that of pXO2 (Figure 6.3; Tinsley et al., 2004). The level of protein similarity, combined with the conservation of gene order,

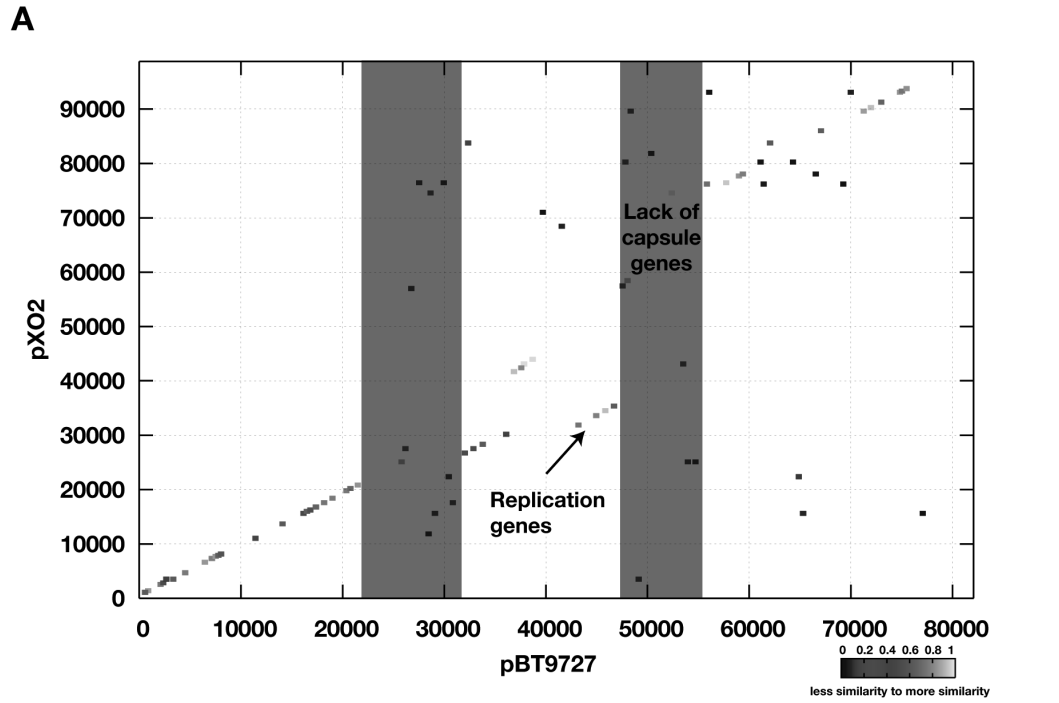

Figure 6.3 Examination of the homology between pXO2-like plasmids. Panel A contains the PSR plot comparing pBT9727 and pXO2, demonstrating that the plasmids are highly conserved except for the plasmid region with encoding the capsule biosynthesis genes and one other region. Panel B is a detailed view of the nucleic acid sequence of the proposed plasmid origin in this group of pXO2-like plasmids. The gray box is the conserved origin and the arrow indicates the nick site. See color insert.

suggests that these plasmids may have recently diverged. There are significant differences between the two plasmids (Figure 6.3a). The pXO2 region encoding for the poly-γ-D-glutamic acid capsule biosynthetic genes has been replaced in pBT9727 with genes encoding hypothetical proteins and putative mobile elements. This replacement suggests that pBT9727 might have evolved to fulfill other functions than providing this isolate with capsule biosynthetic genes. One additional difference is that pXO2 is not self-transmissible whereas pBT9727 appears to be (Van der Auwera et al., 2008).

CONCLUSIONS

Based on sequence analysis of the plasmids of the *B. cereus* group of organisms, it is evident that plasmid-borne genes dictate the significant differences observed in pathogenicity and host range. The plasmid content of *B. anthracis* is extremely restricted,

consisting of only pXO1 and pXO2; however, this plasmid content provides a view into the lifestyle of *B. anthracis*. The *B. anthracis* plasmid content may be limited because there is limited opportunity to exchange genetic material from the time a spore germinates in the macrophage, rapidly grows in the bloodstream of the host, kills the host, and returns to a dormant state of a spore. This is in contrast to the *B. cereus* group, which can replicate outside the host in many different environments with ample opportunity for genetic exchange. From the distribution of the homologous plasmids, the pXO1-like plasmids are restricted to the *B. cereus* species, whereas the pXO2-like plasmids are restricted to the *B. thuringiensis* species. Is it possible that a progenitor acquired a plasmid from each of the other species to form the highly virulent clone now known as *B. anthracis*?

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Chapter 7

Iron Acquisition by *Bacillus anthracis*

Gleb Pishchany and Eric P. Skaar

INTRODUCTION

Iron is an essential element for most forms of life as it is required for numerous cellular processes including nucleic acid synthesis, protection against oxidative stress, respiration, metabolism, and gene regulation. The acquisition of iron is therefore crucial to the success of most bacterial pathogens including *Bacillus anthracis*. In keeping with this, bacterial genomes encode systems dedicated to iron acquisition and storage. The importance of iron to the pathogenesis of bacterial infection is demonstrated by the fact that inactivation of iron uptake systems decreases the virulence of most microbial pathogens (Crosa et al., 2004). Considering the requirement for iron availability during infection, systems involved in its acquisition have been recognized as viable targets for novel antimicrobials. The primary systems by which bacterial pathogens acquire iron from their vertebrate hosts involve siderophore-mediated iron capture and the direct acquisition of heme iron from host hemoglobin.

Siderophores are small secreted molecules that are produced by bacteria and bind iron with a remarkably high affinity, allowing them to outcompete host-iron sequestration molecules (Crosa et al., 2004). Upon binding to iron, siderophores are imported into the bacterial cytoplasm through the action of dedicated transport systems. Siderophores are typically produced and secreted in response to low-iron environments, and the transcription of siderophore synthesis genes is typically regulated by the canonical iron-dependent repressor Fur (Lee and Helmann, 2007). In high iron, Fur binds to DNA sequences known as Fur boxes and represses transcription of the downstream genes. When iron is low, Fur dissociates from the Fur boxes and derepresses transcription (Escolar et al., 1999; Hantke, 2001). The importance of Fur-mediated iron-dependent gene regulation to *B. anthracis* physiology is underscored by the fact that *fur* mutations are lethal (Gat et al., 2008).

In addition to siderophore-mediated iron capture, bacteria employ mechanisms of acquiring iron directly from heme complexed to hemoglobin, which is the most abundant form of iron within vertebrates (Wilks and Burkhard, 2007). Each hemoglobin protein contains four heme molecules, and each heme molecule is composed of a tetrapyrrole ring encircling a single iron atom. Bacteria acquire heme iron through toxin-mediated

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erythrocyte lysis followed by the binding of liberated hemoglobin. Upon hemoglobin capture at the microbial surface, heme is removed from the globin portion of hemoglobin and internalized into the cytoplasm, where it is degraded to release nutrient iron (Reniere et al., 2007).

The importance of iron to the pathogenesis of *B. anthracis* is highlighted by the observation that the genome of *B. anthracis* contains significantly more iron acquisition systems than non-pathogenic members of the genus *Bacillus* (Read et al., 2003). Specifically, the genome of *B. anthracis* strain Ames encodes 15 putative iron-specific ABC transporters and two distinct siderophore synthesis systems (Read et al., 2003). Moreover, many of these systems are expressed during disease as illustrated by the presence of antisera specific to iron acquisition systems within immunized humans and rabbits (Gat et al., 2006; Kudva et al., 2005). Despite the significant amount of genomic information dedicated to iron acquisition by *B. anthracis*, the machinery and mechanisms involved in iron acquisition are largely unexplored. In this chapter, we review current knowledge regarding *B. anthracis* siderophore and heme-based acquisition strategies to acquire iron during the pathogenesis of anthrax.

HOST-IRON METABOLISM

Iron is thought to be the most critical elemental nutrient in determining the outcome of host–pathogen interactions (Prentice et al., 2007). This is because the lack of available iron within vertebrates provides a nutritional barrier to colonization by microbial invaders. There are two primary reasons for the scarcity of free iron within vertebrate tissue. First, iron is insoluble at physiologic pH. Second, vertebrates take immense measures to carefully regulate iron homeostasis by sequestering it via host molecules with very high binding affinities. These measures both protect the host from iron toxicity and ensure that the concentration of free iron within tissues is orders of magnitude below that required to support bacterial growth and virulence. The active sequestration of nutrient metals in order to defend against microbial growth is a process termed nutritional immunity. It should be noted that the host is forced to maintain a certain level of bioavailable iron for cellular metabolic needs and thus employs intricate mechanisms to maintain iron balance. This is demonstrated by the observation that depriving cells of intracellular iron leads to rapid cell death (Edison et al., 2008). Conversely, if dietary or genetic factors shift the balance to a condition of iron overload, mammals are less able to resist infections (Bullen, 1981). Among the most well-documented pieces of evidence supporting the role of iron in infections is that tuberculosis patients supplemented with iron are much more susceptible to the pathogenesis of *Mycobacterium tuberculosis*, and individuals with iron overload diseases such as hemochromatosis routinely suffer reinfections (Bullen et al., 1991; Prentice, 2008). Furthermore, animal models have revealed that *B. anthracis* and other pathogens inactivated for iron acquisition systems display significantly reduced virulence (Abergel et al., 2006b; Cendrowski et al., 2004; Crosa et al., 2004; Reniere and Skaar, 2008; Skaar et al., 2004b; Torres et al., 2006). Taken together, these findings underscore the importance of nutritional immunity in protecting against bacterial infections.

In order to appreciate the sources of iron that are available to invading pathogens during infection, it is important to first understand the physiology of iron in a healthy vertebrate host. Under normal conditions, 1–2 mg of iron enters the body of an adult human through absorption in the duodenum and small intestine. This process maintains the total amount of iron within a healthy adult at approximately 3–4 g (Ganz and Nemeth, 2006). Upon absorption, iron is bound by transferrin, which is secreted into the plasma and traf-

ficked to tissues so that iron can be delivered throughout the body. In a healthy individual, virtually all extracellular iron is bound by transferrin, accounting for about 0.1% of total iron within the organism (Edison et al., 2008). Due to the high affinity of transferrin for iron and the fact that only about 30% of transferrin is iron saturated at any given time, the extracellular concentration of free iron within vertebrates is extremely low (Sarkar, 1970). Upon cellular contact, transferrin-iron is taken up by cells via interaction with a transferrin receptor through clathrin-mediated endocytosis. Once endocytosed, iron is released from transferrin in a pH-dependent process mediated by the influx of protons into the endosomes. The vast majority of absorbed iron (~80%) is taken up by erythroid precursors in the bone marrow and is incorporated into heme, which is bound by hemoglobin, the primary oxygen transport protein of vertebrates. In addition, a significant amount of absorbed iron (~10%) is incorporated within heme into the oxygen storage protein myoglobin which is found within muscle cells known as myocytes (Edison et al., 2008). Excess intracellular iron is stored within the protein ferritin to protect against the reactivity of free iron. Ferritin can be found in the cytoplasm, nucleus, and mitochondria, and its multimeric form can contain up to 4500 atoms of iron (MacKenzie et al., 2008). Finally, a small fraction of iron is bound by the antimicrobial protein lactoferrin found in secretions such as breast milk and tears. The remaining iron is used by different cell types and iron-binding proteins with roles in a variety of physiological processes. During infection, the primary sources of iron available to invading bacterial pathogens include transferrin iron, heme iron bound to hemoglobin or myoglobin, and iron complexed to ferritin.

IRON WITHIN MACROPHAGES

A hallmark of anthrax is the ability of *B. anthracis* to grow within macrophages and exploit macrophage trafficking in order to disseminate throughout the host. This intracellular lifestyle necessitates that *B. anthracis* acquire and metabolize iron within infected macrophages. In this regard, the unique role of macrophages in iron homeostasis affects the struggle for iron during infection in this intracellular niche. In addition to a conventional transferrin receptor, macrophages express a cell-type-specific receptor: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which triggers transferrin internalization through an endosomal route (Raje et al., 2007). Further, macrophages are responsible for recycling hemoglobin-iron contained within senescent erythrocytes. Approximately 20 mL of senescent erythrocytes accounting for up to 20 mg of iron are terminated daily (Ganz and Nemeth, 2006). Splenic and reticuloendothelial macrophages are responsible for salvaging iron contained within erythroctyes through a process called erythrophagocytosis, whereby these specialized macrophages engulf erythrocytes and degrade the released hemoglobin (MacKenzie et al., 2008). The heme cofactor of degraded hemoglobin is further catabolized by heme oxygenase leading to the release of iron, which is then exported from macrophages by ferroportin. Exported iron is complexed by transferrin and is distributed throughout the body (Ganz and Nemeth, 2006). In addition to erythrophagocytosis, macrophages uptake haptoglobin-bound hemoglobin released from lysed erythrocytes through haptoglobin recognition by CD163 (Kristiansen et al., 2001). Considering these pathways for uptake and recycling within macrophages, iron in the form of transferrin, ferritin, and hemoglobin is likely targeted by *B. anthracis* during the pathogenesis of anthrax.

How *B. anthracis* acquires iron within host macrophages is poorly understood, and current models are largely based on studies carried out with other macrophage-associated intracellular pathogens. Upon phagocytosis of microbial invaders, macrophages take

measures to starve the pathogen of iron. Studies with intracellular bacteria have demonstrated that vacuoles containing engulfed bacteria are initially enriched for iron; however, iron is depleted from these vacuoles upon interferon (IFN)-γ or tumor necrosis factor (TNF) exposure (Wagner et al., 2005). The origin of iron within these vacuoles is unclear; however, it is likely to have been brought through an endosomal route with transferrin. In support of this, *M. tuberculosis* has been shown to localize with phagolysomes containing transferrin (Clemens and Horwitz, 1996). Phagocytosed *B. anthracis* colocalizes with LAMP1, which is a marker for lysosomal vacuoles (Hu et al., 2006). Further, the phagosomal iron exporter Nramp1, which protects against intracellular bacterial outgrowth, is associated with vacuoles containing ingested microbes (Gomes and Appelberg, 1998; Searle et al., 1998). *B. anthracis* spore germination and translocation of toxins occur within endosomes of infected macrophages (Dixon et al., 2000; Guidi-Rontani et al., 1999, 2000, 2001); however, it is unclear whether iron availability affects the germination process of *B. anthracis*. Therefore, it remains to be determined whether iron deprivation within the lysosomal compartment affects *B. anthracis* pathogenesis. Upon germination, the vegetative cells escape from the phagolysomes and multiply within the host cytoplasm (Ruthel et al., 2004). Although it is conceivable that iron is not required for germination, iron acquisition is invariably required for bacterial multiplication necessitating that *B. anthracis* employ iron acquisition strategies within the host cytoplasm (Cendrowski et al., 2004; Lee et al., 2007; Maresso et al., 2006). Free iron is available at low concentrations within the cytoplasm of macrophages (Appelberg, 2006); however, this pool of free iron is significantly reduced upon IFN-γ activation. This is at least in part achieved through downregulation of the number of transferrin receptors on the cell surface (Byrd and Horwitz, 1989). In the absence of free iron, the most likely iron source for *B. anthracis* in the cytoplasm is ferritin, although the ability of *B. anthracis* to utilize ferritin iron has not been reported. *Neisseria meningitidis* can induce iron release from ferritin by stimulating autophagy, allowing for bacterial replication within the cytoplasm (Larson et al., 2004), and it is possible that *B. anthracis* induces a similar process. It is not yet known which iron sources are utilized by *B. anthracis* during the pathogenesis of anthrax; however, the iron sources that support *B. anthracis* growth *in vitro* have been studied in detail. In this regard, it has been shown that *B. anthracis* can acquire iron from the siderophores desferal and ferrichrome, as well as from hemoglobin and transferrin, but not lactoferrin (Garner et al., 2004). The systems that allow *B. anthracis* to acquire iron from these diverse sources are discussed below.

B. ANTHRACIS IRON ACQUISITION STRATEGIES

Siderophores

Siderophores are small molecules that are secreted by bacteria and bind iron with affinities that outcompete host-iron sequestration measures. Typically, siderophore synthesis operons are upregulated in response to low levels of available iron. The genome of *B. anthracis* encodes for two known siderophore synthesis systems, each of which synthesizes a distinct siderophore (Cendrowski et al., 2004; Wilson et al., 2006). Transcription of each of these siderophore systems is upregulated during macrophage infection at least five-fold compared to expression in iron-replete culture medium (Bergman et al., 2007). This upregulation occurs during germination and vegetative outgrowth within macrophages, indicating a role for siderophore-dependent iron acquisition during the intracellular phase of *B. anthracis* lifecycle.

Bacillibactin

Bacillibactin (anthrabactin) is synthesized by proteins encoded by the *bac* operon (Cendrowski et al., 2004; Wilson et al., 2006). Genes of the *bac* locus (*B. anthracis* catechol) are 79% identical to the *dhb* siderophore synthesis locus of *B. subtilis* and are also present in other *Bacillus spp.* Fur recognition sequences upstream of the *bac* operon are identical to the Fur binding regulatory sequences that control the expression of *dhb*. This suggests that the expression of both *bac* and *dhb* is under the control of iron-dependent Fur regulation. Bacillibactin is based on a 2,3-dihydroxybenzoyl catechol and binds iron with a remarkably high affinity (Dertz et al., 2006; Rowland et al., 1996; Wilson et al., 2006). The biosynthesis pathway of bacillibactin has been characterized in other bacteria; therefore, we omit its description here (May et al., 2001). Bacillibactin is not required for the virulence of *B. anthracis* in animal models of infection, and studies of bacillibactin from *B. anthracis* have primarily focused on the regulation of the *bac* operon (Cendrowski et al., 2004).

In addition to being controlled by iron availability, bacillibactin production is sensitive to temperature fluctuations (Koppisch et al., 2005). In the presence of high levels of CO_2, which induces toxin synthesis, the levels of bacillibactin expressed by *B. anthracis* grown at 37°C are reduced as compared to bacteria grown at 30°C (Koppisch et al., 2005). In addition, the *B. anthracis bac* operon is upregulated upon oxidative stress irrespective of iron concentrations through a mechanism which is thought to involve Fur (Passalacqua et al., 2007). This oxidative stress-dependent upregulation of the *bac* operon is even more pronounced in strains inactivated for the superoxide dismutase gene *sodA*. Paradoxically, bacillibactin is not produced by *B. anthracis* in iron-rich conditions regardless of the presence of oxidative stress (Passalacqua et al., 2007). In iron-deplete conditions, bacillibactin accumulates intracellularly as well as being secreted into the medium, but upon oxidative damage, bacillibactin is barely detectable in wild-type cells and is undetectable in the cytoplasm of *sodA* mutants. Secreted bacillibactin levels also decrease upon oxidative damage (Passalacqua et al., 2007). These observations suggest that oxidative stress inhibits siderophore synthesis while simultaneously increasing transcription of the siderophore synthesis genes. This phenomenon is even more pronounced in a *sodA* mutant whereby *bac* expression is significantly upregulated but less bacillibactin is produced.

Petrobactin

B. anthracis produces a second siderophore which is synthesized by genes encoded by the *asb* operon (*a*nthrax *s*iderophore *b*iosynthesis) (Figure 7.1; Cendrowski et al., 2004; Garner et al., 2004; Wilson et al., 2006). This siderophore is not found in *Bacillus subtilis* and has received considerable attention from the scientific community due to its unique features and its contribution to the pathogenicity of *B. anthracis*. *B. anthracis* requires *asbA* for growth in iron-deplete conditions, and *asbA* mutants are defective in virulence in murine anthrax infections as well as impaired for growth within and killing of macrophages (Abergel et al., 2006b; Cendrowski et al., 2004; Garner et al., 2004; Lee et al., 2007; Pfleger et al., 2007, 2008). The proteins encoded by the *asb* operon synthesize an unusual siderophore that has been named petrobactin. Similar to bacillibactin, petrobactin is a catecholate siderophore. However, unlike other known catecholate siderophores, which are based on 2,3-dihydroxybenzoic acid, petrobactin is based on an unusual 3,4-dihydroxybenzoic acid (3,4-DHB; Garner et al., 2004). The biosynthesis pathway of

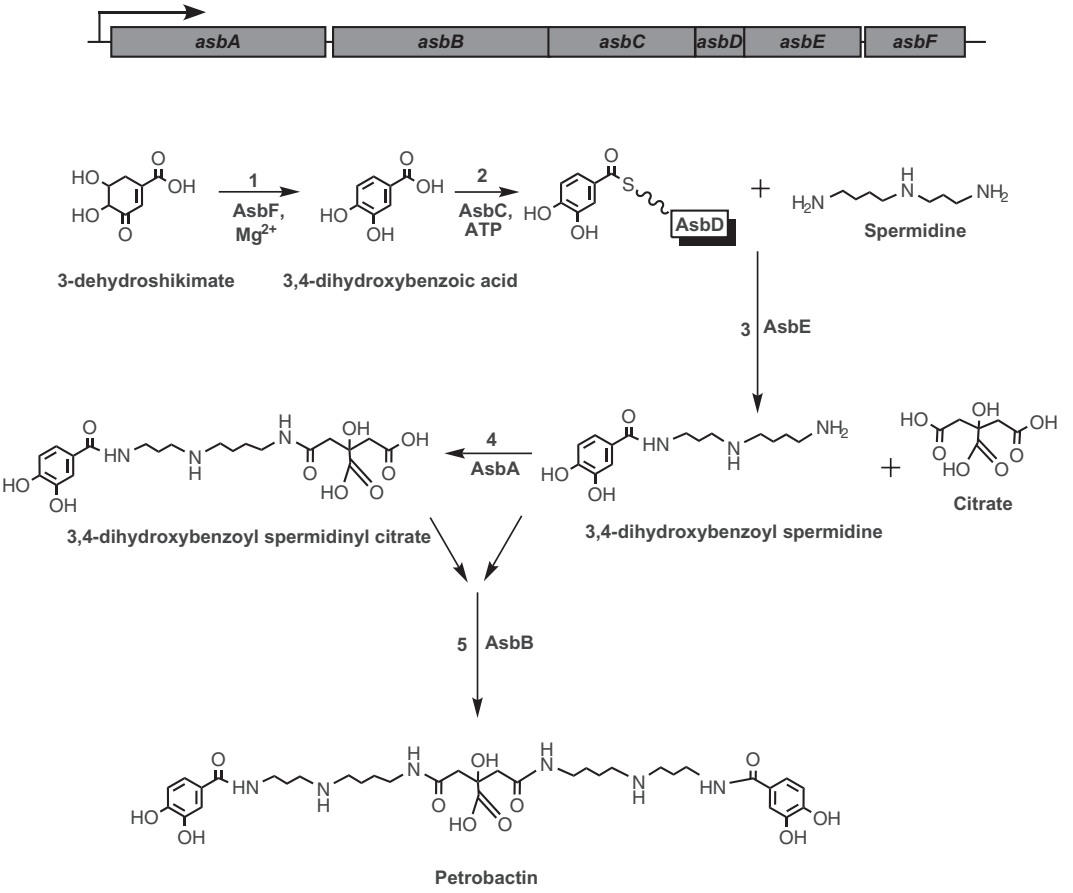

Figure 7.1 *asb* operon and petrobactin synthesis pathway. Petrobactin synthesis involves the genes of the *asb* operon (Cendrowski et al., 2004; Garner et al., 2004; Koppisch et al., 2005; Wilson et al., 2006) and occurs in the following steps: (1) AsbF mediates conversion of 3-dehydroshikimate to 3,4-dihydroxybenzoic acid (Koppisch et al., 2008; Pfleger et al., 2008); (2) AsbC adenylates 3,4-dihydroxybenzoic acid and transfers it to AsbD; (3) AsbE condenses AsbD-bound 3,4-dihydroxybenzoic acid with spermidine to form 3,4-dihydroxybenzoyl spermidine (Pfleger et al., 2007); (4) AsbA condenses 3,4-dihydroxybenzoyl spermidine with citrate to form 3,4-dihydroxybenzoyl spermidinyl citrate; and (5) AsbB condenses 3,4-dihydroxybenzoyl spermidine formed in step 3 with 3,4-dihyroxybenzoyl spermidinyl citrate from step 4 to form petrobactin.

petrobactin is depicted in Figure 7.1. Petrobactin is produced by *B. cereus*, and the unrelated marine bacterium *Marinobacter hydrocarbonoclasticus*, but not by *Bacillus lichenformis, B. subtilis*, or *Bacillus thuringiensis* (Barbeau et al., 2002; Koppisch et al., 2005). Petrobactin from *M. hydrocarbonoclasticus* restores the growth defect of *B. anthracis asb* mutants, confirming the similarity of this molecule across genera. Interestingly, although petrobactin is not synthesized by *B. subtilis,* it can be utilized by this bacterium as an iron source for growth (Abergel et al., 2008).

Mutants in any genes of the *asb* locus are not capable of petrobactin synthesis, leading to a growth defect in low iron that can be overcome by addition of petrobactin into the growth medium (Lee et al., 2007). Interestingly, in low-iron conditions where even wild-type spores are unable to outgrow, provision of exogenously added petrobactin is capable

of promoting vegetation and growth (Lee et al., 2007). Petrobactin binds both di- and trivalent iron ions, although it binds Fe^{3+} with a higher affinity (Liu et al., 2007). Petrobactin has been shown to remove iron from both transferrin and the iron chelator ethylene-di(*o*-hydroxyphenyl)acetic acid through its higher affinity for iron (Abergel et al., 2008; Garner et al., 2004).

One significant advantage that petrobactin confers on *B. anthracis* is the ability to circumvent components of the host innate immune response. A primary method used by vertebrates to prevent infection is to combat siderophore-mediated iron acquisition by production of the host protein siderocalin (Abergel et al., 2006a; Goetz et al., 2002). Siderocalin is secreted by neutrophils, whereupon it binds to bacterial siderophores and prevents siderophore trafficking and uptake by invading bacteria. Siderocalin can bind multiple distinct bacterial siderophores, including bacillibactin, thus inhibiting iron uptake by *B. anthracis*. However, siderocalin does not bind petrobactin, likely due to the distinct 3,4-DHB-based structure of petrobactin (Abergel et al., 2006b). Thus, by secreting petrobactin, *B. anthracis* can avoid the host countermeasure of siderocalin production, allowing *B. anthracis* to acquire iron during infection.

Transcription of *asb* and, accordingly, synthesis of petrobactin, is upregulated in low-iron conditions most likely through release of Fur inhibition although Fur binding sites upstream of *asb* are not entirely conserved (Wilson et al., 2006). As seen with bacillibactin, petrobactin levels drop in response to an increase in temperature from 30°C to 37°C (Garner et al., 2004). This is in contrast to the effect of temperature on the levels of 3,4-dihydroxybenzoic acid, whose levels increase in the same conditions, suggesting that a downstream step in the synthesis of petrobactin is affected by fluctuations in temperature. Unlike the *bac* genes, transcription of the *asb* operon is largely unaffected by oxidative stress, suggesting distinct regulation between these loci (Passalacqua et al., 2007). Although the transcription of the *asb* locus is unaffected by oxidative stress, the levels of petrobactin decrease upon oxidative stress, and this decrease is exacerbated in a *sodA* mutant (Passalacqua et al., 2007).

Heme Acquisition

The majority of iron within the mammalian host is in the form of heme, which is primarily bound by hemoglobin. Based on the abundance of erythrocyte hemoglobin within the vertebrate bloodstream, heme is likely a valuable iron source to extracellular *B. anthracis* following macrophage escape. In agreement with this supposition, *B. anthracis* can lyse erythrocytes and is capable of utilizing heme and hemoglobin as a sole iron source for growth through a system dedicated to the acquisition of heme iron from hemoglobin (Gat et al., 2008; Klichko et al., 2003; Maresso et al., 2006, 2008; Skaar et al., 2006; Zink and Burns, 2005). This system, known as the iron-regulated surface determinant (Isd) system, was originally identified in the related bacterium *Staphylococcus aureus* (Mazmanian et al., 2003). The Isd system is present in members of the *Cereus* group but is absent in all other members of the *Bacillus* genus. The Isd system consists of eight proteins: IsdC, IsdX1 (also known as IsdJ), IsdX2 (also known as IsdK), IsdE, IsdE2 (also known as IsdF), IsdF (also known as IsdX), sortase B (SrtB), and IsdG (Figure 7.2). IsdC is covalently anchored to the cell wall, IsdX1 and IsdX2 are secreted proteins, while IsdE, IsdE2, and IsdF encode for a putative membrane-localized ABC-type transporter (Gat et al., 2008; Maresso et al., 2006, 2008). Sortase B is a transpeptidase dedicated specifically to anchoring IsdC to the cell wall, and IsdG is located in the cytoplasm (Maresso et al., 2006; Skaar

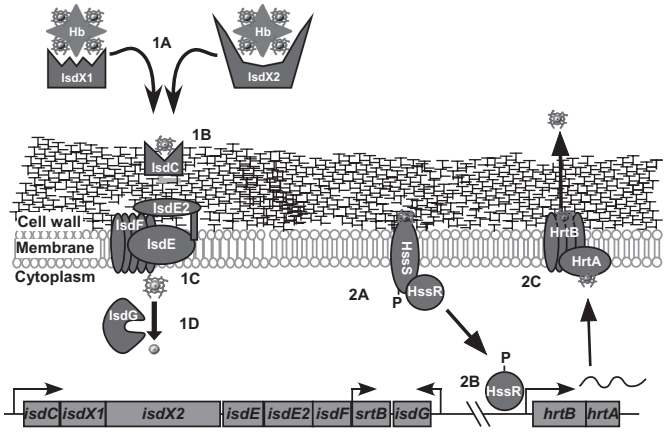

Figure 7.2 Heme homeostasis of *B. anthracis*. (1) Heme-iron acquisition: (1a) Hemoglobin is bound and heme is extracted by hemophores IsdX1 and IsdX2 (Gat et al., 2008; Maresso et al., 2008); (1b) IsdX1 and IsdX2 pass heme to IsdC, which transports heme through the cell wall and passes it to IsdEE2F* (Maresso et al., 2006); (1c) IsdEE2F passes heme through the membrane into the cytoplasm*; and (1d) IsdG degrades heme to release free iron for use as a nutrient source. (2) Heme detoxification: (2a) Excess heme that is not degraded is sensed by HssS, which subsequently activates HssR; (2b) HssR binds the promoter region upstream of *hrtAB*, leading to increased expression of the HrtAB efflux pump; (2c) HrtAB relieves heme toxicity. See color insert.

* Steps 1b, 1c, and 2c are not experimentally proven.

et al., 2006). The cell wall anchored and secreted components of the Isd machinery are expressed during infection and induce a robust antibody response (Gat et al., 2006, 2008; Kudva et al., 2005). The *isdCX1X2EE2F* operon contains two transcription termination sequences and encodes for two detectable transcripts. One transcript encodes the entire operon while the other includes only *isdC*, *isdX1*, and *isdX2* (Gat et al., 2008). The reasons for these overlapping transcripts are not yet clear. The genes encoding SrtB and IsdG are encoded as individual transcripts and are located adjacent to the *isdCX1X2EE2F* operon. The properties of IsdC, IsdX1, IsdX2, and IsdG have been investigated in *B. anthracis* and are discussed below.

IsdX1 and IsdX2 have been described as the first hemophores identified in gram-positive bacteria. Hemophores are secreted proteins that remove heme from host hemoproteins and then shuttle back to cognate receptors on the bacterial cell allowing for heme capture. Consistent with this, IsdX1 and IsdX2 contain a signal peptide but lack a canonical cell wall anchoring motif and thus are secreted into the extracellular milieu (Gat et al., 2008, Maresso et al., 2008). Upon binding hemoglobin, IsdX1 is capable of extracting heme. Heme binding by IsdX1 is of lower affinity than that of hemoglobin, suggesting that IsdX1 binding to hemoglobin must somehow alter the hemoglobin–heme interaction leading to heme release (Maresso et al., 2008). IsdX1 preferentially binds holo-hemoglobin, suggesting a mechanism whereby IsdX1 binds hemoglobin containing heme and upon removal of heme releases apo-hemoglobin (Maresso et al., 2008). IsdX1, IsdX2, and IsdC contain putative NEAT (NEAr Transporter) domains. These domains were originally identified in proteins whose genes are located proximal to genes encoding for iron transporter systems (Andrade et al., 2002). NEAT domains have been shown to bind heme and hemoglobin in the Isd system of *S. aureus* (Dryla et al., 2007; Grigg et al., 2007; Pilpa

et al., 2008; Sharp et al., 2007). Therefore, binding of hemoglobin and heme by IsdX1 is likely dependent on the NEAT domain. IsdX1 also binds myoglobin, but with a lower affinity than hemoglobin (Maresso et al., 2008).

IsdX2 is required for the utilization of hemoglobin as a sole iron source, but it is dispensable when heme is the only available iron source (Gat et al., 2008; Maresso et al., 2008). IsdX2 contains five NEAT domains and is very effective at extracting heme from hemoglobin. IsdX2 is also capable of binding myoglobin; however, IsdX2 binds myoglobin with a lower affinity than hemoglobin (Maresso et al., 2008). Interestingly, although lacking a canonical sequence necessary for covalent cell wall anchoring, IsdX2 is both cell wall localized and secreted. It is suggested that the NSKTA sequence found within the C-terminus of IsdX2 is recognized as an anchoring motif by sortase leading to inefficient cell wall anchoring of IsdX2 (Gat et al., 2008). Similar to siderophore synthesis proteins, the *isd* locus is regulated by iron availability in a Fur-dependent manner; however, the *isd* locus does not appear to be regulated by temperature (Gat et al., 2008; Maresso et al., 2008), and it is downregulated in high CO_2 concentrations (Gat et al., 2008). It is possible that the demand for iron in *B. anthracis* decreases under low oxygen conditions due to a diminished reliance on oxidative phosphorylation, which requires heme as a cofactor for cytochromes of the electron transport chain.

IsdC is required for acquisition of iron from both heme and hemoglobin (Gat et al., 2008). IsdC carries a unique NPKTG cell wall anchoring motif that is recognized by SrtB (Maresso et al., 2006). SrtB cleaves IsdC between the threonine and glycine residues of the NPKTG motif and anchors it to the peptide cross bridge of peptidoglycan (Maresso et al., 2006). IsdC has been found in both cell wall and supernatant fractions of *B. anthracis* cultures (Gat et al., 2008; Maresso et al., 2006). The presence of IsdC in the supernatant is likely due to autolysis of *B. anthracis* or cell wall turnover that is the result of normal cellular physiology. IsdC can extract heme from hemoglobin and is required for the acquisition of iron from heme (Gat et al., 2008; Maresso et al., 2006). Accordingly, strains lacking *srtB*, and hence unable to anchor IsdC to the cell wall, are also defective in heme-iron acquisition. IsdC appears to be the only target of SrtB in both *S. aureus* and *B. anthracis* as there are no other cell wall proteins with a similar anchoring motif in either of the organisms (Zhang et al., 2004). The fact that both *S. aureus* and *B. anthracis* encode sortases devoted specifically to anchoring heme-iron acquisition systems stresses the importance of heme-iron acquisition for these pathogens. Another indication of the importance of this system for *B. anthracis* is that a mutant lacking *srtB* is defective for growth in macrophages (Zink and Burns, 2005). Interestingly, in low-iron media lacking heme supplementation, strains inactivated for *isdC* and *isdX2* display increased growth compared to wild-type *B. anthracis*. One possible explanation for this is that expression of these proteins places a burden on bacteria, emphasizing the importance of tightly regulating these genes (Gat et al., 2008). Current data indicate that IsdC, IsdX1, and IsdX2 are dispensable for *B. anthracis* virulence (Gat et al., 2008).

Once inside the cytoplasm, heme is acted on by the cytoplasmic protein IsdG. *B. anthracis* IsdG is 35% identical to IsdG and IsdI from *S. aureus*, two heme degrading monooxygenases responsible for the liberation of iron from heme (Reniere and Skaar, 2008; Skaar et al., 2004a, 2006; Wu et al., 2005). Notably, *B. anthracis* IsdG contains a catalytic NWH triad identical to the one involved in heme degradation by staphylococcal IsdG (Skaar et al., 2006). *B. anthracis* IsdG binds heme in a 1:1 stoichiometry and, consistent with its predicted function, can degrade heme in the presence of a reducing agent. Further, IsdG is required by *B. anthracis* for growth on heme as a sole iron source and provides protection against heme toxicity (Skaar et al., 2006). *B. anthracis* Sterne strains

lacking *isdG* are not attenuated for virulence in the A/J mouse model (Skaar et al., 2006). However, the inconsistencies between the A/J mouse model and the pathogenesis of anthrax infection make it difficult to draw conclusions regarding the contribution of heme degradation to anthrax.

HEME TOXICITY

Heme presents a paradox to bacterial pathogens. Although heme is an abundant nutrient iron source, the reactivity of heme makes this molecule toxic at high concentrations (Everse and Hsia, 1997). Different organisms have evolved diverse mechanisms to avoid heme toxicity, including heme degradation (heme oxygenases in animals) and heme sequestration (hemozoin in parasites). *B. anthracis* can reach cell densities as high as 10^9 CFU/mL in plasma during bacteremia, and *B. anthracis* secretes hemolysins that lyse erythrocytes, liberating hemoglobin. In addition, *B. anthracis* encodes systems dedicated to the rapid and efficient acquisition of heme iron. In keeping with this, *B. anthracis* must express systems dedicated to resisting heme toxicity. Early experiments investigating bacterial resistance to heme toxicity demonstrated increased heme resistance of *B. anthracis* when compared to other species (Heyningen, 1948). A mechanistic explanation for the impressive heme resistance of *B. anthracis* has recently been described. The first protein to be reported as contributing to heme detoxification in *B. anthracis* is the heme oxygenase IsdG discussed earlier in this chapter. IsdG mediates resistance to heme toxicity through heme catabolism. In addition to IsdG, a second system has recently emerged as playing a vital role in the resistance to heme toxicity. This system, named the heme-regulated transporter (HrtAB), allows *B. anthracis* to adapt to high heme levels and presumably contributes to growth within vertebrates (Figure 7.2) (Stauff and Skaar, 2009).

Prior exposure of *B. anthracis* to subinhibitory concentrations of heme increases the resistance of this organism to heme concentrations that are normally toxic. This fact suggests the existence of a process by which *B. anthracis* adapts to heme toxicity. The mechanism by which *B. anthracis* adapts to heme toxicity is by sensing heme through a two-component system known as the heme sensor system (HssRS) (Stauff and Skaar, 2009). HssRS-mediated heme sensing leads to upregulation of HrtAB, which alleviates heme toxicity and allows growth in high heme levels. More specifically, the sensor histidine kinase HssS detects heme toxicity through as-yet-unidentified mechanisms and is autophosphorylated at a conserved histidine residue. This phosphate is then rapidly transferred to an aspartate residue of the DNA binding response regulator HssR. Phosphorylated HssR binds to a conserved direct repeat sequence in the promoter region of *hrtAB* and activates transcription of genes encoding this ABC-type transporter, which is made up of an ATPase (HrtA) and a permease (HrtB) (Stauff and Skaar, 2009). Sequence analyses suggest that HrtAB functions as an efflux pump; however, this has not been proven experimentally. Regardless of its specific function, the expression of HrtAB is required to protect *B. anthracis* against heme toxicity. The importance of HrtAB-mediated heme detoxification is highlighted by the observation that *Bacillus* species that encode this system are resistant to high levels of heme while those that are devoid of the system are unable to survive exposure to low levels of heme. In addition, deletion of the genes encoding for HssRS or HrtAB leads to a dramatic decrease in the heme resistance of *B. anthracis*. Although the contribution of HssRS and HrtAB to anthrax has not been evaluated, *B. anthracis* expresses *hrtAB* within vertebrates, suggesting that bacterial pathogens experience heme stress during infection (Stauff and Skaar, 2009).

CONCLUSION

Iron is believed to be the single most important element during host–microbe interactions, and the struggle for iron is one of the decisive factors determining the outcome of infection. In this regard, both host and pathogen have evolved intricate mechanisms to perpetuate this arms race for nutrients. Bacterial pathogens with multiple life cycles must have overlapping mechanisms of acquiring iron from the diverse ecological niches which they occupy within the host. Conversely, the host has evolved intracellular and extracellular mechanisms to prevent unrestricted iron acquisition by a diverse array of microbial pathogens. *B. anthracis* utilizes at least two defined strategies to appropriate host iron: siderophore secretion and heme acquisition. Siderophores can outcompete host proteins for iron, while heme is the most abundant form of iron within vertebrates. Studies focused on *B. anthracis* iron acquisition have identified some features which are specific for this organism. These include (1) the novel siderophore petrobactin that is resistant to siderochalin and (2) IsdX1 with IsdX2 which are the only known hemophores identified in gram-positive bacteria. To cope with the effectiveness of its heme-iron acquisition strategies, *B. anthracis* has evolved a mechanism to adapt to heme toxicity through heme sensing by HssRS and the alleviation of heme toxicity by HrtAB. Acting in concert, these systems provide *B. anthracis* with a finely tuned arsenal for acquiring and metabolizing iron from a diverse array of host sources. Based on the pivotal role of iron acquisition in this and other pathogens, the generation of novel antimicrobials that target iron acquisition has tremendous therapeutic potential.

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Chapter 8

Anthrax Toxins

Mahtab Moayeri and Stephen H. Leppla

Bacillus anthracis carries two plasmids which encode its essential virulence determinants. The anthrax toxin proteins are encoded by the pXO1 plasmid, while the proteins that synthesize the poly-D-glutamic acid capsule are encoded by the pXO2 plasmid. While the capsule is an essential virulence determinant which is particularly important in the establishment of anthrax, the fatality and symptoms associated with this disease are primarily the result of systemic septicemia and production of large quantities of the toxins. Antibiotics can clear bacteria from infected hosts, but if sufficient levels of the toxins have already been secreted by the organism, the disease is fatal even after treatment. Thus, anthrax toxins comprise the minimum essential requirement for the lethality associated with anthrax, and immunization against the protective antigen (PA) component of these exotoxins is sufficient for full protection against spore infections in most animal models. Therefore, the vaccine currently approved for human use consists principally of PA. An understanding of the function of anthrax toxins and the mechanism by which they manifest their *in vivo* effects is essential to deciphering the pathogenesis of anthrax disease.

BRIEF OVERVIEW OF ANTHRAX TOXIN RESEARCH

In 1954, Harry Smith and colleagues made the observation that filtered serum from *B. anthracis*-infected guinea pigs killed animals when injected intravenously (Smith and Keppie, 1954; Smith et al., 1955). This finding proved that an exotoxin activity was responsible for the lethal effects of anthrax. The same filtrates could also induce subcutaneous edema in experimental animals. These investigators later purified two toxin activities with a central common receptor-binding protein (Smith and Stanley, 1962; Stanley and Smith, 1961; Stanley et al., 1960), now known as PA. Two different enzymes, the adenylate cyclase, edema factor (EF), and the protease, lethal factor (LF), can combine with PA to produce the two anthrax toxins known as edema toxin (ET) and lethal toxin (LT) which were characterized in those early animal studies by Harry Smith's group.

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

Following a productive period of investigations into the *in vivo* effects of these toxins in animal models during the 1960s (not discussed in this chapter), almost no research on anthrax was performed until the early 1980s, when the advent of molecular biology led to the cloning of the toxin genes and advances in purification of the proteins (Leppla, 1988, 1991; Robertson and Leppla, 1986; Robertson et al., 1988; Vodkin and Leppla, 1983; Welkos et al., 1988). The availability of pure proteins facilitated research and led to the discovery that EF is a potent adenylate cyclase (Leppla, 1982). Advances in gram-positive genetics in the early 1990s made it possible to create isogenic mutants in the toxin genes, which in turn allowed researchers to implicate LT as a major virulence factor for anthrax (Cataldi et al., 1990; Pezard et al., 1991). The discovery of the striking LT-mediated lysis of macrophages (Friedlander, 1986; Friedlander et al., 1993) led to many studies that focused on LT effects on eukaryotic cells and structure–function analyses of PA. Interestingly, ET remained mostly ignored for almost two decades of anthrax toxin research. Since the 2001 anthrax attacks in the United States, the volume of published research on anthrax LT has increased exponentially each year (and still outnumbers ET studies by 10 to 1). In this chapter, we introduce the reader to a range of topics that encompass the major findings in anthrax toxin research through early 2009.

PA

PA is the essential component of both ET and LT, without which they cannot implement any of their effects in the host. It is also the primary vaccine and therapeutics target for anthrax. This section will review the structure and function of this protein.

Structure

PA binds cell surface receptors, and is responsible for translocation and delivery of LF and EF through the endocytic pathway to the cytosol. PA is produced as an 83-kDa protein. The crystal structures of the protein alone and complexed with its cellular receptor have been reported, allowing for structure-driven functional analyses (Lacy et al., 2004a; Petosa et al., 1997; Santelli et al., 2004). PA has four domains. An amino terminal domain 1 (aa 1-258) contains a cleavage site (aa 164-167) that allows generation of a 63-kDa form of the protein (PA63) that is essential for the ability to heptamerize. The PA63 heptamer is the actual functional form that can bind and transport LF and EF. PA83 domain 2 (aa 259-487) cooperates with domain 4 (aa 597-735) in receptor binding (Lacy et al., 2004a; Santelli et al., 2004). The conformational rearrangement of this domain at low pH is also crucial for the heptamer to form a 14-strand beta barrel pore and for freeing the region needed for insertion into endosome membranes (Qa'Dan et al., 2005). The intervening domain 3 is involved in both oligomerization of PA63 molecules and ligand binding (for detailed review, see Young and Collier, 2007).

Function

Binding to Receptors

PA Receptors PA binds to two receptors on eukaryotic cells (Bradley et al., 2001; Scobie et al., 2003). Tumor endothelial marker 8 (TEM8, also referred to as ATXR1) and

capillary morphogenesis protein 2 (CMG2, also referred to as ATXR2), which have physiological roles that are not yet well understood but appear to depend on their binding to collagen a3 (6) and collagen IV or laminin, respectively. These binding partners are indicative of the receptors' potential interactions with the extracellular matrix (Bell et al., 2001; Dowling et al., 2003; Hotchkiss et al., 2005; Nanda et al., 2004; Werner et al., 2006). TEM8 has been shown to mediate actin-dependent cell spreading (Werner et al., 2006) and angiogenic functions (Hotchkiss et al., 2005; Nanda et al., 2004; Rmali et al., 2005). Mutations in CMG2 lead to two genetic disorders, juvenile hyaline fibromatosis and infantile systemic hyalinosis (Dowling et al., 2003; Hanks et al., 2003), possibly by altering trafficking and causing receptor retention in the endoplasmic reticulum (ER; Deuquet et al., 2009).

The relative abundance and tissue distribution of TEM8 and CMG2 are not well known. Although these genes are believed to be transcribed in a wide range of tissues as multiple alternative splicing isoforms (Bradley et al., 2001; Scobie et al., 2003), information is lacking on the relative abundance of each functional receptor in different tissues and cell types. TEM8 is overexpressed on tumor endothelium, and is considered a tumor marker candidate (Carson-Walter et al., 2001). That this protein can play a role in endothelial cell function and angiogenesis (Hotchkiss et al., 2005; Nanda et al., 2004; Rmali et al., 2005) also supports its expression on the vasculature. Antibodies to the TEM8 splice variants show its preferential expression on epithelial cells in select organs (Bonuccelli et al., 2005). The use of a fluorescent PA in binding studies comparing CHO-K1 cells to different monocytic cell lines and human umbilical vein endothelial cells (HUVECs) has measured the highest level of PA binding on endothelial cells, with combined PA binding sites ranging from 8000 to 274,000/cell depending on cell type (Deshpande et al., 2006). Earlier reports put the number of binding sites on CHO-K1 cells at about 10,000 (Escuyer and Collier, 1991), and for multiple macrophage cell lines at 8000 (Singh et al., 1989), although a recent study using radioactive PA reports up to 66,000 binding sites on the J774 cell line (Dadachova et al., 2008). The same study reports on the biodistribution of the radioactive PA in mice, which appears to simply follow blood flow distribution in each organ and is likely not reflective of actual PA binding sites. Interestingly, ET induces expression of both anthrax receptors *in vitro* in macrophages and dendritic cells, accompanied by increased rates of toxin internalization (Maldonado-Arocho et al., 2006). It is unclear if this event occurs *in vivo* during anthrax infection and what effect it would have on pathogenesis, as it appears to be monocyte-lineage specific. The idea that ET upregulates its own receptor on select cell types is an intriguing one.

PA binds to a globular extracellular domain in the receptors which can be purified as a separate domain. The PA binding domain in the extracellular domain of the PA receptors is very similar to Von Willebrand factor type A (VWA) domains (Bradley et al., 2001; Scobie et al., 2003). Within this structure there is a metal ion dependent adhesion site (MIDAS domain), similar to that found in alpha integrin receptors, which has been shown to be essential for PA binding (Bradley et al., 2003; Rosovitz et al., 2003). Structural analyses of receptor binding to PA show that this interaction has clear conformational interaction similarities to the normal physiological interactions of this integrin family of receptors (Lacy et al., 2004a,b; Santelli et al., 2004).

A range of very different binding affinities have been reported for the receptors' interaction with PA. Surface plasmon resonance (SPR) measurements using the purified VWA domain of CMG2 and PA83 have measured Kd values of 170 pM (in Mg^{+2}) or 780 pM (in Ca^{+2}) (Wigelsworth et al., 2004). These affinities are surprising, in that they

are orders of magnitude higher than those reported for other VWA domain-ligands, as well as what has been reported for TEM8 by various methods. The interaction between purified TEM8 extracellular domain and PA83 has been measured by SPR at 130 nM (in Mg^{+2}) or 1.1 μM (in Ca^{+2}), three orders of magnitude lower affinity than that for CMG2 measured by the same method (Scobie et al., 2005). Although this difference in affinity has been suggested as the basis for the lower pH requirement for CMG2-bound PA oligomer to form a pore (Rainey et al., 2005), other measurements of PA binding to receptors have suggested much smaller differences in affinity between the two receptors. Schild plot nonlinear regression analyses of direct competition curves in cell viability assays using uncleavable PA mutant proteins as competitors have allowed measurement of affinities of receptors as they exist on cells. These studies yield only a threefold difference in affinity between TEM8 (3.2–3.5 nM) and CMG2 (9.5 nM) (Liu et al., 2007). Supporting this finding, fluorescent PA-based saturable binding studies on CHO-K1, HUVEC, and various monocytic cell lines which express CMG2 (and possibly TEM8 as well) yielded PA binding affinities ranging from 5.8 nM to 33.3 nM (Deshpande et al., 2006). Another study using the J774 macrophage cell line and radioactive PA reports a binding affinity of 6.7 nM (Dadachova et al., 2008). Thus, it appears likely that the functional affinity for both receptors is in the nanomolar range, with CMG2 binding PA with a higher affinity. Mutagenesis studies that altered amino acids in either PA or the receptors have helped identify key residues that are crucial for the interaction and yielded PA variants having differences in affinity for the two receptors (Chen et al., 2007; Liu et al., 2007; Scobie et al., 2006). Interestingly, recent studies have also suggested that the cytoplasmic domain of the TEM8 receptor can influence PA binding (Go et al., 2009).

LRP6 Identification of lipoprotein-receptor-related protein 6 (LRP6) as a PA co-receptor has been a controversial finding. Initially, this co-receptor for Wnt signaling was identified as necessary for anthrax LT toxicity in a screen using antisense RNAs generated from a library of expressed sequence tags (ESTs) (Wei et al., 2006). Knockdown experiments show LRP6 to be required for PA binding/endocytosis, and antibody to its extracellular domain protected against LT toxicity (Wei et al., 2006). Co-immunoprecipitation experiments also showed interaction between LRP6 and both PA receptors (Wei et al., 2006). However, mouse embryonic fibroblasts from LRP6 (and LRP5) knockout mice are fully susceptible to a cytotoxic LF fusion protein delivered via PA receptors (Young et al., 2007), bringing into question the requirement of LRP6 for PA function. LRP6 knockdown by siRNA in anthrax toxin-receptor-deficient CHO cells expressing either receptor results in no deficiencies in PA uptake (Young et al., 2007). HeLa cell siRNA knockdown further confirms that LRP6 has no effect on PA-mediated LF entry or cleavage of its cytoplasmic substrates in this human cell line (Ryan and Young, 2008). However, very recent work has reconfirmed that LRP6 forms a complex with the anthrax toxin receptors and that RNAi against both receptors leads to downregulation of LRP6 levels and decreased Wnt signaling from this receptor (Abrami et al., 2008). This report also observes that although LRP6 is not required for PA endocytosis, toxin binding to receptors results in phosphorylation of LRP6, its relocalization to lipid rafts, and even its endocytosis (Abrami et al., 2008). Furthermore, the kinetics of heptamer pore formation in endosomes and LF translocation appear to be greatly reduced in cells with LRP6 siRNA silencing, implicating LRP6 in the efficiency of PA receptor endocytosis. Thus, LRP6 is not *required* for PA receptor endocytosis but may facilitate the process.

Cleavage and Cargo Binding

Following binding to receptors, PA83 is rapidly cleaved by cell surface furin to release a 20-kDa polypeptide (PA20) (Gordon and Leppla, 1994; Gordon et al., 1995). Cell-bound PA63 rapidly heptamerizes to form the binding sites for LF and EF. The transmembrane domains of TEM8 molecules may associate to stabilize cell surface oligomers (Go et al., 2006). LF and EF bind only to the PA63 heptamer and not to the monomer (Mogridge et al., 2002b) and both can bind simultaneously to a single heptamer (Pimental et al., 2004). Structural studies clarify that steric hindrance allows binding of up to a maximum of three cargo molecules per heptamer (Mogridge et al., 2002a) because each binding site spans two monomers within the PA63 heptamer (Cunningham et al., 2002; Mogridge et al., 2002b). Thus, earlier reports describing an equal number of moles of LF internalized relative to moles of PA may be inaccurate (Singh et al., 1999). The affinity of EF/LF association with PA63 has been reported to be 1–2 nM (Elliott et al., 2000).

PA20 Generation in Circulation PA83 can be cleaved *in vivo* prior to binding cells (Ezzell and Abshire, 1992; Moayeri et al., 2007; Panchal et al., 2005). An early report showed that serum collected from spore-infected guinea pigs late in an infection had only the cleaved form of PA present, and a follow-up study isolated complexes of PA63-bound LF from plasma of infected rabbits and guinea pigs (Ezzell and Abshire, 1992; Panchal et al., 2005). This was initially thought to be a result of proteases possibly released during the disease process in sick animals. Later studies using purified PA83 and a mutant PA that is unable to bind to cells verify their rapid conversion to PA63 in circulation (Moayeri et al., 2007). The significance of PA cleavage by a protease in circulation is currently unknown. A recent study proposes PA20-mediated gene regulatory activity on peripheral blood leukocytes (Hammamieh et al., 2008), the significance of which during a real infection remains unclear.

Endocytosis

PA cleavage and heptamerization after binding to receptors are very rapid events (Liu and Leppla, 2003). Heptamerization is essential for and drives receptor-mediated endocytosis, as uncleavable PA mutants do not undergo endocytosis or form the endosome-restricted sodium dodecyl sulfate (SDS)-resistant oligomers which can only result from conformational changes at lower pH (Abrami et al., 2003; Beauregard et al., 2000; Liu and Leppla, 2003). PA63 heptamerizes with equal efficiency with or without cargo, but cleavage and heptamerization are essential for its movement to lipid rafts and subsequent clathrin-mediated endocytosis (Abrami et al., 2003). The heptameric PA63 complex is transferred to intraluminal vesicles within early endosomes (Abrami et al., 2004), undergoing a conformational change that leads to membrane insertion and pore formation. As channel formation first occurs in these intraluminal vesicles, EF/LF release to cytoplasm is suggested to involve a back-fusion between these intraluminal vesicles and late endosome membranes (Abrami et al., 2004). Interestingly, PA63 heptamer bound to CMG2 requires a lower pH for effective pore formation than TEM8-bound oligomer (Rainey et al., 2005). This is possibly due to the fact that PA domain 4 must dissociate from its receptor to allow pore formation, and dissociation from CMG2 may require more acidic conditions, perhaps due to the higher affinity of this receptor for PA (see above section on PA–receptor affinities). Thus, translocation of cargo through the PA pore may occur at different stages of

the endocytic pathway, and receptors plays a role in the pH-dependent conformational change that is required for translocation (Lacy et al., 2004a).

Palmitoylation and ubiquitination of the cytoplasmic tail of PA receptors play a role in controlling their endocytosis (Abrami et al., 2006). Palmitoylation of cysteine residues in the cytoplasmic tail of the PA receptors has been shown to alter the extent of their association with lipid rafts, and as it is only the ubiquitination of the tail following movement to rafts (where they can be proximal to their ubiquitin ligase) that allows rapid endocytosis of the receptor, palmitoylation can be thought of as a mechanism for prevention of receptor endocytosis in the absence of PA binding. The PA binding event triggers ubiquitination of the receptors and their uptake (Abrami et al., 2006). Paradoxically, a series of cytoplasmic tail truncated TEM8 mutants, including one with a complete deletion of the cytoplasmic tail expressed in a receptor-deficient cell line, are equally efficient for PA-mediated delivery of a cytotoxic LF fusion protein, implying that the TEM8 cytoplasmic tail is not required for efficient endocytic function (Liu and Leppla, 2003). In fact, TEM8 anchored with the glycophosphatidylinositol (GPI)-anchoring sequence of an unrelated protein still functions with equal efficiency as a PA receptor in toxicity studies using a cytotoxic LF fusion protein (Liu and Leppla, 2003). An alternate route of PA endocytosis independent of clathrin and dynamin has also been suggested because blocking dynamin function did not protect against PA delivery-mediated toxicity (Boll et al., 2004). Although unlikely, a secondary endocytosis mechanism may potentially explain the observed differences in these studies. Recent studies have also suggested that the cytoplasmic domain of TEM8 receptor can influence PA binding (Go et al., 2009).

Finally, through a completely unknown function, ARAP3 (Arf-GAP and Rho-GAP with ankyrin repeat and PH domains 3), a GTPase-activating protein, also appears to play a role in receptor endocytosis (Lu et al., 2004). It is hypothesized that this required protein either acts through ARF6 (ADP-ribosylation factor 6) which is involved in clathrin-mediated uptake, or through an unknown Rho GTPase.

LF/EF Translocation

As previously noted, the PA heptamer formed at the cell surface following cleavage of PA83 to PA63 is not SDS-resistant (pre-pore) (Milne et al., 1994). A conformational change that results in heptamer conversion to the SDS-resistant form (pore) made of an extended 14-stranded beta-barrel capable of translocating LF and EF only occurs at low pH (similar to conditions found in endosomes). The PA pore is cation-selective. Dominant negative PA mutants can be generated in which mutation of certain residues lining the lumen of the pore renders PA oligomers inactive for translocation. Heptamers formed from a mixture of these mutant PA molecules and wild-type PA can bind receptor and LF/EF, but the presence of the mutant PA within the heptamer "poisons" it and blocks acid-dependent translocation of the cargo (Sellman et al., 2001; Yan and Collier, 2003).

Elegant studies have identified Phe427 of PA as a solvent-exposed residue in the lumen of the pore that is crucial for cargo transport (Krantz et al., 2005b). The seven Phe residues play a role in the pre-pore to pore transition, and in the final pore they form a phenylalanine "clamp" that catalyzes translocation (Krantz et al., 2005b; Sun et al., 2008). Translocation through the pore is believed to occur by a Brownian ratchet mechanism linked to protonation status involving this Phe-clamp (Krantz et al., 2005a). LF or EF must unfold in order to be accommodated in the beta-barrel of the pore as an extended polypeptide (Krantz et al., 2004; Wesche et al., 1998). Translocation has been shown to be N-terminal to C-terminal, requiring the first 36 residues of the cargo, with the number of

cargo molecules bound to heptamer not affecting the efficiency of translocation (Zhang et al., 2004a,b).

Some studies have suggested that LF translocates freely into the cytosol while EF may remain associated with endosomal membranes following transit through the PA pore (Guidi-Rontani et al., 2000), although these results have not been verified and raise questions about how a membrane-associated EF could refold and associate with its essential co-factor, calmodulin.

The search for cytosolic chaperones required in the transport and refolding of LF/EF, (similar to those required for diphtheria toxin translocation) (Ratts et al., 2003), has not yielded definitive results. Recently, however, it has been reported that a sequence in the N-terminal regions of LF and EF is required for translocation by virtue of interaction with zeta-COP and beta-COP of the COPI coatomer complex (Tamayo et al., 2008). Interestingly, the need for a functional COPI coatomer complex for PA63 pore formation in cells has also been reported (Abrami et al., 2004).

Finally, although PA heptamer is primarily thought of strictly as the vehicle for LF and EF transport, and PA itself is not normally toxic to cells or in animals, it has been shown that macrophages manipulated to overexpress PA receptors succumb to PA. The toxicity of PA to these cells requires its binding, translocation to endosomes, and acidification of the endosomes, suggesting a possible ability for endosomal permeabilization by PA when very high numbers of heptamers form (Salles et al., 2006). Whether these findings have relevance to toxin action during the infectious process is not known.

LF

Structure and Enzymatic Function

LF is a 90-kDa zinc metalloproteinase (Klimpel et al., 1994) which cleaves the N-terminus of a number of mitogen-activated protein kinase kinases (MAPKKs, or Meks), including Mek1, 2, 3, 4, 6, 7 (Duesbery et al., 1998; Pellizzari et al., 1999; Vitale et al., 1998, 2000). Determination of the LF crystal structure shows it to have four domains (Pannifer et al., 2001). The N-terminal domain 1 (known as LFn, residues 1-254) binds to PA and when fused to other proteins is sufficient for their translocation to the cellular cytosol (Arora et al., 1992; Arora and Leppla, 1993, 1994; Milne et al., 1995). The "passenger polypeptide" can be at the amino or carboxy terminus of LFn (Arora and Leppla, 1994; Milne et al., 1995), although fusions having LFn at the N-terminus are generally used. LF domain 2 is made of two regions (residues 263-297 and 385-440) and is conformationally similar to the *B. cereus* VIP2 toxin, but is mutated for residues that are essential for catalytic function of that ADP-ribosylating toxin. Mutations in domain 2 have shown it is important for association with the Mek substrates (Liang et al., 2004) at a site C-terminal to the Mek cleavage sequence (Chopra et al., 2003). Domain 3 is important for LF activity, as a deletion of residues 308-326 makes LF nontoxic (Arora and Leppla, 1993). Domain 4 contains the zinc binding site and cooperates with domains 2 and 3 to form the catalytic core (Pannifer et al., 2001).

The sequences in the Mek proteins that are cleaved by LF lie in the domains that dock to their substrate kinases (Bardwell et al., 2001, 2003), and the cleavage event prevents binding and phosphorylation of these downstream partners (Chopra et al., 2003). Thus, the LF cleavage of Meks disrupts the very important ERK1/2, JNK/SAPK, and p38 signaling pathways, which are each involved in a multitude of cellular functions from

proliferation and cell cycle regulation, to immune modulation and defense against a variety of stresses. It is not surprising, therefore, that many of the pathways that have been so widely studied by pharmacological or genetic inhibition of the Mek proteins are similarly effected in the same cells with LT treatement.

Not much is known about the mechanisms by which LT finds Mek substrates after translocation through the PA heptamer pore in endosomes, but a recent paper suggests a role for Rho GTPases in the trafficking of LF to their substrates (DeCathelineau and Bokoch, 2009). Following translocation to the cytosol, however, the N-terminal sequence of LF determines its stability and potency in cell and animal toxicity (Gupta et al., 2008; Verma et al., 2008). This potency is likely linked to the half-life of LF in the cell as determined by the "N-end rule" of targeting proteins for proteasomal breakdown (Varshavsky, 1997).

Cytotoxic and Immunomodulatory Effects

The early discovery that LT induces a rapid and striking lysis of murine macrophages (Friedlander, 1986) led to this cell type becoming the major focus of LT research in eukaryotic cells. Since the discovery that LT targets the Mek proteins, researchers have broadened their focus to include the multitude of other immunomodulatory, non-cytototoxic effects this toxin induces in a range of other cells. The contribution of LT action on macrophages to anthrax pathogenesis is still an area of interest, although the relevance of the classic rapid lysis of murine macrophages to human anthrax disease is much in question, especially as human macrophages are now recognized as not being susceptible to rapid lysis by LT. In any event, a significant part of the research on LT still focuses on the "macrophage" question, so this section will review LT effects on this particular cell type, and relate these data to studies on other cell types. LT lethality to animals, however, is likely to result from the toxin's effects on a number of cell types and tissues.

Monocytes/Macrophages

Cytotoxicity: The Unusual Rapid Lysis of Murine Macrophages Different strains of inbred mice have macrophages with distinctly different sensitivities to LT. Some macrophages lyse rapidly in under 90 min (using saturating doses of fully active LT), while others are resistant to rapid lysis (Friedlander, 1986; Friedlander et al., 1993). Mouse macrophage cell lines derived from these mice follow the same sensitivity pattern. A survey of macrophages from many inbred mice has divides them into LT-sensitive (examples include Balb/cJ, C3H/HeJ, CBA/J, FVB/NJ, SWR/J) and LT-resistant groups (examples include DBA/2J, AKR/J, SJL/J, A/J, and C57BL/6J mice) (Roberts et al., 1998). Macrophages that are resistant to the rapid lysis by LT do undergo a slow apoptotic death dependent on Mek cleavage (Muehlbauer et al., 2007; Park et al., 2002). There have also been reports of an apoptotic death in LT-sensitive macrophages when sublytic doses of LT are used (Park et al., 2002; Popov et al., 2002b). Thus, the ability to undergo apoptosis may exist in both macrophage types but may not be apparent in the LT-sensitive macrophages, where the rapid lysis/necrosis pathway dominates. Although to date all macrophage preparations isolated from humans have not exhibited rapid lysis like that seen in the LT-sensitive inbred mouse macrophages, most studies on LT-mediated cell death still focus on this unique, rapid lysis. Rats can similarly be divided into groups according to

whether they harbor LT-sensitive or LT-resistant macrophages (Nye et al., 2007). For the purposes of this chapter, "LT-sensitive" will refer to macrophages that can undergo the rapid lysis (also referred to as *Nalp1b*^{S} macrophages following the next section). Furthermore, "LT-mediated macrophage death" will refer to the rapid lysis/necrotic death, unless specifically identified as apoptosis.

Nalp1b, Caspase-1, and the Inflammasome Early studies showed that there is no correlation between Mek cleavage and macrophage sensitivity to LT (Pellizzari et al., 1999, 2000). The most important advance in the understanding of LT-mediated macrophage lysis came with the identification of *Nalp1b* as the LT sensitivity locus (Boyden and Dietrich, 2006). Macrophage sensitivity to LT was initially mapped to a single locus on mouse chromosome 11, Ltxs1 (Roberts et al., 1998), which was first misidentified as *Kif1c* (Watters et al., 2001), and later definitively proven to be the closely-linked *Nalp1b* (Boyden and Dietrich, 2006). Sequence analysis of *Nalp1b* from 18 inbred mouse strains identifies five polymorphic alleles in mice. All mice carrying sensitive macrophages with one exception carry what is now defined as allele 1. The CAST/EiJ mouse is the only strain with LT-sensitive macrophages that carries allele 5. The other three identified alleles always correlate with macrophage resistance to LT. Introduction of a BAC (bacterial artificial chromosome) containing the *Nalp1b* locus derived from a mouse with sensitive macrophages into a resistant background confers macrophage sensitivity and demonstrates that the sensitivity allele is dominant (Boyden and Dietrich, 2006).

Nalp1b is homologous to human *Nalp1* and is a member of the growing NLR (Nod-like receptor) family of intracellular pattern recognition receptors that sense bacterial products or danger signals in the cytoplasm (Martinon and Tschopp, 2007; Tschopp et al., 2003). These proteins contain a nucleotide-binding domain (NACHT), a leucine-rich repeat domain (LRR), and either a caspase recruitment domain (CARD) or pyrin domain (PYD), or both. They function in a complex known as the "inflammasome" which is responsible for recruitment and proteolytic activation of caspase-1, which in turn activates the proinflammatory cytokines IL-1β and IL-18 (Martinon and Tschopp, 2007). The caspase-1 inflammasomes are now known to be essential not just for innate immune responses, but also for the induction of death pathways in response to many different microbial signals (Yu and Finlay, 2008). LT activates caspase-1 in LT-sensitive (*Nalp1b*^{S}) but not LT-resistant (*Nalp1b*^{R}) macrophages (Boyden and Dietrich, 2006; Cordoba-Rodriguez et al., 2004), mirroring the *in vivo* observation of early transcription-independent IL-1β release in LT-injected mouse strains harboring sensitive macrophages (but never in those with resistant macrophages) (Moayeri et al., 2003, 2004). In LT-sensitive (*Nalp1b*^{S}) macrophages, LT induced caspase-1 activation involves the movement of pro-caspase-1 from a smaller membrane-associated 200-kDa complex to a large 800-kDa membrane-associated complex containing Nalp1b, α-enolase and caspase-11, but not the adaptor protein ASC, which is found in many other inflammasome complexes (Nour et al., 2009). Interestingly, heat shock-induced protection against both Nalp1b (and Nalp3) caspase-1 activation in sensitive macrophages involves the "trapping" of caspase-1 in a high molecular weight complex (Levin et al., 2008).

The rapid macrophage lysis/necrosis death in response to LT treatment absolutely requires caspase-1 activation, which occurs at a step subsequent to the cleavage of Mek substrates in the cytoplasm (Muehlbauer et al., 2007; Wickliffe et al., 2008b). Formation of the Nalp1b inflammasome also requires LT-mediated potassium efflux, which has been described as a general signal sensed by other inflammasomes (Fink et al., 2008;

Petrilli et al., 2007; Wickliffe et al., 2008b). Early studies characterizing LT-mediated macrophage lysis reported increases in permeability to ^{22}Na and ^{86}Rb as a relatively early event, at 45 min post-LT treatment, accompanied by conversion of ATP to ADP/AMP, while leakage of lactate dehydrogenase and morphological changes marking onset of cell lysis occurred later, after 75 min of LT treatment (Hanna et al., 1992). These early studies on timing of permeability alterations in macrophages, combined with protection of lysis by medium isoionic for Na+ and K+ (suggestive of altered activity of the Na+/K+ pump or other ion channels), may have been the first indication of the K+ efflux necessary for activation of the Nalp1b inflammasome.

The Nalp1b inflammasome induced by LT is unique in that unlike other characterized inflammasomes, its formation requires proteasome activity (Fink et al., 2008; Squires et al., 2007; Wickliffe et al., 2008b). The early finding of macrophage protection from LT-induced lysis by proteasome inhibitors (Tang and Leppla, 1999) can thus now be directly linked to the requirement for proteasome activity in LT-mediated caspase-1 activation. The breakdown of a protein (or a number of proteins) by the proteasome appears to be upstream of most events induced by LT that eventually lead to caspase-1 activation minutes prior to cell death (Wickliffe et al., 2008b). Loss of mitochondrial membrane potential induced by LT is an earlier event that occurs shortly after Mek cleavage but can be reversed by proteasome inhibition (Alileche et al., 2006). In fact, addition of proteasome inhibitors well after cleavage of Mek proteins still allows rescue of macrophages from LT-mediated death, apparently by preventing late steps, including caspase-1 activation (Alileche et al., 2006; Squires et al., 2007; Wickliffe et al., 2008b). The mechanisms by which LT activates the Nalp1b inflammasome and caspase-1 and by which caspase-1 activation causes macrophage lysis are unknown, although osmotic protectants can protect cells from lysis (Hanna et al., 1992). Mek cleavage occurs equally in LT-sensitive and resistant macrophages, but it is possible that an event downstream of this common cleavage event is "sensed" differently by the $Nalp1b^S$ and $Nalp1b^R$ proteins and that cleavage of these particular substrates is still the first requirement for macrophage lysis. Alternatively, LT may target a different unidentified substrate, perhaps one that acts as an inhibitor of inflammasome activation, allowing its targeting to the proteasome.

A recent report identifying a NOD2-Nalp1-caspase-1 complex has also suggested a potential role for NOD2 in LT-mediated caspase-1 activation (Hsu et al., 2008). The interpretation of the LT portion of these studies is difficult, as the investigators used LT only in cells from the C57BL/6J mouse which harbors the *Nalp1b*^{R} allele and has been shown by many groups not to activate caspase-1 or release IL-1β in response to LT treatment (but does so in response to bacterial infections). In this recent report on NOD2, *B. anthracis*-induced IL-1β secretion from LT-resistant macrophages depends on NOD2 and Nalp1 and is attenuated when LT-deficient mutant bacteria are used in infections. It remains to be seen, however, if NOD2 is required for caspase-1 activation by LT in macrophages from mice harboring the *Nalp1b*^{S} allele associated with the rapid necrosis of macrophages.

Other Events Associated with Macrophage Lysis The discovery of Nalp1b and the requirement for caspase-1 activation in LT-mediated macrophage lysis has directed attention to the role of inflammasome activation in cell death. This presents both an opportunity and a challenge to revisit a substantial body of data accumulated over a number of years on the biochemical aspects of the LT-induced lysis for potential clues to understanding the process. In this section we will review the pertinent findings from those earlier studies.

Mitochondria and Antioxidants LT induces superoxide production in sensitive macrophages, and antioxidants protect against LT toxicity (Hanna et al., 1994). It would be interesting to see if reactive oxygen species (ROS) production plays a role in activation of caspase-1 and if they are linked to the mitochondrial membrane potential loss that occurs in response to LT (Alileche et al., 2006). The mitochondrial Bcl-2 family proapoptotic Bnip3 proteins that have been implicated in both apoptotic and rapid necrotic macrophage death pathways may actually play a role in caspase-1-mediated lysis, as their levels seem to correlate with induction of resistance in macrophages harboring the *Nalp1b*^{S} allele (Ha et al., 2007a) (see further discussion of LT and BNIP3 in later sections).

Protective Treatments Protection against LT-mediated macrophage death has long provided clues as to pathways that may be involved in cytotoxicity. The inhibitors that may prove most informative in deciphering the intracellular events leading to macrophage death are those which do not effect LF binding, entry, and translocation (as measured by no effect on cleavage of the Mek1 substrates in cytosol), but instead manifest their protective function downstream of LF cleavage of these proteins. The proteasome is now known to be required for the LT-mediated activation of caspase-1, but the manner of its involvement and/or its substrate remains unknown. Established proteasome inhibitors (Tang and Leppla, 1999) and new compounds shown to prevent proteasome function such as celastrol (a quinine methide triterpene) (Chapelsky et al., 2008) protect against LT lysis and always act downstream of Mek cleavage. The proteasome substrate(s) involved in LT-mediated lysis are targeted by the N-end rule of protein breakdown, as type-2 destabilizing amino acid derivative inhibitors of this pathway (e.g., phenylalanine amide) also protect against cell death (Wickliffe et al., 2008a). Bestatin methyl ester, an aminopeptidase inhibitor, can synergize with these amino acid derivatives in protection (Wickliffe et al., 2008a). One protein identified as an N-end rule substrate broken down in sensitive bone marrow-derived macrophages in response to LT treatment is c-IAP1, a member of the *i*nhibitor of *a*poptosis *p*rotein (IAP) family. However, the breakdown of this protein is dependent on caspase-1 activation by LT, indicating it is not the crucial upstream event required for activation of the Nalp1b inflammasome. Furthermore, this protein is not broken down in immortalized LT-sensitive macrophage lines such as the RAW274.6 and only degrades as an N-end rule substrate in primary and bone marrow-derived macrophages (Wickliffe et al., 2008a).

Not surprisingly, caspase-1 inhibitors (the specificity of which is often suspect) also protect against LT with a range of potencies, although complete protection is rarely achieved (Muehlbauer et al., 2007; Wickliffe et al., 2008b). Recently, it has been shown that heat shock can inhibit LT-mediated activation of the Nalp1b inflammsome through formation of a large complex containing pro-caspase-1. Interestingly, heat shock effects on pro-caspase-1 may be universal, as LPS/nigericin-mediated activation of the Nalp3 inflammasome is also inhibited by heat shock (Levin et al., 2008). Identification of the protective proteins in complex with caspase-1 could help to understand inflammasome-mediated activation of this enzyme.

Many compounds protect through inhibition of proper PA63 oligomer formation or by blocking translocation of LF to cytosol, either through inhibition of receptor-mediated endocytosis or acidification of endosomes (Artenstein et al., 2004; Menard et al., 1996; Moayeri et al., 2006; Sanchez et al., 2007). Other inhibitors such as phosphatase inhibitors identified in two separate studies have been implicated in interfering with LF cleavage of Mek substrates through unknown mechanisms (Kau et al., 2002; Panchal et al., 2007).

Calcium channel blockers and antagonists, phospholipase A2 inhibitors, the tyrosine-specific protein kinase A inhibitor genistein, neomycin, protein kinase C inhibitors, and phospholipase C inhibitors have all also been reported to protect against LT macrophage lysis, although the doses used in some of the studies were inappropriate, and the steps at which these inhibitors function is unknown (Bhatnagar et al., 1989, 1999; Shin et al., 1999, 2000).

Other Studies Finally, although a number of publications have analyzed transcriptional and translational alterations following LF treatment, it is clear that continued protein synthesis is not needed for LT-induced lysis to occur (Levin et al., 2008; Pellizzari et al., 1999). Many studies of this type assess events at time points where macrophages are well beyond the point of no return, mostly dead, even by each study's own death response curves, and thus are describing the sequelae of severe stress and cell death rather than LT-specific events (Comer et al., 2005b; Kuhn et al., 2006; Sapra et al., 2006). Even when attempts are made to investigate events at time points where a smaller percentage of cells are dead, the fact that any portion of cells have succumbed is clearly indicative that most of what is being studied is the macrophage stress responses to the lytic event (Chandra et al., 2005). The only such study investigating responses at very early times after LT treatment found altered expression of genes under GSK-3β (glycogen synthase kinase 3β) regulation, but it is unlikely these changes play a role in the caspase-1-mediated rapid lysis induced by LT, despite the observation that inhibition of this kinase sensitized both *Nalp1b*^{S} and *Nalp1b*^{R} macrophages (Tucker et al., 2003). The upregulation of genes controlled by GSK-3β may be a survival response in the apoptotic death pathway LT induces in "resistant" macrophages (see below), as GSK-3β inhibition is normally a *protective* event. GSK-3β inhibition has also been linked to protection from Mek-cleavage mediated cell arrest induction by LT (Ha et al., 2007b) (see later sections).

Cytotoxicity: Apoptotic Death of "Resistant" (Nalp1b^R) Macrophages

Macrophages from mice harboring only *Nalp1b*^{R} alleles succumb to LT over a 16- to 72-h period through an apoptotic pathway dependent on Mek cleavage (Muehlbauer et al., 2007; Park et al., 2002). The apoptosis induced by LT in *Nalp1b*^{R} macrophages may involve blocking signaling via ERK to the RSK-C/EBPβ (ribosomal S6 kinase-2/CCAAT enhancer binding protein β) pathway. (It should be noted here that the status of the *Nalp1b* alleles of the knockout mice used in the study implicating this pathway was not assessed, but despite a backcross to the FVB-*Nalp1b*^{S} mouse, was likely to still be *Nalp1b*^{R}) (Buck and Chojkier, 2007).

Interestingly, LT treatment of LPS-primed *Nalp1b*^{R} macrophages sensitizes them to a more rapid 8-h apoptotic death through removal of the p38 protective pathway, while LT alone does not induce apoptosis in resistant macrophages during this time frame (Park et al., 2002). Inexplicably, LPS-primed *Nalp1b*^{S} macrophages in these studies were also shown to undergo apoptosis after LT treatment, possibly because the LT was used at sublytic concentrations (Park et al., 2002). A second report on sensitization of resistant *Nalp1b*^{R} macrophages finds sensitization to LT with various microbial products through a TNF-α-dependent pathway (Kim et al., 2002). In contrast to the previous study, however, sensitized macrophages, while not undergoing the rapid lysis associated with caspase-1-dependent death in *Nalp1b*^{S} macrophages, succumb via a unique necrotic pathway distinctly different from apoptosis. Pan-caspase inhibitors do not protect against this unique LT-mediated necrotic death in sensitized *Nalp1b*^{R} macrophages, while rapamycin appears to protect through unknown mechanisms (Kim et al., 2002). In another contrasting result

to the first study, at sublytic doses of LT, TNF-α-mediated sensitization of *Nalp1b*^{S} macrophages does not occur (Kim et al., 2002). Interestingly, LT-resistant human macrophages are sensitized by the treatments used in this study. These sensitization studies suggest the intriguing possibility that in a real infection, macrophage sensitization to LT could occur, but macrophage death would be apoptotic or a variation of necrotic death not associated with caspase-1 activation or rapid IL-1β release.

Resistant Nalp1b^{S} Macrophages A small population of cells in sensitive macrophage cell lines such as RAW246.7 (*Nalp1b*^{S}) are resistant to LT, but this resistance can be reversed by cell passage (Ha et al., 2007a). Furthermore, small subpopulations of *Nalp1b*^{S} macrophages can be made LT-resistant through repeated low-dose treatments of LT (Salles et al., 2003). This phenomenon is called "toxin induced resistance" (TIR).

The analysis of RNA transcripts from *Nalp1b*^{S} macrophages in the phenotypically "resistant state" produced through either of these procedures shows significantly reduced expression of two related proteins, mitochondrial Bcl2/adenovirus E1B-interacting proteins Bnip3 and Bnip3L (Ha et al., 2007a). The overexpression of these proteins confers protection to sensitive cells, while their downregulation in resistant macrophages (by siRNA) sensitizes them. Interestingly, the continued cleavage of Meks by LT is required for continued resistance in these cells, because p38 shutdown is essential for resistance. In fact, p38 inhibitors induce LT resistance in RAW246.7 cells through reduction of the Bnip3 proteins. Thus, a sublethal dose of LT (to inhibit p38), or p38 inhibitors can lead to resistance of *Nalp1b*^{S} macrophages through mechanisms that have not yet been mechanistically connected to caspase-1-mediated events. For example, it is unknown if Bnip3 proteins' hypothesized function in controlling LT-induced mitochondrial dysfunction occurs downstream of Nalp1b-mediated activation of caspase-1 by LT, or if LT directly results in mitochondrial changes which can subsequently be modified by Bnip3, and it is these early mitochondrial events which subsequently lead to potassium flux, inflammasome activation, and caspase-1 activity. Bnip3 proteins are unique in that they have been implicated in both apoptosis death pathways as well as in a novel necrotic cell death which is preceded by mitochondrial membrane permeabilization, production of ROS, and sudden loss of plasma membrane integrity reminiscent of the lysis seen in *Nalp1b*^{S} macrophages (Vande Velde et al., 2000). The role of these proteins in LT-mediated cell death certainly deserves further study.

Human Monocytic Cell Apoptosis Thus far, human macrophages have been found to be resistant to the rapid LT-mediated lysis observed in certain inbred mice, but activated human peripheral blood mononuclear cells do undergo apoptosis following inhibition of cell division (Popov et al., 2002a). It would be interesting to see if multiple human Nalp1 alleles exist, and if any are similar to the *Nalp1b*^{S} alleles in mice. The apoptosis induced by LT in human mononuclear cells has been verified to depend on their activation state. Human monocytic cell lines (U-937, HL-60, and THP-1) undergo cell division arrest with LT treatment over a course of days, without cell death (Kassam et al., 2005). The differentiation of these cells to macrophages can also be prevented by LT through inhibition of Mek pathways. When differentiated to macrophages, these cells become sensitive to a slow caspase-1-independent apoptosis (over 24 h or longer). This apoptosis is independent of the Mek inactivation which occurs similarly in nondifferentiated and mature cells. The differentiation-associated death of these cells is different from the TNF-α-mediated "sensitization" of resistant mouse macrophages (Kassam et al., 2005). A similar report in dendritic cells (discussed in the next section) also supports a role for differentiation/activation in their LT

susceptibility (Reig et al., 2008). Interestingly, maturation of multiple human monocyte lines has also been associated with higher levels of PA binding (Deshpande et al., 2006).

Other studies in the same human monocytic cell lines, which depend on Mek1 activity for growth, show that in the absence of activation, LT induces cell arrest through breakdown of cyclin D1/D3, a process which can be reversed by activation of the phosphatidylinositol 3-kinase/Akt pathway. Interestingly, Akt provides protection from cell arrest by inhibition of glycogen synthase kinase-3b (Ha et al., 2007b). The induction of cell cycle arrest by LT is not unique to macrophages, as cell proliferation and/or differentiation has been shown to be inhibited by LT in lymphocytes that require ERK activity for these functions (discussed in next section) (Fang et al., 2005, 2006; Paccani et al., 2005). The sequelae of LT-mediated cell cycle arrest could explain host death in experimental animals that do not harbor LT-sensitive macrophages (Moayeri et al., 2003). LF is stable in circulation for long periods of time unless removed by excess PA (M. Moayeri and S.H. Leppla, unpublished data) and more importantly, following entry into cells, it appears to be stable in the cellular cytoplasm for many days (Ha et al., 2007a), potentially allowing continued prevention of cell division. Ha and colleagues have also reported that LT can cleave Mek1 up to 7 days in various tissues from mice after only a single bolus administration, supporting the notion of toxin stability in various cell types, many of which may be subjected to cell arrest (Ha et al., 2007b). Whether most cells *in vivo* can mount a recovery in the face of continued Mek1 cleavage by activating protective responses such as the PI3K/Akt pathway remains to be seen. It is interesting to note that many of the published studies reporting cell "death" in response to LT treatment in a variety of cell types are actually studying proliferation arrest relative to untreated controls, and not the induction of a death pathway. Care must be taken to distinguish between cell arrest and apoptotic death when investigating the sensitivity of new cell types.

Modulation of Macrophage Function Almost all of LT's immunomodulatory effects in macrophages and other cells are a result of Mek cleavage and inhibition of the Mek1/2-Erk1/2, Mek 4/7-SAPK-JNK, and Mek3/6-p38 signaling pathways. LT-mediated inhibition of the cytokine production induced in response to classic stimuli is well documented in LT-treated cell lines (Erwin et al., 2001; Pellizzari et al., 1999) and in comparisons of macrophages and mice infected with wild-type and LF-deficient spores (Bradburne et al., 2008; Drysdale et al., 2007). Non-cytokine responses, such as nitric oxide production in response to LPS, are also inhibited by LT (Pellizzari et al., 1999). Similar LT effects have also been demonstrated in human monocytes. Following LT treatment, activated human peripheral mononuclear cells are inhibited in their bactericidal ability and cytokine responses to bacterial cell wall preparations (Popov et al., 2002a). LT-treated nonhuman primate alveolar macrophages retain full phagocytic function but are impaired in cytokine secretion and bactericidal activity against both *B. anthracis* Sterne and Ames strains (Ribot et al., 2006). Guinea pig alveolar macrophages also lose bactericidal ability when LT-treated, possibly through inhibitory effects of the toxin on secretory type IIA phospholipase (Gimenez et al., 2004).

Dendritic Cells (DCs)

Interpreting studies of the effects of LT on DCs is made complicated by the fact that the isolation method and tissue source of these cells lead to different activation states, and LT-mediated effects appear to be highly dependent on the maturation state of DCs. Thus, care should be taken when reviewing this literature. DCs are the only other cell type that

have been reported to succumb to LT through a rapid proteasome-dependent lysis when cells harbor the *Nalp1b*^{S} allele (Alileche et al., 2005; Muehlbauer et al., 2007). Murine *Nalp1b*^{R} DCs and human DCs behave in the same manner as macrophages, undergoing a slow (24–72 h) apoptosis in response to LT (Alileche et al., 2005). While in one study bone marrow-derived DCs (immature) and spleen-derived activated DCs have equal LT sensitivity (Alileche et al., 2005), another study finds that the sensitivity of *Nalp1b*^{R} DCs is altered with activation state (Reig et al., 2008). In the latter report, LT kills 50% of immature murine *Nalp1b*^{R} DCs over 24 h by apoptosis, and inhibits their maturation and LPS-triggered cytokine release (Reig et al., 2008). In contrast, cells that are first activated are protected against apoptosis. Furthermore, DCs from mice with the *Nalp1b*^{S} allele in which caspase-1 is knocked out (eliminating the rapid necrosis pathway and allowing the apoptosis pathway to dominate) are also protected against LT by maturation signals. Although Mek1/2 cleavage is not affected by maturation state, inactivation of these kinases by LT is required for apoptotic death, since maturation also protects against Mek1/2 inhibitor-mediated cell death. Strangely, it appears that Nalp3 deficiency can exacerbate LT-mediated killing of the *Nalp1b*^{S} DCs which already succumb through the rapid caspase-1-dependent pathway (Reig et al., 2008). The link between Nalp3 and Nalp1b is unknown.

Despite the apoptosis and rapid cell death reported for DCs, there are numerous studies in which human DC (Maldonado-Arocho et al., 2006), murine *Nalp1b*^{R} DC (Tournier et al., 2005), and even *Nalp1b*^{S} DC (over 48 h of toxin treatment!) (Agrawal et al., 2003) have been found to be resistant to LT-mediated cell death. In other studies, the DCs include a resistant subpopulation such that LT lytic dose effects on *Nalp1b*^{S} DC could be assessed over 24 h (Chou et al., 2008). It is unclear if in these studies the activation state or other changes in DC signaling pathways played a role in the reported resistance to LT.

Similar to macrophages, LT inhibits DC cytokine secretion in response to classic stimuli (Agrawal et al., 2003; Cleret et al., 2006; Tournier et al., 2005), and prevents their maturation (Agrawal et al., 2003; Reig et al., 2008), shutting down their ability to function as antigen-presenting cells or to provide co-stimulatory molecules for lymphocyte activation (Agrawal et al., 2003). Even infection of DC with *B. anthracis* spores that produce LT has a clear inhibitory effect on DC innate cytokine responses while infection with toxin-deficient strains does not have the same effect (Cleret et al., 2006). A new report suggests that even the toxin inhibitory effects on DC function and cytokine production are dependent on the source of DC and the pathway through which the cells are stimulated (Chou et al., 2008). Although the findings in this study for the most part support the previously reported LT suppression of cytokine production in response to various stimuli (Chou et al., 2008), the LT-mediated enhancement of MHC Class-II and CD86 activation markers in lung DCs is in contrast to the suppression previously reported for splenic DCs (Agrawal et al., 2003). In summary, it is not surprising that LT manifests many effects in DCs similar to those seen in macrophages. In future years, *in vivo* investigations using murine models may provide insight as to how these cells are affected by toxin during infection.

Endothelial Cells and Vasculature

Because LT induces a unique vascular collapse in animal models (see next section), endothelial cells have long been considered a logical candidate target for this toxin. The first report of LT-mediated endothelial cell apoptosis in HUVECs (Kirby, 2004) measured viable cell numbers relative to untreated cells grown in parallel for the same number of days, making it difficult to discern if proliferation arrest rather than actual toxin-induced

apoptotic death was assessed. Proliferation arrest by LT in these cells is a distinct possibility since ERK pathway inhibitors could replicate LT effects and ERK1/2 are required for proliferation of these (and many other) cells. Other investigators have also described LT-mediated proliferation arrest in HUVEC (Huang et al., 2008) and SV40-transformed mouse endothelial cells (Depeille et al., 2007). However, in the previously mentioned initial study of HUVEC sensitivity (Kirby, 2004), cell surface annexin V staining at 18 h after LT treatment (11% vs. 4% in untreated cells) and FITC-ZVAD staining accompanied by protection using caspase inhibitors indicates at least a modest degree of apoptotic death. Subsequent reports of LT effects on HUVECs and human lung microvascular endothelial cells (Paddle et al., 2006; Pandey and Warburton, 2004) are also unclear as to whether apoptosis or cell arrest is being measured.

A number of laboratories have been unable to induce apoptosis in HUVECs (Batty et al., 2006; Warfel et al., 2005) but do note proliferation arrest (Batty et al., 2006) and LT-induced loss of barrier function in the absence of any endothelial cell death (Warfel et al., 2005). Thus, it remains unclear whether HUVECs actually undergo apoptosis in response to LT, but LT-induced loss of barrier function could clearly play a very important role in vascular collapse induced by this toxin *in vivo*. The rapidity with which LT induces vascular leakage in a modified Miles-assay in mice also seems to support a mechanism of altered endothelial barrier function that is independent of cell death (Gozes et al., 2006).

LT can also modulate endothelial cell responses to LPS and cytokine stimuli, examples of which include enhancement of TNF-induced VCAM-1 expression and monocyte adhesion to endothelial cells (Steele et al., 2005; Warfel and D'Agnillo, 2008) and inhibition of IL-8 production through transcript destabilization (Batty et al., 2006). Interestingly, these two effects would have opposing results in a classic cell-mediated endothelial cell damage model during infection *in vivo*. These effects may be less relevant to LT-mediated lethality or vascular collapse, which occur in the absence of cytokine responses or classic immune cell margination-associated endothelial damage (Cui et al., 2004; Moayeri et al., 2003). However, they may certainly play an important role in bacterial infections that are accompanied by strong cytokine production. The classic vasculitis associated with anthrax infections (Fritz et al., 1995; Grinberg et al., 2001; Vasconcelos et al., 2003; Zaucha et al., 1998) that is absent with LT treatment alone (Moayeri et al., 2003) is likely to be affected by such modulations of endothelial cell responses. It is interesting to note that many of LT-mediated effects on endothelial cells, such as the inhibition of IL-8 (Batty et al., 2006), or tissue factor expression (Rao et al., 2004) would theoretically be inhibitory to classic shock vasculitis.

LT and Vascularization Independent of the LT-mediated vascular shock that kills mice and rats, LT effects on vasculature *in vivo* can be seen in its inhibition of angiogenesis and targeting of tumor vasculature (Alfano et al., 2008; Depeille et al., 2007; Duesbery et al., 2001; Liu et al., 2008). Similar to what has been reported for endothelial cells, where LT-induced alteration of barrier function is not accompanied by endothelial cell death (Warfel et al., 2005), LT has also been shown to induce endothelial cell-death independent permeability in zebrafish vasculature with associated pericardial edema (Bolcome et al., 2008). Interestingly, these changes were replicated using a Mek1/2 inhibitor, making this one of the few studies linking Mek1/2 inhibition directly to *in vivo* vascular dysfunction. However, as Meks are cleaved equally in both sensitive and resistant mice and rats, it is difficult to know why the consequences of Mek cleavage in endothelial cells would have such different results in different strains. Thus, the role of Mek cleavage in endothelial cell dysfunction deserves further study.

Neutrophils

Although there is general agreement that LT is not cytotoxic to neutrophils, decreased proliferation and survival of these cells has been noted after several days of toxin treatment (Xu et al., 2008). Most of the studies of LT effects on neutrophils have focused on superoxide responses. Unfortunately, the literature on LT effects on neutrophil superoxide responses is confusing with seemingly contradictory findings that report both stimulatory and inhibitory effects of the toxin. On the proinflammatory side, Xu and colleagues have reported that LT treatment results in a higher and more sustained superoxide production by neutrophils in response to classic stimuli (Xu et al., 2008). Using comparisons of pharmacological Mek inhibitions and assessment of LT effects on Mek signaling pathways, the authors show that the combination of full Mek1/2 blockade but incomplete Mek 4/7 inhibition allows a more robust superoxide response from neutrophils (Xu et al., 2008). Older studies using shorter LT treatment periods report that LT blocks neutrophil priming and superoxide production from neutrophils (Wright and Mandell, 1986). Interestingly, Crawford et al. report inhibition of receptor-mediated neutrophil NADPH oxidase activity (fMLP or C5a-mediated) but not receptor-independent phorbol myristic acetate (PMA) induction of superoxide formation (Crawford et al., 2006), suggesting a possibility of stimulus-dependent effects. It is possible that LT has both inhibitory and stimulatory effects on neutrophils depending on dose and length of treatment and that these effects become primarily stimulatory with longer treatment times (Xu et al., 2008). Furthermore, with the exception of the study reporting the stimulatory effect of LT on neutrophil superoxide production (Xu et al., 2008), almost all studies have been performed with human neutrophils which are now known to produce alpha-defensins that can inhibit LT activity (Kim et al., 2005).

LT affects on neutrophil chemotaxis are also well studied but are seemingly contradictory. More recent reports have shown that LT inhibits neutrophil actin assembly and chemotaxis (During et al., 2005) through blocking Hsp27 phosphorylation as a consequence of p38 pathway shutdown (During et al., 2007). This result is interesting in that LT inactivation of Hsp27-mediated actin function may also affect other cell types, such as endothelial cells. An older study, however, shows that LT stimulates chemotaxis of neutrophils (Wade et al., 1985).

With much of the work on LT effects on neutrophils having focused primarily on superoxide responses and chemotaxis, modulation of cytokine responses has been little studied, with a lone report that LT treatment of human neutrophil-like NB-4 cells leads to decreases of numerous chemokines (Barson et al., 2008).

Finally, the discovery that alpha-defensins produced from human neutrophils can efffectively neutralize LT (Kim et al., 2005) is interesting in its own right while also calling attention to the existence of species-based differences, in this case because mice do not produce these small cationic peptides. It is unclear whether LT or ET inhibit production of these alpha-defensins, and whether the peptides can reach effective concentrations *in vivo* in locations where LT acts, including within cells. *In vitro* studies with human neutrophils would benefit from an assessment of the secreted levels of these peptides in the presence of toxin treatments.

Lymphocytes

LT is not toxic to lymphocytes *in vivo* (Alileche et al., 2005; Moayeri et al., 2003) and *in vitro* (Comer et al., 2005a; Fang et al., 2005) but inhibits proliferation, activation, and

cytokine production in these cells through Mek pathway shutdown (Comer et al., 2005a; Fang et al., 2005). One possible mechanism for LT modulation of T-cell functions is the toxin's blocking of NFAT and AP1 transcription factor activation (Paccani et al., 2005). Other sequelae of Mek cleavage in lymphocytes include chemotaxis inhibition by disruption of the chemokine receptor signaling pathways that require Erk1/2 activation (Paccani et al., 2007). ET can act synergistically with LF in manifesting inhibitory effects on T-cell function (Paccani et al., 2005, 2007). Very few studies have been performed on LT effects on B cells. LT has been reported not to be cytotoxic for B cells but to reduce B-cell proliferative responses and IgM production in response to various ligands both *in vitro* and *in vivo* (Fang et al., 2006).

It is interesting to ask why targeting of B cells or T cells might play a role during the course of an acute disease like anthrax. The effects of LT on lymphocytes may be a bystander effect resulting from the shutdown of the Mek pathways which affect virtually every type of host cell in some way. However, it is also possible that *B. anthracis* benefits from targeting adaptive immunity in non-acute, cutaneous anthrax infections. It is perplexing, however, that a combination of PA and active LF elicit a stronger antibody response in mice than the separate proteins (M. Moayeri and S.H. Leppla, unpublished data). The fact that the fully toxigenic nonencapsulated Sterne strain is used as a human vaccine in some countries and can induce robust antibody responses in animal models does bring into question the potency with which LT targets the adaptive immune response.

Other Cells

Platelets LT has been reported to suppress human whole blood clotting, platelet aggregation, P-selectin expression, and endothelial cell interaction (Kau et al., 2005), although it is unclear if similar events would occur in mice. The absence of hemorrhaging and fibrin deposits in mice treated with LT makes it unlikely that platelets play a role in the vascular insufficiency induced by LT alone (Moayeri et al., 2003), but platelet dysfunction may have great implications for LT's alteration of the host response to bacterial infection.

Epithelial Cells, Keratinocytes, Melanoma Cells Human small airway epithelial cells may undergo cell arrest or apoptosis in response to LT (Paddle et al., 2006). The rat epithelial cell line A549, however, is resistant to LT toxicity even over days of toxin treatment (Pandey and Warburton, 2004). LT is also not cytotoxic to human keratinocytes but upregulates the chemokine RANTES in these cells (Kocer et al., 2008). This is a rare instance of LT proinflammatory action. Interestingly, although LT-treated melanocytes have not been studied, LT is very effective at inducing apoptosis in human melanoma cells, which has prompted consideration of its use as an antitumor agent (Koo et al., 2002). Finally, in a surprising recent study in human lung epithelial cells, PA-mediated activation of TLR2/TLR6 and induction of cytokine production was reported, through a process that could be blocked by the presence of LF (and thus LT). Because the authors do not use a nonbinding PA control in these studies, it remains possible that the effects observed could be due to *B. anthracis* components present in certain PA preparations rather than to PA itself (Triantafilou et al., 2007).

Glucocorticoid Receptor Inhibition

The hypothalamic-pituitary-adrenal (HPA) axis and the glucocorticoid receptor (GR) response pathways control inflammatory responses during infection. LT, through its effects

on the p38 pathway, inhibits GR-mediated activation, but not GR-mediated repression of NF-kB or other transcription factors (Webster and Sternberg, 2005; Webster et al., 2003). Inhibition of GR activation has been demonstrated in mice (Webster et al., 2003), and it is likely that this LT effect is universal to many cell types as a downstream consequence of p38 inhibition. Evidence of the involvement of the endocrine system in LT sensitivity comes from studies in which resistance to LT in mice is reversed by adrenalectomy or glucocorticoid feeding/injections (Moayeri et al., 2005). Endocrine perturbation-based sensitization of LT-resistant mice may also be responsible for the variation in mouse sensitivities observed for the same strain of mouse in different animal facilities, perhaps through undetected underlying infections. Laboratories have reported varying sensitivities in mice dependent on stress, re-localization, and animal housing conditions. The additional loci that play a role in LT-mediated mouse susceptibility may involve endocrine-based pathways (McAllister et al., 2003; Moayeri et al., 2004). The implication of LT-mediated inhibition of the GR response for actual infections cannot be underestimated, especially in light of the preferred steroid treatment for inflammatory responses in anthrax shock in the clinical setting.

Lethality in Animals

Mice have been shown to succumb to toxin-deficient encapsulated *B. anthracis* strains (by intratracheal route as a spore, or by IV route following germination) with the same rate and LD_{50} as virulent parental strains (Heninger et al., 2006), making them a poor model for *B. anthracis* bacterial infection studies assessing the role of toxin. In the nonecapsulated Sterne strain (pXO1+, pXO2–), however, toxin deficiency leads to severe attenuation in both subcutaneous and aerosol murine models (Pezard et al., 1991). While rodents may be poor models for the study of anthrax infection, LT and ET can each replicate major symptoms associated with anthrax disease (such as pleural edema) in these animals, making them ideal for a reductionist approach in assessment of *in vivo* toxin pathogenic mechanisms (Ezzell et al., 1984; Firoved et al., 2005; Moayeri et al., 2003).

Study of LT effects in animals was made difficult for years because recombinant PA and LF preparations were typically produced in *Escherichia coli,* and it is very difficult to remove endotoxin from such preparations. Even when standard endotoxin measurement assays do not detect this contaminant, overnight treatment of macrophage lines with PA or LF prepared from *E. coli* clearly show a cytokine response profile that differs from toxin made from *B. anthracis* (which does not induce cytokine responses).

LT induces an atypical vascular collapse in mice and rats without classic hallmarks of endotoxic shock, marked by absence of hemorrhaging or cytokine involvement (Cui et al., 2004; Moayeri et al., 2003). The timing of lethality is strikingly different in the mouse and rat models, or even among different mouse strains. In sensitive rat strains (including Fischer, Sprague Dawley, and Brown Norway), death can occur as rapidly as 37 min (Ezzell et al., 1984; Gupta et al., 2008) and seldom exceeds 60 min when saturating LT doses (100 μg of each toxin component) are accurately injected IV. Susceptible inbred mouse strains, however, succumb to toxin at different rates but over a course of days (48–120 h). Almost all histopathological observations in LT-treated mice are sequelae of vascular insufficiency, with no direct LT-mediated pathology in any organ system as assessed at the light microscopy level. Not surprisingly, in the rat, there are no histopathological changes associated with the rapid LT-induced death. Despite early studies attributing LT-induced shock to the lysis of macrophages and TNF-α induction by LT

(prepared in *E. coli*) (Hanna et al., 1993), we now know that cytokines are not required for LT-mediated vascular collapse in rodents (Cui et al., 2004; Moayeri et al., 2003). The mechanism by which LT induces vascular collapse is not known, although alteration of endothelial cell function is an often-mentioned and reasonable hypothesis. Additionally, the heart may be the primary target for LT in rodents; it has been suggested that the pulmonary edema classically associated with LT treatment in rats may simply result from severe heart failure induced by LT targeting of cardiomyocytes and depressed left ventricular systolic function (Kuo et al., 2008; Watson et al., 2007a,b). Recent electron microscopy analyses have identified striking LT-mediated pathological changes in murine heart myocytes and endothelial cells and changes in cardiac damage biomarkers are the earliest events measurable in toxin-treated mice (Moayeri et al., 2009).

Over recent years, a Sprague Dawley rat infusion model has also provided information on the novel vascular shock induced by LT, showing that it differs in almost every respect from cytokine-mediated endotoxic shock (Cui et al., 2004, 2006; Sherer et al., 2007). Despite the clear changes LT induces in blood pressure and heart rate, no cytokines, nitric oxide, or histopathological changes are found in rats over 12–24 h of toxin infusion. Norepinephrine treatment, while beneficial to other shock states, has no effect on survival (Li et al., 2009). Amazingly, fluid support worsens outcome in LT-mediated rat shock (Sherer et al., 2007), indicating another potentially cautionary finding for clinicians dealing with anthrax infections.

The Role of Macrophage Sensitivity in LT-Mediated Death

In 1993, Hanna et al. reported that silica-based depletion of macrophages from LT-sensitive Balb/c mice (which harbor *Nalp1*^{S} macrophages) resulted in resistance to LT. An endotoxic-shock like death due to cytokine release following LT-mediated macrophage lysis in mice was hypothesized after antiserum to IL-1β and TNF-α protected mice from LT. Unfortunately, these early studies were performed with PA and LF produced from *E. coli*, and the TNF-α produced in these mice may have been a result of endotoxin contamination. The discovery of the targets of LF, the Meks, makes it clear that LT actually abrogates TNF-α production in response to stimuli such as LPS (Erwin et al., 2001; Pellizzari et al., 1999). This finding has also been verified in LT-treated mice (Moayeri et al., 2003). Explaining the correlation found between lethality and sensitive macrophages, however, is more difficult. LT does induce vascular shock in mice with resistant macrophages (Moayeri et al., 2003, 2004). While mice with *Nalp1b*^{S} macrophages certainly have a strong IL-1β release following lysis of their macrophage population *in vivo*, with subsequent transcriptional increases in a subset of defined cytokines, this cytokine burst rapidly subsides after macrophage depletion (Moayeri et al., 2003, 2004). The cytokine burst does not occur in mice having resistant *Nalp1b*^{R} macrophages, yet both *Nalp1b*^{S} and *Nalp1b*^{R} mice succumb to LT-induced shock. Macrophage sensitivity certainly can exacerbate the lethal effect, but only in certain mouse backgrounds, such as the C57 background, where providing *Nalp1b*^{S} results in a more rapid death almost identical to the Balb/cJ mouse (Moayeri et al., 2004). In many strains, however, macrophage sensitivity does not influence lethality or time to death (Moayeri et al., 2004), supporting the idea that multiple loci are involved in animal sensitivity to LT (McAllister et al., 2003), while only a single locus controls macrophage sensitivity to this toxin (Boyden and Dietrich, 2006). It is possible that the IL-1β response in some *Nalp1b*^{S} mice accelerates LT effects, but other loci provide a level of protection in other *Nalp1b*^{S} mice which are far more resistant (such as all C3H strains) (Moayeri et al., 2004).

In support of the involvement of multiple loci in LT sensitivity, the normally highly resistant DBA/2J (*Nalp1b*^{R}) mouse can be made sensitive to LT by asymptomatic underlying bacterial infections or by manipulation of its endocrine system, while maintaining fully LT-resistant macrophages (Moayeri et al., 2005). Studies using recombinant inbred mice derived from DBA/2J and Balb/cJ mice suggest that at least three loci control mouse sensitivity (McAllister et al., 2003). One locus is the *Nalp1b* locus. A second chromosome 11 locus has been hypothesized to be inducible nitric oxide synthase (iNOS), although mouse knockout studies have clearly shown this locus plays no role in LT susceptibility in the C57BL/6J background (Moayeri et al., 2004).

Rats can also be divided into LT-sensitive and LT-resistant groups with much more clarity than mice, in that sensitive animals succumb to 10 μg PA + 10 μg LF in 60–90 min, while resistant animals cannot be killed with 25-fold higher doses (Moayeri and Leppla, unpublished data). Survival appears to be controlled by a single locus (Nye et al., 2007), and a correlation to macrophage sensitivity has been noted, although the susceptibility locus has not yet been mapped. The rapid death in rats clearly involves mechanisms that are both similar (such as any leading to induction of pleural edema) and very different (those leading to a 37-min death) from those in mice. The identification of LT susceptibility loci in additional animal species requires further study.

LT, Spores, and Macrophages in Early Infection

Although many studies show germination of spores taken up by macrophages as an early stage of anthrax infection, the fate of the germinated bacteria and the role of toxin in their survival is a matter of dispute. Some investigators have shown intracellular division of germinated Sterne strain spores following uptake by RAW264.7 macrophages (Dixon et al., 2000), while others do not find bacterial replication within macrophages and report macrophage sporicidal activity (Guidi-Rontani et al., 2001; Hu et al., 2006; Welkos et al., 1989). Toxin's role in these early stages of infection is also unclear. Some reports suggest that toxin action is required for release of the large numbers of dividing intracellular bacteria from cells (Dixon et al., 2000), while others show toxin as necessary for the spore to survive macrophage killing (Guidi-Rontani et al., 2001) but do not find any division of germinated bacteria in macrophages. Anti-PA antibodies bind to spore surfaces and may enhance spore uptake by macrophages (Stepanov et al., 1996; Welkos et al., 2001), leading to both increased germination and killing of spores (Welkos et al., 2002). Thus, the real situation *in vivo* may be a complicated balance between macrophage killing of spores, germination, and dissemination after macrophage killing. One group has proposed direct translocation of LT produced by germinating spores from phagolysosomes into cytosol, resulting in the killing of macrophages by LT which may allow the escape of germinated bacteria (Banks et al., 2005). A follow-up study using a toxin-receptor negative variant of the LT-sensitive RAW264.7 macrophage cell line shows these cells are able to provide better protection against a challenge with Ames spores in mice than their receptor-expressing counterparts, suggesting a protective role for macrophages that bacteria can overcome with toxin (Cote et al., 2008). In a way, these studies provide an indirect comparison of LT-sensitive and LT-resistant macrophages in their ability to protect against spores, and it appears that LT-resistance of macrophages (in this case through absence of receptor) would allow better killing of spores and control of infection. Paradoxically, however, macrophage sensitivity to LT has been shown to have an inverse correlation to spore sensitivity in animals (Lincoln et al., 1967; Welkos et al., 1986), suggesting

LT-mediated macrophage killing may be detrimental to establishing infection. Either way, it seems likely that toxin is important in controlling early infection steps, including germination and survival in macrophages, prior to any outgrowth and manifestation of systemic effects. It is unlikely, however, that LT-mediated macrophage lysis plays a role, since a majority of anthrax-susceptible animals have LT-resistant macrophages.

EF

EF is a potent calmodulin-dependent adenylate cyclase (Leppla, 1982) which although initially characterized for the remarkable edema it caused in animals when injected subcutaneously (Stanley and Smith, 1961) is now known to modulate a large number of cell signaling pathways through cAMP production.

While the last decade has seen a great increase in the volume of literature on ET-mediated *in vitro* and *in vivo* effects and especially exciting discoveries on EF structure, studies on this toxin still lag behind those on LT. This is surprising, since it is expected that the multitude of cellular processes known to be influenced by cAMP would be subject to perturbation by ET. The massive levels of cAMP induced in nearly all types of cells will affect many pathways, so the challenge then becomes to identify those targets that are relevant to anthrax pathogenesis. As for LT, the possibilities for immunomodulation and subversion of signaling pathways by ET through cAMP production are myriad, so the hope is that relevant targets and functional effects can be identified in the near future.

Structure and Enzymatic Function

EF is a 92-kDa protein which requires the calcium sensor protein calmodulin (CaM) for enzymatic activity. Following the seminal papers reporting EF's enzymatic activity (Leppla, 1982, 1984) and mutagenesis studies identifying the catalytic and CaM binding domains of EF (Labruyere et al., 1990, 1991), almost everything known about the structure/function of this protein has come from a set of elegant structural analyses performed by a single laboratory (see below). This began with the determination of crystal structures of the enzyme with and without bound CaM, as well as with ATP and cAMP analogs (Drum et al., 2002), allowing for an understanding of the structural role of CaM in activation of the enzyme. The EF structure changes substantially upon binding of CaM. CaM has been shown to bind EF in an extended form through novel bindng sites, different from the compact conformations reported for CaM's many mammalian binding partners that bind at very similar CaM sites (Drum et al., 2000, 2002). Strangely, CaM binding to EF is also quite different from the binding surfaces used by the adenylate cyclase of *Bordetella pertussis* (Guo et al., 2008). Thus, EF seems to have evolved to use a completely different binding region in CaM. Calcium ions bound to CaM are crucial for the high-affinity (Kd 5–20 nM) interactions of EF and CaM (Shen et al., 2002), with affinities in low calcium concentrations being many orders of magnitude lower (Kd >10 μM). ET may trigger increased Ca^{+2} influx into cells to achieve concentrations optimal for activity, although the response within seconds is inconsistent with EF internalization by endocytosis (Kumar et al., 2002). Unlike many CaM binding partners, EF can bind Ca^{+2}-free CaM with low affinity through the CaM N-terminal domain, but Ca^{+2} is required for the higher affinity interaction with the C-terminal domain (Ulmer et al., 2003). In fact, Ca^{+2} binding at the two sites in the N-terminal domain of CaM is not necessary for maximal EF activity, while Ca^{+2} binding at the two sites in the C-terminal domain is essential. The conformational

changes that convert the enzyme from inactive to active state upon binding of CaM do not involve changes in the position of residues involved in enzymatic function, but rather in those that allow proper and tight binding of substrate ATP (Drum et al., 2002). In fact, adefovir diphosphate, a potent inhibitor of EF activity, has 10,000-fold higher affinity for EF-CaM than ATP does, and crystal structure of this inhibitor bound to EF-CaM reveals the altered residue interactions at the catalytic site that are crucial for the improved substrate binding (Shen et al., 2004). A two-metal ion catalytic mechanism similar to that used by mammalian adenylate cyclases has been described for EF catalysis (Shen et al., 2005). His-351 has been implicated in active site catalytic function by multiple structural studies (Drum et al., 2002; Shen et al., 2005) and functional analyses (Gupta et al., 2005).

Early reports on ET showed that this toxin differed from the *Bordetella pertussis* adenylate cyclase toxin in that it required receptor-mediated endocytosis and delivery through an acidic compartment, in a manner paralleling what was found for LF delivery (Gordon et al., 1988, 1989). These findings were later verified by fluorescence imaging of EF activity in the cytosol (Dal Molin et al., 2006). The recently developed methods for imaging of cAMP activity in living cells using a fluorescence resonance energy transfer between two subunits of PKA (each fused to a different fluorescent protein) whose proximity depends on cAMP-mediated activation of PKA indicate a timing of activity visualization supporting a release of EF from late endosomes, with a primarily perinuclear staining of activity (Dal Molin et al., 2006). The results from these studies indicate a gradient of cAMP activity from endosomes to cytosol, but cannot clarify whether the EF molecules remain endosome membrane associated after translocation through the PA pore, as has been suggested by one group of invetigators (Guidi-Rontani et al., 2000).

Immunomodulatory Effects

ET does not kill cells, a result consistent with the known effects of cAMP. Thus, with the exception of one report (Voth et al., 2005), ET has been shown to be nontoxic to all cells on which it has been tested, including DC (Chou et al., 2008; Tournier et al., 2005), macrophages (Comer et al., 2006), T cells (Paccani et al., 2005), neutrophils (Crawford et al., 2006), and human microvascular endothelial cells (Hong et al., 2007). For the most part, ET manifests its many immunomodulatory effects through increase of cellular cAMP and subsequent activation of the PKA/CREB or Epac/Rap1 pathways in a manner independent of apoptosis or cell death, although a recent report suggests that ET can induce cell cycle arrest in macrophages through activation of nuclear GSK-3β (Larabee et al., 2008).

Very early reports showed that as might be expected from a toxin producing cAMP (which has both stimulatory and inhibitory effects on numerous transcription pathways), ET treatment of monocytes can induce production of some cytokines (IL-6) while modulating LPS-mediated production of other cytokines (Hoover et al., 1994). Recent reports have confirmed the suppressive as well as stimulatory effects of ET on cytokine responses from DC as well (Chou et al., 2008; Tournier et al., 2005). Studies using spores of strains mutated in toxin component genes verify positive and negative regulation of cytokine production from DC by ET (Cleret et al., 2006; Tournier et al., 2005). ET-exposed bone marrow-derived macrophages show increased production of VEGF (Kim et al., 2008), while ET effects on T-cell activation, proliferation, chemotaxis, and cytokine responses are primarily inhibitory (Baldari et al., 2006; Comer et al., 2005a; Paccani et al., 2007). ET also downregulates secretory type IIA phospholipase A2 from alveolar macrophages, circumventing its protective effect against *B. anthracis* infection (Raymond et al., 2007).

Thus, it appears that while LT inactivation of Mek pathways is almost invariably suppressive to most innate immune response pathways, cAMP's role as a powerful second messenger results in a complex set of pro- and anti-inflammatory effects. Furthermore, the dosing of ET relative to other stimuli and to LT must play an important role in outcome of this toxin's effects. As an example, a recent report on ET-mediated CREB activation in Jurkat T cells demonstrates an initial rapid activation of this transcription factor followed by an eventual impairment of CREB responsiveness after prolonged ET treatment (Puhar and Montecucco, 2007).

ET's effects on activation of PKA-regulated pathways and the CREB transcription factor will likely have many other manifestations, as reflected by the multitude of transcriptional changes in response to this toxin (Comer et al., 2006). One such striking effect of ET involves targeting the cytoskeleton. The effects of ET in inducing drastic cell shape changes (rounding) in CHO-K1 are well-known (Leppla, 1982), and similar effects induced by ET via PKA have also been reported in Y1, 293T, and mouse embryonic fibroblast cells (Hong et al., 2005). More recently, it was discovered that ET targeting of syndecan-1, which is regulated by CREB, results in dramatic increases in macrophage migration through cytoskeletal changes (Kim et al., 2008). ET enhancement of neutrophil chemotaxis has been reported *in vitro* (Wade et al., 1985) as well as *in vivo* using isogenic toxin-deficient mutants of the Ames strain (Drysdale et al., 2007) and may involve both cytoskeletal changes as well as cytokine induction through the PKA pathway. ET activation of the Epac/Rap1 pathway, however, has been observed to inhibit endothelial cell chemotaxis and induce a different morphological change (flattening rather than rounding) in treated microvascular endothelial cells (Hong et al., 2007).

Other reported activities of ET include the inhibition of human neutrophil and macrophage phagocytic activity (possibly again through cytoskeletal effects) (Comer et al., 2006; O'Brien et al., 1985) and reduction of NADPH oxidase and superoxide production in response to stimuli (Crawford et al., 2006). Thrombin-induced platelet aggregation and clotting function has also been reported to be suppressed by ET (Alam et al., 2005), likely contributing to the hemorrhaging associated with ET in animal models (Firoved et al., 2005). ET can directly induce vascular leakage, but not likely through alterations in endothelial permeability, as trans-endothelial electrical resistance of endothelial monolayers is increased in response to ET (Tessier et al., 2007). Instead neurokinins, prostanoids, and histamine may be involved in ET-induced vascular permeability (Tessier et al., 2007).

Lethality in Animals

ET can be lethal to mice at certain doses, through induction of shock (Firoved et al., 2005). However, considering the lower levels of EF produced relative to LF during infection (Dal Molin et al., 2008), it is not known whether the amounts of EF produced during an infection approach those needed to kill. The pathology associated with lower, nonlethal doses of toxin, such as extreme changes found in adrenal glands, are more likely to be relevant to pathogenesis during infection. The striking ET-induced pathologies observed in mice include hemorrhagic lesions in a wide range of organs and extensive tissue damage accompanied by both hypotension and bradycardia (Firoved et al., 2005). This response contrasts with the lack of hemorrhagic pathology during LT-induced shock (Moayeri et al., 2003).

ET also induces shock in rats, but in a manner distinctly different from that caused by LT. ET hemodynamic dysfunction in the rat model is also accompanied by hemorrhage (Kuo et al., 2008), increased heart rate (Cui et al., 2007; Watson et al., 2007a), and reduced

cardiac output, but without any effect on cardiac ejection fraction (and myocardial contractility) (Watson et al., 2007a,b). Thus, it is likely that ET effects on the heart are secondary to general loss of circulatory fluids in many tissues.

LT/ET Cooperation

Although the reductionist approach of using LT and ET toxins *in vitro* and *in vivo* separately has provided useful information, these toxins are presumably produced *in vivo* at the same time. In a recent report of *in vivo* toxin production levels, a subcutaneous spore infection model in rabbits results in a range of LF (10–35 μg/mL) after 48 h, with the LF : EF ratio always round 5:1 (Dal Molin et al., 2008), similar to what has been previously reported *in vitro* (PA : LF : EF, 20:5:1) (Sirard et al., 1994) and paralleling findings in another *in vivo* study of Ames spore infection in rabbits (Mabry et al., 2006).

Recent studies have examined the combined effects of LT and ET, although the doses and timing of toxin administration were not based on *in vivo* studies, and therefore the results may be of limited relevance to an *in vivo* infection. Interpretation of findings of such experiments is also made difficult by the fact that ET can increase PA receptors on monocytic cell populations (Maldonado-Arocho et al., 2006). In some of the few studies that have investigated the combined effects of the toxin, ET has been found to accentuate and complement the LT inhibitory effects in T cells and DC (Chou et al., 2008; Paccani et al., 2005; Tournier et al., 2005). However, just as it seems odd that LT has evolved to suppress the multitude of cytokine pathways that are induced by *B. anthracis* bacterial infections, it is equally odd that many ET signaling pathways negate LT signaling pathways or vice versa. ET strongly activates CREB, which is a macrophage survival factor that lies downstream p38 (Park et al., 2005). LT inhibits the p38 pathway through Mek3/6 cleavage and many of LT's effects in cells result from blockade of this pathway. ET activation of CREB has been shown to promote survival of LT-treated macrophages that normally would undergo apoptosis (Park et al., 2005). All this points to a need for the study of these toxins' effects *in vivo*, in the real infection setting.

There have been only a few *in vivo* studies of LT and ET synergy. Low doses of ET which do not induce pathological changes sensitize LT-resistant mice to LT (Firoved et al., 2007). We now know that LPS may contribute to this sensitization effect by ET (M. Moayeri and S.H. Leppla, unpublished data). Sensitization to LT effects may occur through ET's striking effects on the adrenal gland (Firoved et al., 2005) and the resulting endocrine perturbation, which is known to alter LT sensitivity in murine models (Moayeri et al., 2005). In the rat infusion model, ET increases heart rate (unlike LT, which decreases heart rate), but the toxins cooperate to worsen shock (Cui et al., 2007). Again, the doses of ET used in most studies are unlikely to accurately reflect those in a bacterial infection, so that care must be taken in interpreting results. A better understanding of the contribution of each toxin and their interactions (antagonist, synergistic, etc.) may eventually be reached by comparing the behaviors and effects of toxin-isogenic mutants in infection models, although even then the results will need to be interpreted with caution.

FINAL REMARKS

Anthrax LT and ET are the essential virulence factors for lethality in anthrax disease as evidenced by the full protection afforded against infection through targeting of their common component, PA. Advances in recent years have allowed a better understanding

of these toxins' cytotoxic and immunomodulatory effects on many cell types *in vitro* and *in vivo*. The range of cellular events induced or inhibited by these toxins is indeed immense. However, the discoveries that LF and EF act in the cell cytosol to specifically alter the Mek and cAMP pathways has provided a biochemical basis from which researchers can seek to build molecular explanations for the physiological and pathologic changes in animals and humans infected by *B. anthracis*. The decades of productive research on the Mek and cAMP signaling pathways provides a firm basis on which to design such research, but care must be taken to avoid simply confirmatory (and expected) investigation of the toxins' effects on these pathways. We have to accept that many of the discovered effects in a range of cell systems are simply bystander results of the toxins' targeting of these ubiquitous signaling pathways.

The challenge now facing those anthrax toxin researchers who wish to decrease the threat of this disease is to extend their analyses so as to understand how the toxins contribute to human infections. Important questions needing analysis include: Is Mek cleavage essential or sufficient for LT-induced lethality? If so, what is the target cell in which Mek cleavage matters? If not, what other LF substrates mediate its effects? How does LT induce vascular collapse? Is there an evolutionary advantage to harboring a sensitive *Nalp1b* allele, and are studies on the unique LT-mediated mouse macrophage lysis helpful in understanding the disease process in human hosts? Does the *Nalp1b* allele status determine the response in other cell types? What are the relevant intersections of the pathways perturbed by LT and ET during infection, especially in the context of the multitude of other pathways altered during bacterial infection? Can the many immunomodulatory findings *in vitro* be correlated to relevant cellular events during anthrax infection?

Given the complexity of the human body and its response to infection, we can expect the dramatic progress in anthrax toxin research to lead to improved vaccines and therapeutics, but only if we now extend studies on pathogenesis to appropriate and relevant animal models, as has been often recommended by Harry Smith (2002), whose fundamental research on the toxins began this quest (Smith and Keppie, 1954; Smith et al., 1955).

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Chapter 9

Bacillus anthracis Virulence Gene Regulation

Jason M. Rall and Theresa M. Koehler

INTRODUCTION

Studies of *Bacillus anthracis* gene regulation have centered primarily on expression of genes associated with the most well-established virulence factors, anthrax toxin, composed of protective antigen (PA), lethal factor (LF), and edema factor (EF), and capsule, composed of poly-D-glutamic acid (PDGA). The structural genes for the toxin proteins, *pagA*, *lef*, and *cya*, are located at distinct loci on the 182-kb plasmid pXO1 (Read et al., 2003), while the capsule biosynthesis operon *capBCADE* is on the 96-kb plasmid pXO2 (Candela et al., 2005; Makino et al., 1989; Okinaka et al., 1999). Anthrax toxin is an "A-B type" toxin with unique structural and enzymatic features (Turk, 2008; Young and Collier, 2007). Two distinct catalytic proteins, LF, a zinc-dependent metalloprotease, and EF, a calmodulin-dependent adenylate cyclase, share a common binding/translocating protein, PA. The overall effect of anthrax toxin activity is subversion of the host immune system via disruption of signaling pathways.

The capsule represents the other major virulence factor of the species (Drysdale et al., 2005b). As is true for the capsules of many bacterial pathogens, the *B. anthracis* capsule has an antiphagocytic role in infection (Keppie et al., 1953; Makino et al., 1989; Ovodov, 2006; Scorpio et al., 2007), but unlike the capsules of most other bacteria, the *B. anthracis* capsule is not polysaccharide. The capsule is comprised solely of D-glutamic acid residues that are gamma-linked to form homopolymers of over 215 kDa (Bruckner et al., 1953; Record and Wallis, 1956). Proteins associated with capsule synthesis, assembly, and transport are encoded by the *cap* operon (Green et al., 1985; Makino et al., 1989; Okinaka et al., 1999). Genetic investigations of the *capBCADE* locus and biochemical analyses of the Cap proteins and homologous proteins in other *Bacillus* species have led to a model for capsule formation (Richter et al., 2009). CapB is an ATP-dependent ligase that, together with CapC, makes gamma-linked polymers of D-glutamic acid in the cytoplasm (Gardner and Troy, 1979; Kimura et al., 2004; Makino et al., 1989; Troy, 1973a,b). CapA and CapE are required for transport of the polymers across the cell membrane

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

(Ashiuchi et al., 2004, 2006; Candela and Fouet, 2005; Candela et al., 2005). CapD, also known as Dep, generates amide bonds with peptidoglycan cross bridges to anchor the capsular material into the cell envelope (Candela and Fouet, 2005; Makino et al., 2002). In addition, CapD can cleave the polymers to release low molecular weight fragments from the cell surface (Candela and Fouet, 2005; Uchida et al., 1993). These fragments have been proposed to interfere with host defense (Uchida et al., 1993). Recent reports have shown that PDGA is shed into body fluids in high concentrations during a murine model of pulmonary anthrax (Kozel et al., 2004; Sutherland and Kozel, 2009). Interestingly, PDGA has been shown to complex with lethal toxin in the bloodstream of infected animals (Ezzell et al., 2009). The impact of this association on pathogenesis is unclear, but it is likely that the role of the *B. anthracis* capsule extends beyond a simple antiphagocytic function.

A summary of current understanding of virulence gene expression by *B. anthracis* is presented here. There is a considerable body of knowledge regarding regulation of the classic virulence factors, toxin, and capsule, and recent investigations have focused on control of the regulatory genes themselves. Investigations of additional potential virulence genes are in their infancy, but studies have revealed a combination of unique features and regulatory mechanisms that mirror control of homologous genes in other species.

SIGNALS FOR VIRULENCE GENE EXPRESSION

B. anthracis is a soil-dwelling bacterium that upon entry into a mammalian host can rapidly proliferate and cause disease. The viability of the spore and vegetative forms of the bacterium in the environment appears to be relatively low (Lindeque and Turnbull, 1994), and it has been proposed that multiplication of *B. anthracis* occurs almost exclusively in the host (Turnbull, 2002). However, *B. anthracis* is not a particularly fastidious microbe. The bacterium can be cultured in a variety of defined and complex media and has been shown to germinate and establish metabolically active cell populations in the rhizosphere of grass plants (Saile and Koehler, 2006). Thus, it is likely that *B. anthracis* possesses distinct signaling pathways to control gene expression in and out of the host.

Considering its well-established pathogenicity, investigations of gene regulation in this *Bacillus* species have focused on virulence gene expression in conditions considered to be physiologically significant in the context of an infection. During growth of *B. anthracis* in batch culture, the most notable signal for induction of toxin and capsule synthesis is bicarbonate. When cells are cultured in media containing dissolved bicarbonate and incubated in 5% or greater atmospheric CO_2, transcription of the toxin genes and the capsule biosynthetic operon is elevated up to 60-fold compared to growth in air (0.03% CO_2) (Bartkus and Leppla, 1989; Drysdale et al., 2004; Green et al., 1985; Koehler et al., 1994; Sirard et al., 1994). The influence of bicarbonate/CO_2 on virulence gene expression is not related to pH or carbon utilization (Thorne, 1993) and mechanism for bicarbonate-mediated gene induction is unknown. Another host-related signal that influences virulence gene expression is temperature. Toxin gene transcription is four- to sixfold higher when cells are grown at 37°C compared to 28°C (Sirard et al., 1994). No temperature effect has been reported for capsule synthesis. It is easy to consider these signals as physiologically significant during infection of a mammalian host with a body temperature of 37°C and concentrations of 15 to 40 mM bicarbonate/CO_2 in the bloodstream (Lentner, 1981).

Other studies have examined toxin synthesis in media containing various carbohydrates. Puziss and Wright (1959) determined that when *B. anthracis* is grown in a minimal defined medium containing bicarbonate, calcium, iron, magnesium, manganese, phos-

phates, thiamine, guanine, and certain amino acids, PA synthesis requires the presence of a readily utilizable carbohydrate. They found that PA synthesis was optimal in glucose-containing medium. Independent of the growth medium, the rate of toxin gene expression by *B. anthracis* growing in batch culture is highest during the transition from exponential to stationary phase. This growth phase-associated toxin gene expression led to the determination that the transition state regulator AbrB controls toxin synthesis (Saile and Koehler, 2002) (see below). Given the increase in toxin synthesis as cultures enter stationary phase, van Schaik and coworkers (2007) investigated the role of the stringent response in toxin gene expression. The stringent response is a common bacterial response to starvation in which intracellular accumulation of the hyperphosphorylated guanosine nucleotide (p)ppGpp results in increased expression of genes associated with survival in nutrient-poor conditions. Their studies of the *B. anthracis relA* gene, which is responsible for the synthesis of (p)ppGpp (Mittenhuber, 2001), did not reveal a relationship between the stringent response and anthrax toxin gene expression.

Quorum-sensing systems, by which bacteria control gene transcription in a cell density-dependent manner, have been associated with virulence gene expression in many pathogens. In these systems, bacteria produce and release signals called autoinducers into the extracellular environment as a means of intra- and interspecies communication. The signal concentration is affected by population density. At a critical concentration, cells sense the signal and alter their gene expression to adapt to their environment. For gram-positive bacteria, small peptides commonly serve as signals in quorum-sensing systems (Parker and Sperandio, 2009). There are no reports of a peptide-based quorum-sensing system in *B. anthracis* for control of virulence gene expression. However, *B. anthracis* produces AI-2, a signaling molecule made by a variety of gram-positive and gram-negative species that has been suggested to be important for cross-species communication (Xavier and Bassler, 2003). Synthesis of AI-2 is dependent upon LuxS which cleaves *S*-ribosylhomocysteine into homocysteine, and AI-2 (Schauder et al., 2001). Jones and Blaser (2003) reported that deletion of the *B. anthracis luxS* gene results in a loss of AI-2 activity and a growth defect. They also showed that natural and synthetic AI-2 inhibitors decrease *pagA*, *cya*, and *lef* gene expression in batch culture (Jones et al., 2005). The mechanisms for the AI-2 effect on toxin gene expression and attenuation of growth are not known, but it has been proposed that the role of LuxS in *S*-adenosylmethionine metabolism may result in pleotropic effects on cell physiology and gene expression (Parker and Sperandio, 2009; Winzer et al., 2002). No studies have been published regarding the virulence of a *luxS* mutant in an animal model for anthrax.

ATXA: THE MAJOR REGULATOR OF THE VIRULENCE GENES

AtxA as a Pleotropic Regulator

Transposon mutagenesis and screening for toxin-deficient *B. anthracis* mutants led to the identification of the *atxA* ("*a*nthrax *t*o*x*in *ac*tivator") gene on pXO1 (Koehler et al., 1994; Uchida et al., 1993). Although identified initially as a positive regulator of the anthrax toxin genes, further investigations revealed that AtxA acts as a global regulator. In addition to controlling expression of the pXO1-encoded toxin genes, *pagA*, *cya*, and *lef*, AtxA affects expression of the pXO2-encoded capsule biosynthetic gene operon, *capBCADE*, and a number of other genes located on the plasmids and chromosome

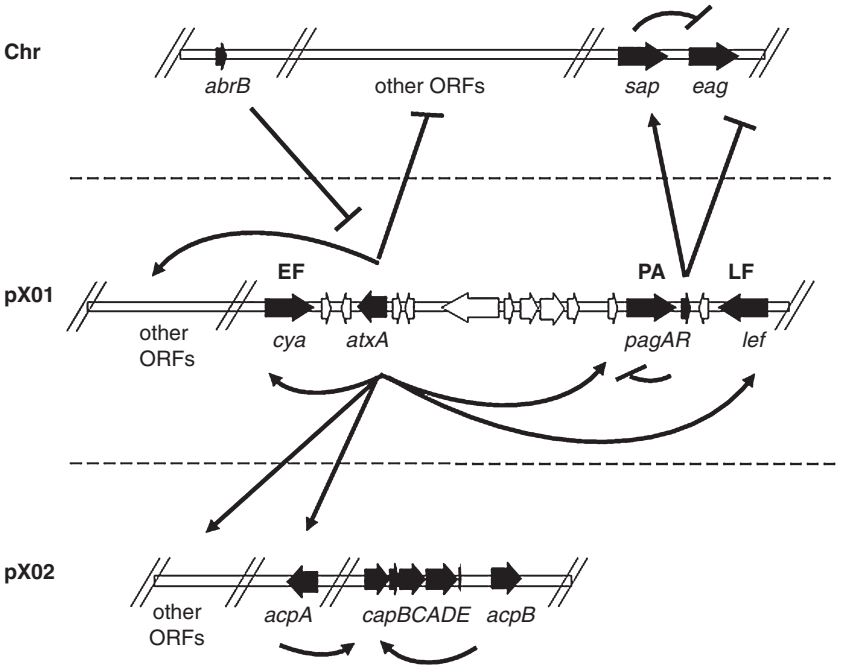

Figure 9.1 Model for plasmid–chromosome crosstalk in gene regulation.

(Bourgogne et al., 2003; Drysdale et al., 2004; Guignot et al., 1997; Hoffmaster and Koehler, 1997; Uchida et al., 1997) (Figure 9.1). The *atxA* gene has been demonstrated to be important for virulence in a murine model for anthrax (Dai et al., 1995). Mice survive infection with an *atxA*-null mutant and show a significantly decreased antibody response to the three toxin proteins, indicating that AtxA also controls toxin gene expression during infection.

Although the gene name implies that AtxA is a direct positive regulator of toxin gene transcription, the mechanism by which AtxA exerts its effects on target gene transcription is unknown. Interestingly, genes located on the plasmids are more highly regulated than those on the chromosome (Bourgogne et al., 2003). In some cases, AtxA controls gene expression via additional downstream regulatory genes such as *pagR* (cotranscribed with *pagA*), *acpA*, and *acpB* (see below).

AtxA Function

The predicted 475-amino acid sequence of AtxA indicates a soluble basic protein, with a calculated isoelectric point of 9.5. *In silico* models generated using Pfam (http://pfam.janelia.org/), Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/), and PredictProtein (http://www.predictprotein.org/) show a number of structural motifs (Figure 9.2). AtxA is predicted to contain two DNA-binding domains: an amino-terminal winged-helix (WH) motif and an adjacent helix-turn-helix (HTH) motif with similarity to that of the DNA-binding regulator Mga of *Streptococcus pyogenes* (Hondorp and McIver, 2007). Nevertheless, specific binding of the AtxA protein to the promoter regions of any of its target genes has not been demonstrated.

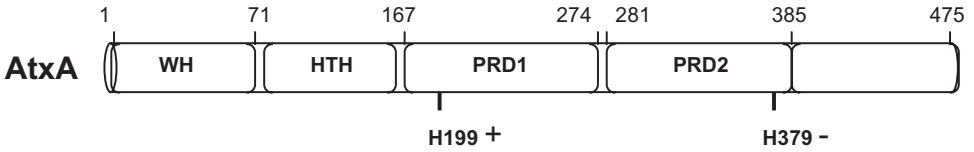

Figure 9.2 AtxA motifs. Phosphorylated histidine residues associated with positive and negative effects are indicated (Tsvetanova et al., 2007). WH, winged helix; HTH, helix-turn-helix; PRD1 and PRD2, PTS regulatory domains.

Modeling programs also reveal motifs for two internal phosphotransferase system regulation domains (PRDs). Tsvetanova and coworkers (2007) demonstrated phosphorylation of specific histidine residues within each of these. Their data indicate that phosphorylation of H199 in PRD1 positively affects AtxA activity *in vivo*, while phosphorylation of H379 negatively impacts AtxA function. Given that PRDs respond to carbohydrate availability (Stulke et al., 1998; Stulke and Hillen, 1998, 2000), the presence of PRDs in AtxA predicts an association between AtxA activity and sugar uptake in *B. anthracis*. Although some other gene regulators have known or predicted PRDs, important distinctions can be made between these proteins and AtxA. LicT, a PRD-containing protein characterized in *B. subtilis*, is an antiterminator that regulates genes related to lichenan utilization (Lindner et al., 2002). Unlike LicT, AtxA appears to affect transcription initiation, rather than termination (Dai et al., 1995). Mga, a virulence gene regulator in the human pathogen *S. pyogenes*, is a specific DNA-binding protein predicted to have two PRDs (Hondorp and McIver, 2007). Transcriptome analyses reveal that Mga directly or indirectly affects not only virulence gene transcription, but also controls expression of multiple genes and operons associated with sugar metabolism. This does not appear to be true for AtxA. Transcriptional profiling studies of *B. anthracis* indicate that the AtxA regulon includes numerous metabolic genes, but few of these have notable association with carbohydrate utilization or uptake (Bourgogne et al., 2003).

Finally, no specific function has been associated with the carboxy-terminal 90 amino acids of AtxA. This region of the protein is predicted to be composed of random coils. Given the presence of PRDs in AtxA, it is intriguing that the C-terminal region has weak similarity to a portion of the EIIB domain of the phosphotransferase system. EIIB phosphorylates incoming sugars passing through the EIIC channel. Some other PRD-containing proteins also have EIIB-like domains that contain phosphorylated histidines (Deutscher et al., 2006; Zeng and Burne, 2009).

atxA Expression

AtxA levels appear to be affected by transcriptional control of the *atxA* gene, but precise measurements of AtxA protein levels in different growth conditions or strains have not been published. Although *atxA* has been associated with bicarbonate-enhanced transcription of target genes, the steady-state level of *atxA* mRNA is increased only approximately 2.5-fold in response to bicarbonate (Drysdale et al., 2005a). The major apparent transcriptional start site (P1) is located 99 bp upstream of the translational start site (Dai et al., 1995), and a second site (P2) is in the center of a divergently transcribed gene, 749 bp upstream of the translational start codon of *atxA* (Bongiorni et al., 2008). Each of these sites is downstream from a consensus sequence for recognition by the housekeeping sigma factor SigA of RNA polymerase (Bongiorni et al., 2008; Dai et al., 1995). Studies of *atxA*

transcription have yielded conflicting results regarding transcription of the gene by specific sigma factors of RNA polymerase. One study indicates that *atxA* transcription is dependent upon the alternative sigma factor SigH (Hadjifrangiskou et al., 2007), but another report shows no affect of SigH on *atxA* transcription (Bongiorni et al., 2008). Since different strains were employed in these investigations, the SigH effect on *atxA* may be strain-dependent.

Although a number of factors have been reported to affect expression of the *atxA* gene, the only regulator that has been shown to interact directly with promoter control sequences is AbrB. The *B. anthracis* AbrB protein is homologous to a transition state regulator that has been well-studied in *B. subtilis* (Banse et al., 2008; Sullivan et al., 2008). In *B. subtilis*, AbrB is associated with growth phase-dependent gene expression. The regulator represses or activates multiple genes as cultures transition from lag to exponential growth phase and again from exponential to stationary phase (O'Reilly and Devine, 1997; Phillips and Strauch, 2002). Results of footprinting experiments indicate that AbrB binds to sequences of the *atxA* promoter that overlap the -35 region of the putative SigA recognition sequence for P1 (Strauch et al., 2005). Thus, AbrB likely competes with RNA polymerase for promoter binding. During growth in batch culture, AbrB represses transcription from the P1 promoter, exerting the greatest effect during exponential growth. A *B. anthracis abrB*-null mutant expresses *atxA* and the toxin genes earlier and at higher levels than an isogenic parent strain (Saile and Koehler, 2002).

Promoter Analysis of AtxA-Controlled Toxin Genes

Transcriptional analyses of the most highly AtxA-regulated genes, the toxin genes, *pagA*, *lef*, and *cya*, have revealed apparent transcriptional start sites and *cis*-acting sequences required for AtxA-mediated expression. The *lef* and *cya* genes appear to have single transcriptional start sites, with 5′ ends mapping 29 and 62 from the translational start codons, respectively (Dai et al., 1995). Two apparent start sites have been mapped for the bicistronic *pagAR* operon. The major transcriptional start site of *pagAR* (P1) is located 58 bp upstream of the translational start codon while the minor site (P2) is 26 bp upstream (Koehler et al., 1994). Transcripts mapping to P1 are dependent upon the presence of *atxA* and are elevated when cells are cultured in bicarbonate. The steady-state level of transcripts mapping to P2 is relatively low and unaffected by *atxA* or bicarbonate. The minimal functional control regions for the *pagAR*, *lef*, and *cya* promoters appear to be of different lengths (168 bp for *pag*, 151 bp for *lef*, and 137 bp for *cya*), and sequence similarities are not apparent. *In silico* modeling and *in vitro* gel mobility experiments indicate significant curvature associated with double-stranded DNA corresponding to the toxin gene promoter regions, suggesting that the structural topology of promoter DNA may play a role in AtxA-mediated control of gene expression (Hadjifrangiskou and Koehler, 2008).

Control of Capsule Synthesis by AtxA

In strains harboring both virulence plasmids, *atxA* controls *capBCADE* expression indirectly, via positive regulation of the pXO2 genes *acpA* and *acpB* (Figure 9.3). As is true for AtxA, the molecular function(s) of AcpA ("*a*nthrax *cap*sule *a*ctivator") and AcpB are unknown, and there is no evidence for direct binding of these regulators to target promoter DNA. Nevertheless, studies of the capsule phenotypes of *atxA*-, *acpA*-, and *acpB*-null mutants and the expression patterns of the regulatory genes with respect to the bicarbonate

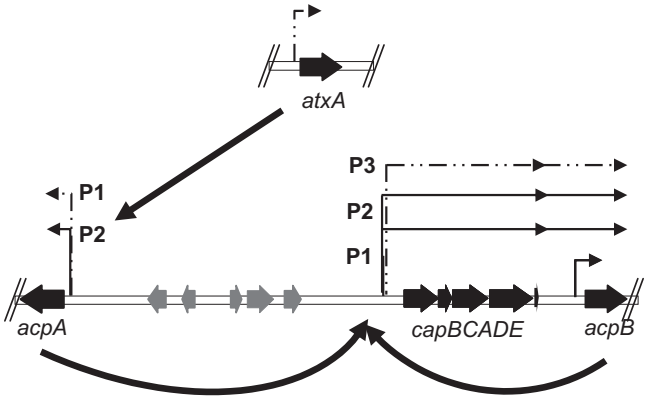

Figure 9.3 Model for regulation of the biosynthetic operon for the capsule. Apparent transcriptional start sites and associated transcripts are indicated by their arrows with solid lines (*atxAlacpAlacpB*-regulated) or hatched lines (relatively low constitutive expression).

signal have elicited relationships between the regulators and this important cue (Drysdale et al., 2004, 2005a, Guignot et al., 1997; Uchida et al., 1997; Vietri et al., 1995). While *atxA* transcription is not affected appreciably during growth in elevated bicarbonate, expression of *capB* is induced 50- to 400-fold in response to the signal (Drysdale et al., 2005a). AcpA and AcpB appear to be limiting for *cap* operon expression. The *acpA* gene has two apparent transcription start sites; one is *atxA*-dependent and is activated approximately 20-fold during growth in elevated bicarbonate. The *acpB* gene has its own constitutive promoter, but transcriptional read-through from the *cap* operon upstream of *acpB* increases transcript levels by nearly 60-fold when cells are grown in elevated bicarbonate. Thus *acpB* positively regulates *capBCADE* expression via a positive feedback loop (Drysdale et al., 2005a). Low-level expression of *acpA* and *acpB* via their constitutive promoters allows some transcription of the *cap* operon, and even in the absence of *atxA* capsule, synthesis can be induced by the bicarbonate signal (Green et al., 1985; Vietri et al., 1995). In agreement with this model, an *atxA*-null mutant is significantly, but not completely, impaired for capsule synthesis. A double mutant, deleted for *acpA* and *acpB* produces no detectable capsule, but the presence of either *acpA* or *acpB* is sufficient for capsule synthesis that is comparable to the parent strain (Drysdale et al., 2004).

The complex control of *cap* operon expression is more intriguing considering studies of capsule synthesis during infection. The *cap* operon is required for virulence in a murine model for inhalation anthrax (Drysdale et al., 2005b). The LD_{50} of a parent strain harboring pXO1 and pXO2 is approximately 10^3 spores. A strain deleted for the *cap* operon does not cause disease at doses up to 5×10^7 spores, and an *acpAacpB* double mutant is equally attenuated. Deletion of *acpA* alone does not result in attenuation, in agreement with the ability of this mutant to produce capsule in culture. However, an *acpB*-null mutant that produces capsule comparable to the parent strain when cultured in elevated bicarbonate is moderately attenuated in the animal model, exhibiting a 100-fold increase in LD_{50}. These data suggest that the positive feedback loop for *cap* operon expression is critical for capsule synthesis during infection, and/or that *acpB* has additional function(s) *in vivo*.

Given that the predicted amino acid sequences of AcpA and AcpB are 62% similar and 39% identical, the partial functional similarity of the two *cap* operon regulators is not

surprising. AcpA and AcpB are also closely related to AtxA, having 47% and 50% overall similarity, respectively (Drysdale et al., 2004). Uchida and coworkers (1997) reported a positive effect of *atxA* on *capB* transcription when the two genes were cloned on separate vectors in a pXO1$^-$ pXO2$^-$ strain, providing evidence that *atxA* can control *cap* operon expression in the absence of *acpA* and *acpB*. Thus, there is functional overlap among all three regulators. It has been proposed that the mechanism for *cap* operon regulation is dependent upon the precise stoichiometry of AtxA, AcpA, and AcpB (Drysdale et al., 2004).

The PagR Repressor

The *pagR* gene of the *atxA*-regulated *pagAR* operon encodes a repressor that is responsible for autogenous control of the operon (Hoffmaster and Koehler, 1999). A weak transcription terminator lies between the *pagA* and *pagR* coding sequences. Attenuation of transcription at this site results in expression of *pagR* and ultimately, decreased levels of PA. PagR is an 11-kD protein that is predicted to have a winged helix topology and to be a member of the ARSR family of transcriptional regulators. PagR appears to be a weak repressor, affecting gene expression only 2- to 7-fold (Hoffmaster and Koehler, 1999; Mignot et al., 2003). Mignot and coworkers (2003) demonstrated that PagR protein not only controls *pagAR*, but also represses the chromosomal genes *sap* and *eag* that encode S-layer proteins. Sap and EA1 are nonessential proteins that form a lattice between the cell wall and the capsule. Although footprinting analyses indicate direct interaction between recombinant PagR and DNA in the *pagAR*, *sap*, and *eag* promoter regions, a consensus sequence for PagR binding is not apparent.

A CONVERGENCE OF COMPLEX REGULATORY SYSTEMS

The link between *atxA* expression and growth phase regulation was the first reported example of the interplay between expression of plasmid-encoded virulence factors and chromosome-encoded proteins associated with basic bacterial physiology (Saile and Koehler, 2002). Recent reports have provided more examples of the interplay between virulence and metabolism. The integration of *B. anthracis* virulence gene control with key metabolic processes is of particular significance for a pathogen in which cell development is tied to the establishment and transmission of disease.

Cell Development

In vivo development (conversion from spores to multiplying vegetative cells) is critical for *B. anthracis* pathogenesis, while *in vitro* development (conversion from vegetative cells to spores) impacts survival of the pathogen outside of the host. Ultimately, the spore-vegetative cell-spore cycle permits transmission to new hosts. There are relatively few published studies regarding *B. anthracis* development, but development of the nonpathogenic *Bacillus* species *Bacillus subtilis* has been well studied (Errington, 2003; Piggot and Hilbert, 2004; Setlow, 2003). In response to certain environmental stresses, including nutrient limitation, *B. subtilis* spores undergo a complex and tightly controlled genetic program resulting in a transition from vegetative cells to spores. Germination occurs upon reintroduction of adequate nutrients and growth conditions. Sensors present

within the spores detect and ultimately transduce the signals, setting off a regulatory cascade that culminates in formation of metabolically active vegetative cells.

Regulation of *atxA* by AbrB represents a direct link between cell development and virulence gene expression. As is true for its homologue in *B. subtilis*, the *B. anthracis abrB* gene is controlled by the response regulator Spo0A (Saile and Koehler, 2002). AbrB and Spo0A are part of a complex regulatory network governing growth phase-related processes that are well established in *B. subtilis*. The master response regulator Spo0A is activated by phosphorylation via a complex signal transduction phosphorelay system (Piggot and Hilbert, 2004). Phosphorylation of Spo0A involves a large number of checkpoints, at which environmental signals are received and interpreted to ensure that sporulation initiation is the optimal means for survival (Hoch, 1993a,b,c; Perego et al., 1996). In batch culture, such signals include cell density and nutrient deprivation (Piggot and Hilbert, 2004), GTP levels within the cell (Sonenshein, 2000; Vidwans et al., 1995), and phosphatase-repressing peptides (Perego, 1998; Perego and Hoch, 1996a,b; Perego et al., 1996). As nutrient levels decrease, the flow of phosphate toward Spo0A increases steadily, leading to gradual activation of Spo0A (Sonenshein, 2000). Among the many roles for phosphorylated Spo0A (Spo0A~P) in gene expression, Spo0A~P represses *abrB*. In a *spo0A*-null mutant of *B. anthracis*, increased AbrB levels result in decreased expression of *atxA* and ultimately reduced toxin synthesis (Saile and Koehler, 2002).

Interesting relationships between *B. anthracis* virulence and sporulation have been revealed in other studies. At the top of the *B. subtilis* sporulation cascade lie five sporulation sensor histidine kinases, KinA-E. In response to appropriate signals, the kinases phosphorylate Spo0F, which transfers the phosphate to Spo0B. Spo0B, in turn, transfers the phosphate to Spo0A, which then activates transcription of critical sporulation genes (Piggot and Hilbert, 2004). Genetic and biochemical analysis of *B. anthracis* sporulation kinase genes indicate that two of the nine kinases identified contain frameshifts in all *B. anthracis* strains investigated (Brunsing et al., 2005). In a pathogenic *Bacillus cereus* strain that harbors the *B. anthracis* toxin plasmid pXO1, a kinase gene also contains a frameshift. These data suggest that acquisition of the virulence genes, via attainment of pXO1, is associated with loss of sporulation sensor histidine kinase activities. Moreover, pXO1 and pXO2 each encode proteins that are highly homologous to the signal sensor domain of a major sensor histidine kinase, GBAA2291, encoded by a *B. anthracis* chromosome gene. Overexpression of the plasmid-encoded proteins in a recombinant *B. subtilis* strain harboring GBAA2291 results in kinase-dependent inhibition of sporulation. It has been proposed that in its native host, the *B. anthracis* kinase is converted to an inhibitor of sporulation by the plasmid-encoded sensor domains (White et al., 2006).

Respiration

Two groups have published investigations implicating control of *atxA* expression by proteins encoded by the *resABCDE* operon. ResD, a response regulator, and ResE, a kinase, comprise a two-component system controlling multiple components of aerobic and anaerobic respiration in *Bacillus* species (Geng et al., 2007; Sun et al., 1996). The ResA, ResB, and ResC proteins form a complex that is associated with cytochrome c biogenesis. Vetter and Schlievert (2007) reported that ResDE controls transcription of the anthrax toxin genes and *atxA*. However, results of a similar study by Wilson and coworkers (2008) indicated that the system has no affect on virulence gene expression. This group showed that *B. anthracis resD* mutants have a propensity to accumulate compensatory mutations and

suggested that such mutations may have impacted the experiments performed by Vetter and Schlievert (2007). Interestingly, in a subsequent study, Wilson and coworkers (2009) screened a transposon library for mutants expressing *pagA* in growth conditions not conducive for toxin synthesis. Increased transcription of *pagA* was associated with enhanced *atxA* expression in mutants harboring insertions in genes encoding two heme-dependent small c-type cytochromes, CccA and CccB. Other mutants exhibiting increased *atxA* and toxin gene expression carried insertions in genes predicted to encode other components of the cytochrome c biogenesis pathway. These included HemL, ResB, and ResC. These intriguing results link cellular redox state and cytochrome level with virulence gene regulation.

DIFFERENTIAL GENE REGULATION IN THE *B. CEREUS* GROUP SPECIES AND PLASMID–CHROMOSOME CROSSTALK

The integration of virulence gene regulation and control of key metabolic processes is interesting from an evolutionary standpoint, especially given the "crosstalk" between the *B. anthracis* chromosome and plasmids. Plasmid content and chromosomal gene regulation differentiate *B. anthracis* from its nearest neighbors. *B. anthracis* is a member of the "group 1 bacilli" also known as the "*B. cereus* group" (Ash and Collins, 1992; Ash et al., 1991). The major species of this group, *B. anthracis*, *Bacillus thuringiensis*, and *B. cereus*, exhibit similar cell structure, physiology, and natural genetic exchange systems, but they are distinct with regard to pathogenicity. While *B. anthracis* is the causative agent of anthrax, *B. thuringiensis* is considered primarily to be a pathogen of *Lepidoptera* (caterpillars), and some *B. cereus* strains produce toxins associated with mild food poisoning. *B. thuringiensis* and *B. cereus* can also cause opportunistic infections in humans (Colpin et al., 1981; Craig et al., 1974; Damgaard et al., 1997; Hernandez et al., 1998; Mahler et al., 1997). Major differences in pathogenicity among the group 1 species are attributed primarily to differences in plasmid content. *B. anthracis* is distinguished by its virulence plasmids pXO1 (anthrax toxin) and pXO2 (capsule) (Candela et al., 2005; Koehler, 2002; Makino et al., 1989; Okinaka et al., 1999). Large self-transmissible plasmids associated with *B. thuringiensis* usually encode proteins toxic for insects (Gonzalez et al., 1982; Schnepf et al., 1998). Strains of *B. cereus* harbor a variety of extra-chromosomal elements not found in the other species. However, there are reports of rare *B. cereus* and *B. thuringiensis* strains harboring plasmids with similarity to pXO1 and pXO2 (Hoffmaster et al., 2004; Klee et al., 2006; Rasko et al., 2007). Some of these unusual strains have been isolated from humans and animals that succumbed to an anthrax-like disease (Avashia et al., 2007; Hoffmaster et al., 2004).

The PlcR Regulon

The chromosomes of *B. anthracis*, *B. thuringiensis*, and *B. cereus* reveal striking sequence similarity and gene synteny (Rasko et al., 2005), but even plasmid-cured strains of the three species exhibit species-specific phenotypes. In part, the phenotypic differences between the species can be attributed to altered gene regulation rather than the gain or loss of genes. The most well-known example of this is the PlcR regulon. First characterized as a "*p*hospho*l*ipase *C* gene *r*egulator" in *B. thuringiensis* (Lereclus et al., 1996), PlcR is now known to control expression of multiple genes in *B. cereus* and *B. thuringiensis*, some of which are associated with pathogenesis (Agaisse et al., 1999; Gohar et al., 2002,

2008; Lereclus et al., 1996). Although homologues of many of PlcR-regulated genes are present in the *B. anthracis* genome, the *B. anthracis plcR* gene contains a nonsense mutation resulting in a nonfunctional protein product. Multiple phenotypic differences between *B. anthracis* and the other two species are attributed to low-level expression of the PlcR regulon in *B. anthracis* (Mignot et al., 2001). Interestingly, expression of the *B. thuringiensis plcR* allowed for the expression of several of these genes (Mignot et al., 2001).

The AtxA Regulon

The AtxA regulon includes *B. anthracis*-specific virulence genes located on pXO1 and pXO2 and some chromosomal genes common to the *B. cereus* group (Bourgogne et al., 2003; Hoffmaster and Koehler, 1997; Sirard et al., 2000). Most *B. cereus* and *B. thuringiensis* strains do not carry the plasmid harboring *atxA* and therefore exhibit differential expression of the AtxA-controlled chromosomal genes. Interestingly, expression of a *B. thuringiensis plcR* gene in a *B. anthracis* strain containing *atxA* resulted in a significant decrease in sporulation, a phenotype that was rescued by deletion of *atxA* (Mignot et al., 2001). These results suggest that the *plcR* and *atxA* regulons in *B. anthracis* are not compatible and that the nonsense mutation within the *B. anthracis plcR* provided a selective advantage for evolution of the species.

β-Lactamase Genes

β-lactamase synthesis is another example of differential gene expression among these species. Susceptibility to β-lactam antibiotics such as penicillin is commonly used to distinguish *B. anthracis* from *B. cereus* and *B. thuringiensis,* which typically exhibit inducible penicillin resistance (Bernhard et al., 1978; Bernstein et al., 1967; Turnbull et al., 2004). Interestingly, all sequenced strains of group 1 species contain β-lactamase (*bla*) genes. β-lactamase expression in all three species is associated with an extracytoplasmic function (ECF) sigma factor, SigP, and its cognate anti-sigma factor Rsp (Chen et al., 2003, 2004; Materon et al., 2003; Ross et al., 2009). The *sigP* and *rsp* genes are required for the inducible penicillin resistance that is common to *B. cereus* and *B. thuringiensis* and for the constitutive penicillin resistance of a rare naturally occurring penicillin-resistant clinical *B. anthracis* isolate. Deletion of the *sigP/rsp* locus abolishes *bla* expression in the penicillin-resistant *B. anthracis* strain and has no effect on *bla* expression in a prototypical penicillin-susceptible strain. Complementation of the mutants with *sigP/rsp* from the penicillin-resistant strain, but not the penicillin-susceptible strain, confers β-lactamase activity upon both mutants. These results are attributed to a nucleotide deletion near the 5′ end of *rsp* in the penicillin-resistant strain that is predicted to result in a nonfunctional protein. Moreover, expression of the *B. cereus* or *B. thuringiensis sigP* and *rsp* genes in a *B. anthracis sigP/rsp*-null mutant confers resistance to β-lactam antibiotics, suggesting that while *B. anthracis* contains the genes necessary for sensing β-lactam antibiotics, the *B. anthracis sigP/rsp* gene products are not sufficient for *bla* induction (Ross et al., 2009).

REGULATION OF OTHER VIRULENCE-ASSOCIATED GENES

In recent years, a number of less well-studied factors have been proposed to be associated with *B. anthracis* virulence. These include proteins predicted to have hemolytic activity,

several secreted proteases (Chung et al., 2006; Delvecchio et al., 2006; Huang et al., 2004; Kudva et al., 2005), iron acquisition systems (Abergel et al., 2006; Cendrowski et al., 2004; Gat et al., 2008; Maresso et al., 2006, 2008; Pfleger et al., 2008; Skaar et al., 2006), and various surface-associated proteins of the vegetative cell wall and exosporium (Ariel et al., 2002, 2003; Gat et al., 2006; Steichen et al., 2003). Although knowledge of the regulation of genes associated with such factors is relatively sparse, investigations related to control of these genes are increasing.

Anthrolysin O (ALO)

ALO is a secreted pore-forming toxin that is a member of the cholesterol-dependent cytolysin (CDC) family (Gilbert, 2002). These proteins bind cholesterol and form pores in the cell membrane leading, among other things, to influx of calcium from outside the cell and release of calcium from intracellular stores such as the endoplasmic reticulum (Gekara et al., 2007). ALO and its *B. cereus* homologue, cereolysin, have hemolytic activity *in vitro* (Bernheimer and Grushoff, 1967; Shannon et al., 2003). The ALO homolog of *S. pyogenes*, streptolysin O, has been shown to be necessary for full virulence in mouse model of infection (Limbago et al., 2000). The precise role of ALO in *B. anthracis* virulence is unclear. An *alo*-null mutant is fully virulent in an intratracheal mouse model for anthrax, but mutants lacking the *alo* gene in combination with the absence of certain phospholipase (PLC) genes are attenuated (Heffernan et al., 2007). Purified ALO kills human neutrophils, monocytes, and macrophages and disrupts barrier function in cultured gut epithelial cells (Bourdeau et al., 2009; Mosser and Rest, 2006). Additionally, antibodies raised against purified ALO have been shown to protect against *B. anthracis* intravenous challenges (Nakouzi et al., 2008).

Regulation of *B. anthracis alo* expression is poorly understood. A canonical PlcR recognition site is present 5′ of the *alo* coding sequence on the *B. anthracis* chromosome. Yet, unlike the homologous gene in *B. cereus*, *B. anthracis alo* expression is not regulated by PlcR due to the nonsense mutation in the *B. anthracis plcR* gene (Mignot et al., 2001; Ross and Koehler, 2006). In batch culture, *B. anthracis alo* expression appears to be highest in rich media and ALO is produced in a growth-phase dependent manner, with highest expression during mid-exponential phase growth (Shannon et al., 2003).

Phospholipases

Phospholipases produced by a number of bacterial pathogens act by cleaving host cell membranes to release second messengers (Schmiel and Miller, 1999). *B. anthracis* secretes three phospholipases: PlcA (phosphatidylinositol-specific PLC), PlcB (phosphatidylcholine PLC), and SmcA (sphingomyelinase). Studies employing an intratracheal model of inhalation anthrax in mice showed that the PLCs were functionally redundant: only the triple mutant lacking *plcA*, *plcB*, and *smcA* exhibited attenuated virulence in a murine inhalation anthrax model (Heffernan et al., 2006, 2007). Little is known regarding control of PLC gene expression in *B. anthracis*. The genes are most highly expressed in cultures grown in anaerobic conditions (Klichko et al., 2003). Homologous PLC-encoding genes of *B. cereus* are regulated by PlcR (Gohar et al., 2008). However, because the *B. anthracis* PlcR is nonfunctional, the *B. anthracis* genes are not subject to this control (Mignot et al., 2001).

Proteases

Zymogen profiles and proteomic analyses of *B. anthracis* culture supernates have revealed at least 16 secreted proteases in addition to LF (Aronson et al., 2005; Chitlaru et al., 2006). Two proteases, Npr599 (neutral protease) and InhA1 (immune inhibitor A1), which have homologues in other *B. cereus* group species, comprise over 90% of the secretome when *B. anthracis* is cultured in NBY (nutrient broth with yeast) medium in air, a condition nonconducive for toxin synthesis (Chitlaru et al., 2006). Notably, when cells are cultured in NBY with elevated CO_2/bicarbonate, Npr599 is not present in the culture supernate and InhA1 levels are decreased. InhA1 has been reported to process a number of host proteins, including several proteins that mediate thrombosis in the host (Chung et al., 2006, 2008, 2009; Kastrup et al., 2008), but there are no published studies of the relative virulence of *B. anthracis* protease mutants in an animal model. If InhA and/or Npr599 contribute to successful *B. anthracis* infection, the negative effect of the bicarbonate signal may indicate a difference in spatial and/or temporal expression of these proteases compared to the anthrax toxin proteins *in vivo*.

Although there is little published information regarding transcriptional control of *inhA1* or the other *B. anthracis* protease genes, one report describes regulation of the *B. thuringiensis inhA1* homologue (Grandvalet et al., 2001). In this closely-related species, *inhA1* expression is repressed by the transcriptional regulators AbrB and SinR. Since *B. anthracis* has homologues to these regulators, it is possible that *inhA1* transcription in *B. anthracis* is controlled in a similar fashion.

Iron Scavengers

Considering that *B. anthracis*, as well as most other bacterial pathogens, requires iron for growth and successful infection of mammalian hosts, microbial factors that facilitate iron acquisition are considered to be virulence factors (Crosa et al., 2004). *B. anthracis* has multiple means of obtaining iron that is tightly bound to carrier and storage proteins in host tissues. In response to low-iron availability, *B. anthracis* secretes the siderophores bacillibactin and petrobactin (Cendrowski et al., 2004; Koppisch et al., 2005, 2008). These ferric iron chelators bind iron with very high affinity and transport it into the bacterium via substrate-binding proteins (SBPs) and other ABC transporter components. Bacillibactin is produced by nearly all *Bacillus* species and is not required for virulence, while petrobactin, which is produced by many prokaryotes, is necessary for *B. anthracis* virulence in a mouse model of infection (Abergel et al., 2006; Cendrowski et al., 2004; Pfleger et al., 2008). The proteins required for bacillibactin and petrobactin synthesis are encoded by genes of the *bacACEBF* and *asbABCDEF* operons, respectively. Transcription of the operons is elevated during growth of the bacterium in low-iron conditions, but the mechanism for transcriptional control is not known. In other bacteria, transcriptional responses to iron levels are commonly mediated by Fur, a regulator whose DNA-binding activity is positively regulated by iron (Baichoo et al., 2002; Escolar et al., 1999). The promoter regions of the *B. anthracis* siderophore operons contain consensus sequences for Fur binding, so it is likely that the *B. anthracis* Fur homologue is a regulator of *bacACEBF* and *asbABCDEF* expression (Cendrowski et al., 2004).

B. anthracis can also scavenge iron from hemoglobin via iron-regulated surface determinant (Isd) proteins (Gat et al., 2008; Maresso et al., 2006, 2008; Skaar et al., 2006). Homologues of *isd* genes are present in all *B. cereus* group members as well as

Staphylococcus aureus. The Isd proteins facilitate the scavenging of iron from heme and heme-containing proteins, including hemoglobin. *B. anthracis* produces three proteins that bind heme, IsdX1 (also known as IsdJ), IsdX2 (also known as IsdK), and IsdC (Maresso et al., 2006, 2008). The proteins contain NEAT (*nea*r iron transporter) domains that are associated with binding to iron-containing ligands (Andrade et al., 2002). Unlike the heme-binding Isd proteins of *S. aureus*, which are all located on the bacterial cell surface, only IsdC of *B. anthracis* is bound covalently to the cell envelope. IsdX1 and IsdX2 are found in cell-free culture supernates. IsdC is anchored to the cell by the transpeptidase SrtB. IsdE1, IsdE2 (IsdF), and IsdF (IsdX) transport the iron-containing complexes into the cell. Ultimately, iron is freed from heme by the monooxygenase IsdG (Skaar et al., 2006).

The Isd and associated proteins are encoded by the 7-gene operon, *isdCX1X2E1E2F-srtB*, and the monocistronic *isdG* gene. As is true for the siderophore operons, the *isd* locus appears to be controlled by Fur because there are four apparent Fur-binding sites in the promoter region of the *isd* operon and two Fur-binding sites in the *isdG* promoter region (Gat et al., 2008). Transcript analysis has revealed two mRNAs associated with the operon, a long transcript corresponding to the entire operon and a shorter transcript mapping to *isdCX1X2* only. Consistent with the presence of the Fur boxes, IsdX2 levels are highest in low-iron conditions and decrease with increasing levels of iron. Interestingly, *isdX2* expression during growth in minimal media is highest during late exponential and stationary growth phases but is repressed by elevated bicarbonate. Although the Isd iron scavenging system of *B. anthracis* clearly functions in acquisition of heme during *in vitro* growth, an *isdCX1X2* mutant is not affected for virulence in a guinea pig model for anthrax (Gat et al., 2008).

Reactive Oxygen Species (ROS)

ROS such as hydrogen peroxide, nitric oxide (NO), and superoxide are part of the mammalian defense system against invading pathogens (Lambeth, 2004). These molecules lead to oxidative damage to DNA, and lipid peroxidation and bacterial factors that ward off the affects of ROS can be critical for *in vivo* survival of the pathogen. *B. anthracis* has a full complement of enzymes designed to protect itself, including catalase, peroxidases, and superoxide dismutase (SOD).

Superoxide is a key player in macrophage defense. Generation of superoxide occurs nearly immediately upon phagocytosis of pathogens. *B. anthracis* has four SODs that convert the harmful superoxide molecule into hydrogen peroxide and oxygen (Passalacqua et al., 2006). The SODs have overlapping function and a quadruple knockout is attenuated in an intranasal mouse model of infection (Cybulski et al., 2009). Regulation of the SOD genes, *sodA1*, *sodA2*, *sodC*, and *sod15*, is not well understood, but the genes appear to be controlled independently. During growth in batch culture in synthetic MG (modified G) medium, expression of *sod15* increases during late exponential and stationary growth phases, while *sodA1*, *sodA2*, and *sodC* expression remains constant (Passalacqua et al., 2006). Expression of the *sodA2* gene is higher in a macrophage model compared to expression in culture (Bergman et al., 2006). Interestingly, a *sodA1* mutant is more susceptible to iron limitation, indicating a connection between iron acquisition and response to ROS (Passalacqua et al., 2007). Finally, although other *B. anthracis* enzymes, including bacillus nitrogen oxide synthase, have been implicated in virulence (Shatalin et al., 2008), there is little information regarding regulation of the genes encoding these proteins.

S-Layer Proteins

Recent publications have shown that vegetative cells of *B. anthracis* can attach to and enter non-phagocytic cells (Kern and Schneewind, 2008; Russell et al., 2007, 2008). Genome analyses and biochemical studies have revealed a number of known and potential surface-associated, or S-layer type proteins of *B. anthracis*. One of these, BslA, has been reported to mediate binding of *B. anthracis* cells to fibroblasts (Kern and Schneewind, 2008). Interestingly, in these studies, adherence required bicarbonate or fetal bovine serum, and transcriptional profiling experiments have revealed that the *bslA* gene (pXO1-90) is positively regulated over 60-fold by AtxA (Bourgogne et al., 2003).

The well-studied S-layer proteins Eag and Sap have not been reported to mediate adherence, but as indicated above, control of the *eag* and *sap* genes is linked to *atxA* via *pagR* (Mignot et al., 2002). PagR is not the only *trans*-acting factor affecting expression of the S-layer genes. The *sap* and *eag* genes are also under growth phase control (Mignot et al., 2002). During batch culture, Sap is produced during exponential growth. As the culture transitions to stationary phase, EA1 is synthesized and *sap* expression decreases. In part, this expression pattern is likely due to recognition of the *sap* promoter by SigA, the housekeeping sigma factor for RNA polymerase, and recognition of the *eag* promoter by SigH, a sigma factor associated with post-exponential phase processes. In addition to their structural role in the *B. anthracis* envelop, Sap and EA1 appear to have intracellular function. The crystallization domains of both proteins can bind directly to the *eag* promoter, and deletion of either gene leads to increased transcription of *eag*, indicating that the proteins have repressor activity (Mignot et al., 2002).

CONCLUSION

Models for regulation of virulence gene expression are most well-established for the major virulence factors of *B. anthracis*, the anthrax toxin and capsule, and are less well investigated for newly proposed factors. Advanced methods for genetic manipulation of the species along with continued genomic and proteomic analyses (Ariel et al., 2003; Bergman et al., 2007; Bourgogne et al., 2003; Chitlaru et al., 2006, 2007; Passalacqua et al., 2009; Read et al., 2003) are likely to facilitate further testing of these models and advance understanding of expression of newly proposed virulence genes. Current research efforts are focused on signal transduction mechanisms affecting virulence gene expression, the interplay between the overall metabolic state of the bacterium and synthesis of virulence factors, and species-specific control of common virulence genes.

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Chapter 10

The Interactions between *Bacillus anthracis* and Macrophages

Susan L. Welkos, Joel A. Bozue, and Christopher K. Cote

The mononuclear phagocytic cells known as macrophages are multifunctional cells of the host immune system with essential roles in the establishment of and response to infection by *Bacillus anthracis*. These cells have been implicated as being important participants in both the successful establishment of lethal infection by *B. anthracis* and in the host innate defense against anthrax. In addition, they have also been the basis of *in vitro* correlate assays of immunity and of models for studies on pathogenetic and host response mechanisms. This review attempts to summarize important findings in the literature associated with the complex topic of *B. anthracis*–macrophage interactions. A complete compilation and review of all the primary literature is beyond the scope of this chapter, and any failure to cite significant sources is unintended.

THE FACILITATION OF SPORE INFECTION BY MACROPHAGES: DEVELOPMENT OF A MODEL

Role of Macrophages as Facilitators of Spore Trafficking and Germination

The major focus of the literature on anthrax has been inhalational anthrax, the most dangerous and lethal form of the disease. Unlike most infections acquired by the pulmonary route, inhalational anthrax is unusual in that inhaled spores normally do not germinate to a significant extent into vegetative bacilli and cause disease initially in the lungs; and thus, there is no primary anthrax pneumonia (possible exceptions will be discussed later). Albeit still a subject of keen debate, according to a widely recognized scenario, the spores appear to be quickly and efficiently phagocytosed by host alveolar mononuclear phagocytes. These phagocytes carry them out of the lungs and to the regional lymph nodes, where the spores germinate into vegetative bacilli. Upon entering the circulation, the bacilli multiply

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

and cause terminal disease. This model incorporates the intensive investigatory efforts by a number of previous researchers (reviewed below), and their efforts established a paradigm that has been described and elaborated upon recently (Dixon et al., 2000; Guidi-Rontani, 2002; Guidi-Rontani and Mock, 2002; Guidi-Rontani et al., 1999b, 2001; Hanna and Ireland, 1999).

Some of the earliest infectious disease microbiologists reported perplexing observations in animals challenged with *B. anthracis* spores by the pulmonary route. Researchers challenged various small laboratory animals (guinea pigs, rabbits, or mice) with concentrated aerosols of spores but were unable to demonstrate the place in the lung from which overt infection ensued. Buchner, and later Ferguson (unpublished data) and Young, attempted to identify the site of spore germination in the lungs of animals exposed to lethal doses but found only a few foci of bacilli in the alveoli and no phagocytes with spores (Buchner et al., 1888; Ross, 1957; Young et al., 1946). Barnes articulated the hypothesis that the lung did not serve as the initial site of infection of inhalational anthrax but instead as a conduit for establishing systemic infection from a different site. Noncellular defenses disposed of a large proportion of the spores which could be recovered from the stomach and presumably arrived there by swallowing or mucocilliary movement (Barnes, 1947). Of the spores remaining in the lungs, about half disappeared within 24 h but could not be recovered from the blood, and their fate was unclear. The conundrum observed by Barnes was explained in an elegant histopathology study by Ross, who explored in detail the course of respiratory exposure to *B. anthracis* spores in guinea pig models (Ross, 1957). Using animals exposed either intratracheally or by inhalation to a fully virulent strain of *B. anthracis*, the following events were observed: many of the spores reaching the bronchi were presumed to leave upper air passages by coughing or swallowing. Spores reaching the alveoli were found in fixed and free alveolar phagocytes within 1 h. Thereafter, spore-infected cells were often observed in the lymphatics leading from lungs to the tracheobronchial lymph nodes after intratracheal challenge, whereas after an aerosol dose, infected phagocytes were only seen near the lymphatic entrance leading to the peribronchial lymph nodes. Intraphagocyte spore germination began in some cases before or during transit. The reduced numbers of spores reaching the lymph nodes might be partly accounted for by macrophage bactericidal activity (Barnes, 1947; Ross, 1957). Spores were found in the regional lymph nodes about 4 h after both intratracheal and aerosol administration. After intratracheal exposure, germinated spores were numerous in lymph nodes at 18 h, and by 24 h, freely multiplying bacilli were seen. Germination was slower after aerosol exposure, but by 24 h, bacilli were found within and leaving the lymph nodes. Polymorphonuclear cells were present in low numbers in the lungs and none were observed in the lymph nodes. The lungs did not exhibit major histopathological changes from normal during this process. These data overall supported the conclusion that the site of germination of inhaled spores is in the regional lymph nodes, where those spores that survive transit from the lungs germinate and spread systemically as bacilli.

Note that this animal model pertains to infections of undamaged lungs of animals not sustaining a procedure-related injury, harboring a preexisting condition, or infection affecting the lungs. Such events might release germinants, permitting local lung germination or other variations in pathogenesis, as has been observed (Dixon et al., 2000; Geiser, 1967; Geiser et al., 1963; Glomski et al., 2007b; Guidi-Rontani, 2002; Heninger et al., 2006; Ross, 1957).

In animal models based on the events as described above (Dixon et al., 2000; Guidi-Rontani, 2002; Ross, 1957), spores were thought to be engulfed by alveolar macrophages which partially served as a "Trojan horse" carrying the spores to the mediastinal lymph

nodes, resulting in bacillary outgrowth and subsequent systemic dissemination. The results of more recent studies have provided further refinements and alternations of this model for the initial events in inhalational anthrax. In an intranasal model employing the unencapsulated toxinogenic Sterne strain of *B. anthracis*, Cleret et al. and others showed that spores are initially phagocytosed primarily by alveolar macrophages; but within a short time, lung dendritic cells take up spores present in the alveoli and appear to be the cell population that transport the spores to the regional thoracic lymph nodes (Brittingham et al., 2005; Cleret et al., 2006, 2007; Tournier et al., 2005). Although these findings should be confirmed in an aerosol model of exposure to a wild-type ($pXO1^+$ $pXO2^+$), virulent *B. anthracis* strain, these data support the concept that *B. anthracis* spores may take control of phagocytic cells encountered in the lungs, inhibiting the production of inflammatory signals, and using them as passive vehicles to promote spread of the infection to the regional lymph nodes.

In contrast, results of other recent murine models using bioluminescent spores suggest a course of events that departs significantly from the prevailing model of spore infection. Glomski showed that local germination and dissemination of the infection can occur at the initial portal of entry without the need for prior transport to regional lymph nodes in inhalational, cutaneous, and gastrointestinal models of infection (Glomski et al., 2007b, 2008). For instance, in mice infected intranasally or by aerosol, tissue invasion and germination in the nasopharynx without early lung involvement was demonstrated. In contrast, Sanz et al. also observed local germination early in the infection, but it was detected in lung tissues and appeared to require contact with macrophages or dendritic cells (Cleret et al., 2007; Guidi-Rontani et al., 1999b; Sanz et al., 2008). Such alveolar bacterial growth has been associated only with infection by unencapsulated toxinogenic strains and with high doses of spores capable of overwhelming cellular defenses (Cote et al., 2008b; Glomski et al., 2008). Several laboratories have shown that the majority of spores are retained within the lungs as dormant spores, sometimes for extended periods (Bozue et al., 2007a; Cote et al., 2006; Friedlander et al., 1993b; Guidi-Rontani et al., 1999b; Heine et al., 2007; Henderson et al., 1956; Heninger et al., 2006; Loving et al., 2007; Pickering and Merkel, 2004; Pickering et al., 2004; Ross, 1957). Nevertheless, it is clear that macrophages can support germination of phagocytosed spores *in vivo* (Banks et al., 2005; Cleret et al., 2006; Cote et al., 2008b; Guidi-Rontani et al., 1999b) and *in vitro* as will be described below. Importantly, Guidi-Rontani observed spore germination within alveolar macrophages but not in bronchioalveolar lavage fluid devoid of these phagocytes (Guidi-Rontani et al., 1999b). To resolve differences in reported findings concerning the initial site of germination *in vivo* and the role of macrophages derived mainly from models with unencapsulated or non-toxinogenic strains in mice and guinea pigs, it will be necessary to study anthrax pathogenesis in models employing fully virulent ($pXO1^+$ and $pXO2^+$) *B. anthracis* in rabbit or nonhuman primate animal models. Although the precise roles of macrophages and other mononuclear phagocytes during the various stages of anthrax pathogenesis have yet to be clarified, their importance remains undisputed.

The Role of Host Phagocytes in the Germination *In Vivo* of *B. anthracis* Spores

The first experiments to demonstrate the phagocytosis of *B. anthracis* and other bacteria by host cells *in vitro* were likely those of early infectious disease investigators, such as

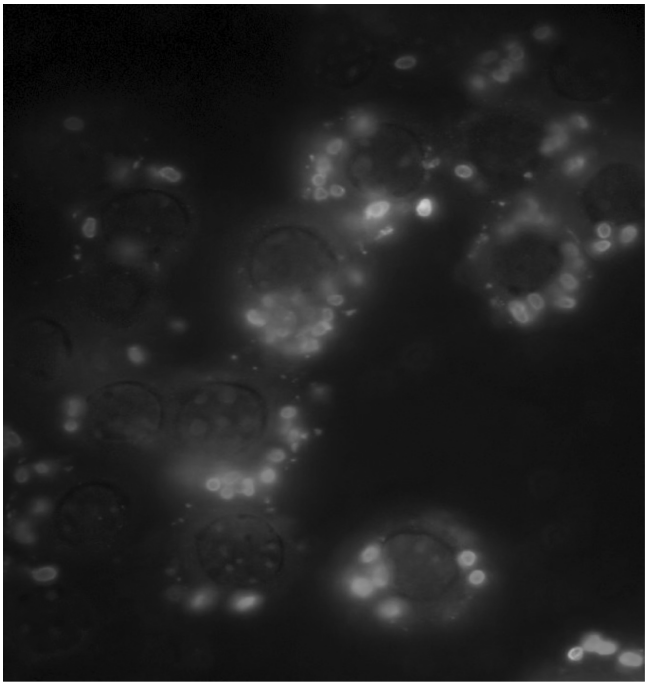

Figure 10.1 Phagocytosis of *B. anthracis* Ames strain spores by macrophages as determined by immunofluorescence microscopy. Macrophages were incubated with spores at the multiplicity of infection of 50. The micrograph illustrates the appearance of macrophages infected with spores as shown by inside/outside staining with FITC-labeled antibodies and TRITC-labeled antibodies, respectively. Specifically, the samples were incubated with a rabbit antibody prepared against whole killed Ames spores that recognized ungerminated and germinated spores, as well as bacilli. These infected macrophages were then incubated with goat anti-rabbit secondary IgG Abs conjugated to red (TRITC) or green (FITC) fluorescent dyes which were added either before or after macrophage permeabilization, respectively. Thus, spores located within the macrophage are stained green, while spores attached to the outside of the macrophage are stained red. Macrophages were visualized by staining with DAPI nuclear stain (blue). See color insert. Reprinted with permission from *Microbes and Infection*, 10, Cote, et al., Early interactions between fully virulent *Bacillus anthracis* and macrophages that influence the balance between spore clearance and development of a lethal infection, 613–619, Copyright Elsevier (2008).

Metchnikoff, who observed the engulfment of these microbes by white blood cells (Metchnikoff, 1905). Numerous studies since then have demonstrated the uptake of *B. anthracis* spores specifically by phagocytes (Figure 10.1) and their germination within the phagosome. Recently, by screening a library of transposon-mutated *B. anthracis*, Barua et al. (2009) identified 18 genetic loci representing a large diversity of functions which are essential for germination of *B. anthracis* within macrophages and emphasizing the highly developed nature of this germination process. However, debate exists on whether host phagocytes are actually required for germination *in vivo*. The absence of germination at early times postinfection as reported by numerous sources (cited above) and the demonstration that spores cannot germinate in cell-free bronchial alveolar lavage fluids (Guidi-Rontani et al., 1999b) would suggest that intracellular residence at some point is essential

for germination to occur. Germination of spores in close association with or within alveolar macrophages was observed very early after intranasal exposure to the bioluminescent recombinant Sterne strain (Sanz et al., 2008). In another Sterne intranasal model, Cleret also reported that alveolar macrophages phagocytose spores in the first 10 min. This initial cellular response was followed by the phagocytosis and trafficking of spores by lung dendritic cells (Cleret et al., 2007).

The results of *in vitro* macrophage phagocytosis studies have supported a role for these cells in the germination observed *in vivo*, as will be discussed below. However, it is also apparent that the close association of spores with macrophages in the absence of phagocytosis can induce germination. The effects of the intra- and extracellular macrophage milieu on germination were initially characterized *in vitro* by Weiner, Ireland, and coworkers. Germination of Sterne and a pXO1-cured derivative of Sterne was greatly enhanced in RAW 264.7 murine macrophage-like cells, and the *gerS*- and *gerH*-encoded germinant receptors were required for this enhancement (Ireland and Hanna, 2002; Weiner and Hanna, 2003). Strains with mutations in *gerS* or *gerH* exhibited a loss of macrophage-associated enhancement of germination. Because nonpathogenic *Bacillus* species did not exhibit this macrophage-enhancing effect, it was proposed that specific host cells were the relevant site of *B. anthracis* germination *in vivo*. More recent studies by Kang et al. (2005) with a *gerH* mutant Sterne strain supported these findings. Nevertheless, direct contact and uptake were not required as spores exposed to the culture milieu of macrophages, but physically separated by a membrane, were able to germinate (Ireland and Hanna, 2002; Weiner and Hanna, 2003). Several *in vivo* studies have provided further evidence that *B. anthracis* spores might be capable of germinating extracellularly during infection. Piris-Gimenez and colleagues (unpublished data) demonstrated the recovery of germinated *B. anthracis* from chambers that had been loaded with spores and implanted intraperitoneally within guinea pigs. The chamber pore size excluded the influx of host cells and egress of the microbes but allowed access to the host milieu. In addition, as reported by Bischof and colleagues, *B. anthracis* has been demonstrated to germinate extracellularly in mouse models of cutaneous anthrax (Bischof et al., 2007). In other studies with mice inoculated by the cutaneous route with spores of the Sterne strain, Hahn and colleagues provided evidence for the apparent ability of the organisms to penetrate undamaged epidermis (Hahn et al., 2005). The major pathway to more invasive infection seemed to be through the hair follicles; however, direct dermal penetration was also seen and resulted in some cases in a lethal infection in the susceptible DBA/2 strain of mice.

Cote and coworkers showed that treating mice with macrophage-depleting regimens did not prevent, but instead, promoted infection with Ames strain spores (Cote et al., 2004, 2006). These results suggested that an association with macrophages might not be required to establish infection and also a protective role for these cells. Recently, using a non-toxinogenic encapsulated *B. anthracis* strain with a *pagA*::*lux* reporter (composed of genes encoding required for luminescence under the control of the *pagA* promoter from *B. anthracis*), Glomski et al. showed that spores can germinate at the primary infection site, that is, in skin, intestines, or nasal passages, without requiring prior transport to lymph nodes (Glomski et al., 2007b). Furthermore, using a model employing mouse strains that are susceptible to infection with toxinogenic, unencapsulated strains ($pXO1^+$ $pXO2^-$), germination and multiplication of spores of *B. anthracis* were confined to the site in the ear of subcutaneous inoculation for an extended period (Glomski et al., 2007a). Protective antigen (PA) vaccination prevented dissemination from this skin site yet did not prevent spore germination (Glomski et al., 2007a).

The Role of Host Cells Other than Macrophages in Spore Trafficking and Early Anthrax Pathogenesis

As described above, the results of *in vivo* studies using macrophage-depleted mice imply that *B. anthracis* potentially germinates and disseminates in the absence of macrophages (Cote et al., 2004; 2006). The precise germination site and route of dissemination in the macrophage-depleted mice are unknown; however, recent evidence suggests involvement of additional host cells in the early spore–host interaction. Mononuclear phagocytic cells, in addition to macrophages, especially dendritic cells (Brittingham et al., 2005; Cleret et al., 2007; Tournier et al., 2007), appear to have a role in spore trafficking and early host responses to *B. anthracis* as detailed above. Moreover, there is growing evidence that epithelial and other nonprofessional phagocytic cells could contribute during the early stages of infection (Bozue et al., 2007b; Chakrabarty et al., 2006, 2007; Oliva et al., 2008; Russell et al., 2008a, 2008b).

Russell et al. (2007, 2008b) showed that *B. anthracis* spores could adhere to and be internalized within phagosomes by cultured human fibroblasts and epithelial cells. Using an alveolar epithelium-like cell line (A549) model, dormant (but not germinated) spores adhered, were internalized, and could traverse A549 cells from the apical side to the basolateral side without disrupting the barrier integrity. These data thus suggested a novel transcellular route of dissemination involving lung epithelial cells used by *B. anthracis*. *In vivo* evidence for the uptake of spores by lung epithelial cells was demonstrated in mice injected intratracheally and examined as early as 2 h postinjection (Russell et al., 2008a). However, this scenario of entry via the bronchoepithelium is reported to be uncommon and appears to be secondary to trafficking via professional phagocytes (Tournier et al., 2009). In agreement with these results, Bozue et al. (2007a,b) showed that, in addition to macrophages, *B. anthracis* can bind to bronchial and other epithelial cells, fibroblasts, and human endothelial cells. They further observed that spores from a BclA-deficient mutant derivative of the Ames strain were able to bind nonprofessional phagocytic cells to a much greater extent than the wild-type (BclA-expressing) spores (Figure 10.2). Such mutants have a deletion of the *bclA* gene which encodes BclA, the major component of the exosporium, the outermost layer of *B. anthracis* (Steichen et al., 2003; Sylvestre et al., 2002). In contrast, the extent of adherence to and uptake by professional phagocytic cells (i.e., macrophages) of the wild type and *bclA* deletion mutant spores were indistinguishable and suggests that BclA might act to reduce nonspecific interactions between *B. anthracis* spores and nonprofessional phagocytic cells in favor of a more directed uptake by phagocytic cells. Oliva et al. (2008) extended these findings by identifying the integrin Mac-1, complement receptor 3, as the specific receptor on professional phagocytic cells involved in binding wild-type (BclA-expressing) *B. anthracis* spores and directing their internalization into professional phagocytes. They also confirmed the presence of an alternative Mac-1-independent pathway that is involved in spore uptake and was revealed only in the absence of BclA.

Considered together with previous reports on the role of dendritic cells (Brittingham et al., 2005; Cleret et al., 2007), the *in vivo* demonstrations that not all spores are phagocytosed by alveolar macrophages (Cote et al., 2006; Guidi-Rontani et al., 1999b), the progression of infection in mice depleted of macrophages (Cote et al., 2004, 2006), and the demonstrated role of the bronchoepithelium as a portal of entry, it is clear that *B. anthracis* may be able to disseminate from the lung by multiple routes. However, the presence of BclA as the major structural component of the exterior nap-like appendages on wild-type spores and its interaction with the Mac-1 receptor suggest that *B. anthracis*

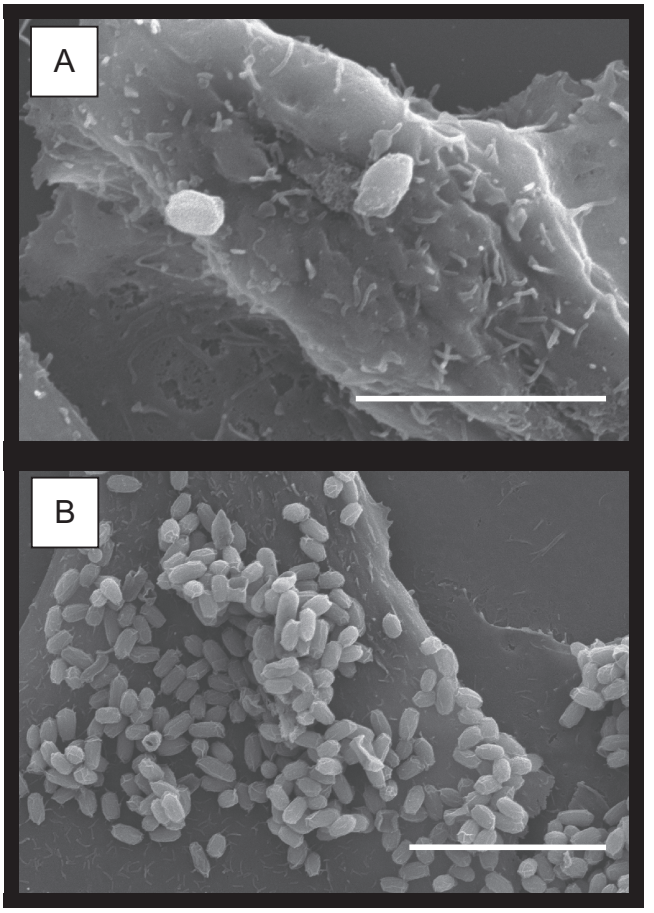

Figure 10.2 Scanning electron micrographs of the interaction between *B. anthracis* spores and bronchial epithelial cells (BEC). BEC were infected with either Ames spores (multiplicity of infection of 38) or *bclA* mutant spores (multiplicity of infection of 44) and incubated as described (Bozue et al., 2007b). A) Adherence of Ames spores to BEC 6 h post-infection. Bar corresponds to 5 µm. B) Adherence of *bclA* mutant spores to BEC 6 h post-infection. Bar corresponds to 10 µm. In contrast to the differences in binding to nonprofessional phagocytic cells (Figure 10.2), both wild type and BclA-deficient spores bind equally well to and are phagocytosed by macrophages (Figure 10.1 and Bozue et al., 2007b).This would support the evidence for a non-Mac1 receptor and the concept that BclA may confer a degree of binding specificity of *B. anthracis* for macrophages.

is preferentially taken up in the lung by professional phagocytic cells, such as alveolar macrophages.

The Cytotoxic and Anti-Host Activities of Spores for Macrophages

The phagocytosis of spores by macrophages is accompanied by production of toxins, enzymes, and other substances by *B. anthracis* that, depending on the spore dose and susceptibility of the cells, can facilitate infection and/or killing of the macrophage. In an

in vitro study with isogenic murine macrophages that expressed or were deficient for the anthrax toxin receptor 2 (ANTXR2/CMG2) gene, Banks et al. showed that anthrax toxin produced by the intracellular germinated spore enables *B. anthracis* to kill the macrophage (Banks et al., 2005). Using mice supplemented with the ANTXR2/CMG2-positive or -negative cells, Cote confirmed a significant role for toxin targeting of macrophages early in infection with *B. anthracis* (Cote et al., 2008b). *In vitro* demonstration of toxin gene expression by germinating spores *in vitro* and within macrophages supported these observations (Cote et al., 2005; Guidi-Rontani et al., 1999b). Also, macrophage depletion experiments in BALB/c mice led Hanna et al. (1993) to conclude that macrophage sensitivity is potentially associated with lethal toxin-mediated toxicity in mice, although this link between macrophage sensitivity and lethal toxin-induced toxicity was disputed in other reports (Moayeri et al., 2004). Several *in vitro* studies with murine, nonhuman primate, and human macrophages and peripheral blood mononuclear cells revealed that toxins inhibit proinflammatory signals of macrophages and dendritic cells (Erwin et al., 2001; Kalns et al., 2002; Pellizzari et al., 1999; Popov et al., 2002a,b; Ribot et al., 2006; Tournier et al., 2005, 2007). Similar *in vivo* findings were reported in studies involving rodent intoxication models (Cui et al., 2004). In contrast, in studies utilizing cell lines and primary murine and human macrophages, several investigators reported infection of these phagocytic cells stimulated proinflammatory cytokine and chemokine production, as will be addressed in the subsequent sections.

In addition to the anthrax toxins, other virulence factors have been identified as having potential roles in macrophage-associated anthrax pathogenesis; and some of the more recent studies will be discussed. The poly-γ-D-glutamic acid capsule has long been known to confer antiphagocytic properties on *B. anthracis* vegetative cells and to be essential for full virulence (Bail, 1914; Drysdale et al., 2005; Ivins et al., 1986; Keppie et al., 1953, 1963; Makino et al., 1989). In recent studies, Scorpio et al. (2007, 2008) showed that CapD, a γ-polyglutamic acid depolymerase encoded on the pXO2 capsule plasmid, could degrade purified capsule and remove the capsule from the surface of *B. anthracis* bacilli. Treatment of vegetative cells with CapD induced macrophage phagocytosis of encapsulated *B. anthracis* and enabled human neutrophils to kill encapsulated organisms. The levels of both phagocytosis and killing corresponded to the degree of enzyme-mediated capsule degradation.

Additional enzymatic proteins and small molecules have been implicated as virulence factors with potential macrophage-associated roles in *B. anthracis* infection. Shatalin et al. (2008) reported that *B. anthracis*-derived nitric oxide is essential for pathogen virulence and survival in macrophages. Sterne strain mutant spores deficient in nitric oxide synthase (bNOS) lost their virulence in mice and were more readily killed upon germination in macrophages. This finding was surprising given the role that nitric oxide, reactive oxygen species, and peroxides have in containing infections. The mechanism of the bNOS-associated resistance to macrophage killing appeared to involve nitric oxide-mediated activation of bacterial catalase and protection against immediate oxidative damage. Thus, bNOS was revealed as an essential virulence factor. Three phospholipase C-encoding proteins (PI-PLC, PC-PLC, SMase) were shown to be required for bacterial growth in macrophages *in vitro* and full virulence *in vivo* in Sterne strain models. The genes encoding all three enzymes had to be simultaneously deleted to demonstrate their effect, and thus, they exhibit redundant activities (Heffernan et al., 2006). PLCs from *B. anthracis* were also reported to be immunosuppressive in that they dampened the responses of dendritic cells to toll-like receptor (TLR) ligands (Zenewicz et al., 2005). *B. anthracis* was also discovered to encode anthrolysin O (ALO), a member of the cholesterol-dependent cytolysin

class of virulence factors. ALO is cytotoxic for several mono- and polymorphonuclear host cells, including macrophages (Cocklin et al., 2006; Mosser and Rest, 2006; Shannon et al., 2003); appears to provoke TLR4-mediated signaling (Park et al., 2004); and, together with lethal toxin, induces apoptosis in macrophages (Shannon et al., 2003). Vaccination with both active and inactivated forms of ALO elicited protection against lethal intravenous challenge with ALO but not against *B. anthracis* in a murine peritoneal infection model (Cowan et al., 2007). Additional secreted proteases, such as one that was capable of inhibiting the activity of the human antimicrobial cathelicidin peptide LL37 peptide (Thwaite et al., 2006), are expressed by *B. anthracis*. Finally, extracellular metalloproteases similar to those expressed by other pathogenic gram-positive bacteria have been detected in *B. anthracis*. The *B. anthracis*-encoded zinc metalloproteases NPR599 and InhA potentially exert direct proteolytic activity against host tissues or indirectly impede innate host defenses by digesting substances, such as extracellular matrix or coagulation proteins, and by perturbing the function of the epithelial barrier system (Chung et al., 2006). Nevertheless, the precise mechanisms and roles of these and other membrane-reactive enzymes and proteases in the virulence of *B. anthracis in vivo* have not been definitively determined.

Bacterial mechanisms have also been described that do not directly counteract host defenses but instead facilitate nutrient acquisition, allow intracellular survival, and promote the infectious process. These mechanisms, which include iron scavenging systems, various enzymes involved in protection against host reactive-oxygen molecules (such as superoxide dismutases), enzymes involved in DNA synthesis, mechanisms that counter antibacterial cationic peptides, and others will be discussed further in later sections.

THE ROLE OF MACROPHAGES IN INNATE HOST DEFENSES

In addition to being a probable cellular target of *B. anthracis* and facilitator of the infection, macrophages appear to be essential in the innate host defense against anthrax. As general innate responders to infections, macrophages are capable of avidly engulfing microorganisms and can kill them by virtue of their TLR recognition of pathogen-associated molecular patterns (PAMPs) (Blander and Medzhitov, 2004); and they also possess specific means for inactivating spores. After exposure to pathogens, macrophages often produce immuno-inflammatory mediators that can recruit and activate other components of the innate immune system as well. Finally, macrophages function as antigen-presenting cells in the later adaptive immune response (Bergman et al., 2005; Pickering and Merkel, 2004; Pickering et al., 2004; Popov et al., 2004; Unanue and DeGregario, 2005).

Macrophages are Sporicidal

The evidence that macrophages are sporicidal is abundant. In the intratracheal guinea pig challenge studies, Ross demonstrated histologically the presence of germinating spores and bacilli within lung macrophages which appeared to have undergone bacteriolysis (Ross, 1957). Various investigators since then have revealed the sporicidal activity of macrophages *in vivo* and *in vitro* using cell lines (e.g., RAW264.7) and primary macrophages from different species and sites (Bozue et al., 2005; Cote et al., 2004, 2005 2006; Guidi-Rontani et al., 1999b; Hu et al., 2006, 2007; Kang et al., 2005; Ribot et al., 2006; Ruthel et al., 2004; Welkos et al., 1989, 2002). For instance, the anthracidal activity of resident and starch-elicited murine peritoneal cells was shown (Cote et al., 2006; Welkos et al., 1989). Hu et al. (2006) and Ruthel et al. (2004) reported the rapid trafficking of *B.*

anthracis spores to phagolysosomes of primary murine bone marrow macrophages with subsequent killing of nearly all the germinated organisms. Ribot showed that alveolar macrophages from nonhuman primates readily phagocytosed and killed the majority of the Ames and Sterne spores when infected at low multiplicity of infection (Ribot et al., 2006).

Although the requirement for macrophages in the *in vivo* germination and outgrowth of *B. anthracis* spores has been a topic of ongoing debate, there appears to be general agreement that the killing requires spore germination. Earlier studies showed that the macrophage was a favorable site for spore germination (Dixon et al., 2000; Guidi-Rontani, 2002; Guidi-Rontani and Mock, 2002; Guidi-Rontani et al., 1999b, 2001; Hanna and Ireland, 1999), and it was suggested that the destruction of the spores within the macrophage was dependent on their germination within the host cell (Welkos et al., 2002). Guidi-Rontani and coworkers (1999b) demonstrated microscopically that the phagolysosomal compartment was the probable site of spore germination. In confirmation of these observations on the role of the phagolysosome, it was demonstrated that pretreating macrophages with inhibitors of phago-endosomal acidification (bafilomycin A and chloroquine) greatly reduces the efficiency of macrophage killing and allows outgrowth and replication of the organisms (Welkos et al., 2002). More recently, Kang et al. established definitively that germination was required for the killing of spores within macrophages by using a *gerH* mutant of *B. anthracis* (Kang et al., 2005). The *gerH* operon encodes germinant sensors expressed in the presence of macrophages and is required for spore germination within the macrophage (Weiner and Hanna, 2003). A mutant deleted for the *gerH* operon was not killed after macrophage phagocytosis. Furthermore, the exosporium, the outermost structure of the ungerminated spores, protected them from macrophage killing, as sonicated spores stripped of their exosporium were killed more readily. In other studies, the *gerX* operon was implicated along with *gerH* in spore germination within macrophages (Guidi-Rontani et al., 1999a; Hu et al., 2007). Additionally, it was shown that spores of an Ames *cotE* mutant strain are significantly delayed in germination and are accordingly more resistant to macrophage killing when compared to survival of the Ames wild-type spores (Giorno et al., 2007). In contrast, spores from a *cotH* mutant Ames strain germinate more rapidly than wild-type spores. After exposure to macrophages, the *cotH* mutant spores are killed more readily than wild-type spores (Giorno et al., 2007). These data thus provide further evidence that the spores must germinate to be killed intracellularly by macrophages.

Although these *in vitro* results revealed important insights on spore germination and its role within host cells, they are not necessarily predictive of *in vivo* infectivity and virulence. For instance, Giorno and coworkers (2007, 2009) showed that mutants having intact germination receptors but lacking major exosporium and coat proteins, including ones devoid of exosporium, are not significantly attenuated in mouse or guinea pig virulence. The minimal extent of spore outer structures required for the spore to remain virulent is unknown, as is the basis of the virulence retention; however, these results ultimately suggest that *in vitro* macrophage models do not completely reflect the environment *in vivo*.

The General Innate Immune Response to *B. anthracis*: Intoxication versus Infection

Numerous studies have been performed to elucidate the impact of the anthrax toxins on immune function. Much of this work has been performed using *in vitro* macrophage assays

to determine the effects of toxins on cytokine production. An early report indicated that while anthrax edema toxin enhanced interleukin-6 (IL-6) expression, edema toxin significantly downregulated lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α expression in human monocytes (Hoover et al., 1994). These data suggested, at least in part, that anthrax toxins could have an immunosuppressive effect on immune cells. Later reports by Pelizarri et al., Erwin et al., and Park et al. provided additional evidence bolstering the relevance of the immunosuppressive effect. These reports demonstrated that anthrax lethal toxin does not induce a proinflammatory cytokine response in a number of macrophage-derived cell lines and that the presence of lethal toxin can suppress the cytokine response normally induced in these cells by the addition of LPS (Erwin et al., 2001; Park et al., 2002; Pellizzari et al., 1999; Pellizzari et al., 2000). These observations were later supported in a report by Ribot et al. (2006) which described similar findings of immune suppression in primary alveolar macrophages harvested from nonhuman primates. Anthrax lethal toxin was again shown to suppress the cytokine response normally induced by LPS (including TNF-α, IL-6, IL-1-β, and IL-8) (Ribot et al., 2006). *In vitro* studies by Gold and coworkers (2004) not only further substantiated these findings but also suggested a non-toxin-mediated mechanism of immunosuppression and host-cell death. This non-toxin-mediated mechanism was proposed as a hypothesis to explain cell death observed early after infection, when toxin levels are less substantial *in vivo*. However, as described earlier, germinating spores have been shown to produce and secrete detectable amounts of anthrax toxins within just minutes to hours after germination initiation (Cote et al., 2005; Guidi-Rontani et al., 1999b).

Besides cytokine expression, anthrax toxins have been shown to alter other aspects of the macrophage immune response. Macrophages produce phospholipase A2, which has been shown to be anthracidal by inducing hydrolysis of bacterial membrane lipids (Piris-Gimenez et al., 2004), as described below. Interestingly, it was demonstrated that levels of this secreted phospholipase in uninfected guinea pig and human bronchioalveolar lavage fluid samples, in some cases, exceeded the amount necessary to kill *B. anthracis in vitro* (Arbibe et al., 1997, 1998; Gimenez et al., 2004). Anthrax lethal toxin can inhibit the production of phospholipase A2 from the alveolar macrophages, suggesting another potentially immunosuppressive mechanism associated with anthrax lethal toxin. While much of the preceding work was accomplished with lethal toxin, edema toxin is also immunosuppressive. Raymond et al. (2007) demonstrated that anthrax edema toxin can downregulate the expression of phospholipase A2 in alveolar macrophages at the transcriptional level. These findings implicate both lethal toxin and edema toxin in the immunosuppressive actions that may provide a means for *B. anthracis* to evade the innate immune response.

Importantly, however, immunosuppression was not found to occur in response to infection with *B. anthracis* spores. These data indicate that *B. anthracis* infection induces a general proinflammatory response in components of the innate immune system. In studies utilizing cell lines and primary murine and human macrophages and dendritic cells, several investigators reported that the phagocytosis and germination of spores stimulated multiple mitogen-activated protein kinase (MAPK) signaling pathways with a consequent activation of genes encoding proinflammatory cytokines and monocyte chemokines (Bergman et al., 2005; Brittingham et al., 2005; Chakrabarty et al., 2006; Pickering and Merkel, 2004; Pickering et al., 2004; Sabet et al., 2006). Levels of the cytokines, such as IL-1α, IL1β, IL-6, and TNF-α, and chemokines, such as the chemoattractant IL-8, were increased. The cytokine release observed *in vitro* in macrophage infection models is reflective of the cytokine stimulation observed in infected mice (Bergman et al., 2005; Pickering

and Merkel, 2004; Popov et al., 2004). The proinflammatory cytokines observed most consistently in different studies to be elevated in the early host responses to spores were TNF-α and IL-6. However, regardless of variations in the identities of stimulated cytokines, these proinflammatory reactions are thought to stimulate an innate cell-mediated immune response to the organisms during the earliest stages of infection.

In studies by Pickering and Merkel (Pickering and Merkel, 2004; Pickering et al., 2004), both cultured murine macrophage-like cells and mice infected with spores demonstrated upregulation of the cytokines tested (to include TNF-α, IL-12, and IL-6). In addition, primary human dendritic cells were observed to have increased levels of TNF-α, IL-6, IL-1β, IL-8, and IL-12 in response to infection by spores. Pickering et al. examined the impact of infection with spores of the toxinogenic Sterne strain on the cytokine response in mice treated with LPS. Interestingly, cytokines induced in response to LPS (to include IL-6, IL-10, IL-12, interferon [IFN]-γ, granulocyte macrophage colony stimulating factor [GM-CSF], and TNF-α) were not significantly inhibited by the concomitant anthrax infection, suggesting the cytokine response to spores can be quite different when compared to that induced by intoxication (Pickering et al., 2004). Popov and coworkers also observed an increase of inflammatory cytokines in mice infected with *B. anthracis* spores (Popov et al., 2004). In this report, differences in the cytokine response were noted in BALB/c compared to C57BL/6 mice, inbred mouse strains that exhibit resistant or intermediate susceptibility to infection with Sterne strain spores (Popov et al., 2004). The greater overall levels of cytokines detected *in vivo* in the BALB/c compared to C57BL/6 mice might be related in part to the presence in macrophages from BALB/c mice of the *nalp1b* sensitivity allele and consequent cytokine release observed *in vitro*, as discussed below. Also, using a human lung slice organ model, Chakrabarty et al. (2006, 2007) demonstrated that the alveolar epithelium, interstitial cells, and macrophages interacted in the production of proinflammatory cytokines and monocyte chemokines upon spore uptake. These and other data suggest that complex cell–cell interactions in response to infection likely occur *in vivo*. Taken together, these data not only underscore the impact of anthrax toxins on macrophage activity and the immune response as a whole but also highlight the importance of examining intoxication in context with infection.

The differences that have been documented in the host responses to spore infection and toxins suggest the following possibility. It can be hypothesized that after a lethal spore challenge, toxin production that is associated with spores germinating inside macrophages (and probably other host cells) infected with a high spore burden facilitates the survival, replication, and release of *B. anthracis* (Banks et al., 2005; Cote et al., 2008b; Heffernan et al., 2006; Ruthel et al., 2004). The toxins produced at later times in infection by the circulating bacilli appear to inhibit activation of immune cells and release of cytokines, abetting the infection and further thwarting an effective host response (Banks et al., 2006; Brittingham et al., 2005; Cleret et al., 2006; Ribot et al., 2006; Tournier et al., 2006, 2007). However, infection with a lower spore multiplicity (or of a more resistant host), might instead allow spore-associated stimulation of protective proinflammatory cytokines *in vivo* and might either retard host cell death or spore destruction. This assumes that any rapid release of mediators does not lead to a cytokine cascade-induced lethal response in the host. The results of a study by Kang and colleagues (Kang et al., 2008) supported these views and provided a partial basis for the differing responses of mice to spores and toxin. Employing murine infection and macrophage models, they showed that both lethal toxin and spores activated the cytokine IL-1β but by two different pathways. Whereas the lethal toxin-associated path led to IL1β-associated cell death, the spore-associated one resulted in IL-1β-induced host inflammatory defensive responses.

Note that in mouse models of anthrax toxin challenge, Moayeri et al. (Moayeri and Leppla, 2004; Moayeri et al., 2003, 2004) proposed that animal susceptibility to lethal toxin is influenced by factors in addition to, and independent of, macrophage sensitivity and cytokine induction. A noninflammatory mechanism that involves hypoxic tissue injury but does not require macrophage sensitivity to toxin was implicated. It is problematical to extrapolate results of a model involving intravenous injection of toxin to anthrax pathogenesis by natural routes, and results of a mouse model might not apply to the pathogenesis of anthrax in higher animals. For instance, *in vitro* studies with primary macrophages obtained from nonhuman primates showed that treatment of these cells with quantities of lethal toxin that kill susceptible murine macrophages had no effect on nonhuman primate alveolar macrophage viability (Ribot et al., 2006). However, in a finding that is consistent with the minimal cytokine response in the murine model, lethal toxin failed to induce a cytokine response in the nonhuman primate primary macrophages (Erwin et al., 2001; Ribot et al., 2006). Nevertheless, these data of Moayeri et al., Ribot et al., and others reemphasize the unresolved nature of the roles of macrophages in host susceptibility to *B. anthracis* and the anthrax toxins.

Specific Mechanisms of the Macrophage Innate Immune Activity Against *B. anthracis*

In addition to the general proinflammatory host response discussed above that is induced by spore infection, specific mechanisms of the innate immune responses of macrophages are reported to have activity against *B. anthracis*. Oxidants and nitric oxide synthase (NOS) are involved in the generation of nitric oxide, reactive oxygen species, and peroxide. The inducible NOS is associated with enhanced antibacterial activity against spores in studies with murine phagocytes, as a NOS2 inhibitor impeded macrophage sporicidal activity (Raines et al., 2006). However, NOS appears to play little if any role in human antimicrobial innate defenses, as the addition of an iNOS inhibitor and subsequent block in nitric oxide synthesis failed to diminish bactericidal activity in the human cells. In addition, cytokines known to induce NO and reduce bacterial survival in mouse macrophages displayed neither activity in human monocyte-derived macrophages (Thoma-Uszynski et al., 2001). Also murine macrophages deficient in the production of superoxide or nitric oxide have been reported to retain their bactericidal capacity (Passalacqua and Bergman, 2006). Thus, mechanisms in addition to the production of reactive oxygen and NO species likely have a role in the innate immune response to *B. anthracis*.

Type IIA phospholipase A2 (PLA2-IIA), which is secreted by macrophages and other inflammatory cells, exhibits potent bactericidal activity against gram-positive bacteria through its ability to hydrolyze membrane phospholipids (Koduri et al., 2002; Weinrauch et al., 1996). Piris-Gimenez and colleagues (2004, 2005) demonstrated the *in vitro* and *in vivo* bactericidal efficiency of sPLA2-IIA against *B. anthracis*. Germinated spores and encapsulated bacilli, but not ungerminated spores, were sensitive to the activity of recombinant sPLA2-IIA *in vitro*. Transgenic mice expressing human sPLA2-IIA were protected against infection with virulent *B. anthracis*; and the human sPLA2-IIA protected mice infected by both peripheral and pulmonary routes and displayed postchallenge therapeutic efficacy.

Finally, cationic antimicrobial peptides, such as the small cysteine-rich defensins and defensin-like peptides represent potentially important innate early responders with direct antibacterial and antitoxic activity against *B. anthracis* (Dawson and Liu, 2008; Ganz

and Lehrer, 1995; Kim et al., 2005; Lehrer, 2004; Patterson-Delafield et al., 1980; Wang et al., 2006; Yang et al., 2007). These peptides are generally expressed abundantly not only in polymorphonuclear leukocytes but also by various bone-marrow-derived mononuclear cells, such as alveolar macrophages (Ryan et al., 1998). They exhibit broad-spectrum antimicrobial activity and were recently shown to inhibit several bacterial toxins (Kim et al., 2005; Mayer-Scholl et al., 2005; Wang et al., 2006). Kim (2005) showed that the human α-defensins (HNP1–3) inhibited the MAPKK proteolytic activity of lethal toxin and protected macrophages from *B. anthracis*-induced cytotoxicity; and their administration to mice prevented lethal intoxication with lethal toxin. The θ-defensin peptides are produced by nonhuman primate polymorphonuclear leukocytes (Tang et al., 1999). Human θ-defensin genes are expressed, but not translated, because their signal sequence domains contain a premature stop codon. Retrocyclins are synthetic θ-defensins whose sequences are based on those encoded in the human θ-defensin pseudogenes. The retrocyclins killed encapsulated bacilli and newly germinated spores of *B. anthracis*, inactivated the enzymatic activity of anthrax lethal factor, and protected murine macrophages from lethal toxin. Treatment of A/J mice with retrocyclins after a lethal exposure to *B. anthracis* strain Sterne prolonged their survival (Beyer, unpublished data). Retrocyclins also partially protected mice against pulmonary infection with spores of the fully virulent Ames strain of *B. anthracis* when treatment was delivered intranasally up to 48 h before infection with the spores (Cote, unpublished data).

Sources of Divergent Findings of Macrophage Models for Anthrax

An important caveat to the macrophage work should be raised at this point. As suggested by the findings discussed above, the literature is replete with papers that describe widely differing results of *in vitro* infection of macrophages with *B. anthracis* spores. For instance, spore infection of murine macrophages has been reported to result in a productive infection with bacillary outgrowth and replication (with or without release of the vegetative organisms and associated cytotoxic effects on the macrophage) (Dixon et al., 2000; Heffernan et al., 2006; Pickering and Merkel, 2004). Guidi-Rontani also observed spore germination and survival; however, no replication was detected within macrophages (Guidi-Rontani et al., 2001). In contrast, macrophages have been shown to be capable of killing *B. anthracis* spores (Bozue et al., 2005; Cote et al., 2005; Hu et al., 2006, 2007; Kang et al., 2005; Welkos et al., 1989, 2002). Welkos and coworkers (2002) observed a decline in viable organisms with time such that by 24 h post-phagocytosis, few spores could be detected intracellularly. The infected macrophages often appeared to be returning to a more quiescent, pre-exposure-like state. The basis of these differences is not always known; however, the variables inherent in the *in vitro* macrophage models can have a major impact on data generated. Thus, it is often difficult to compare the *in vitro* experimental data from different laboratories and to extrapolate it to whole-animal infection.

Some of the major sources of variability and divergent findings include the following: (1) the multiplicity of infection of macrophages by spores and its influence on spore and macrophage survival; (2) assay constituents, such as the culture medium, presence and animal source of serum, presence and concentration of antibiotic in the medium, and the duration of exposure of the macrophages to it; (3) source of the macrophages used (primary or cell line; animal species); (4) the strain of spores used (e.g., Ames or Sterne) and the method of spore preparation; (5) the phagocytosis conditions (e.g., use of centrifugation

to bring spores in proximity of cells, duration of the incubation during the phagocytosis period, and duration/conditions of subsequent incubation of infected cultures); and (6) method of processing infected cultures and procedures used to evaluate levels of germinated and heat-resistant spores, macrophage viability, and presence of residual extracellular spores.

The multiplicity of infection has a major effect on outcome of the experiment. Some laboratories have observed vegetative outgrowth accompanied by macrophage cytotoxicity at a multiplicity of infection of 10 (Heffernan et al., 2006; Ruthel et al., 2004). Also when using a very high multiplicity of infection (44–100), germination with early toxin production and death of macrophages was demonstrated within 8 h of infection (Banks et al., 2005; Cote et al., 2008b). Nevertheless, spore outgrowth and replication has been observed in cells infected at very low multiplicities (Dixon et al., 2000; Pickering and Merkel, 2004). Thus, dose is not the only variable impacting the outcome of infection.

The source of the macrophages and the composition of the assay medium can have a major effect on the results. Spores exposed to medium supplemented with heat-inactivated fetal bovine serum can rapidly initiate germination before macrophage exposure; other sera stimulate little or no germination (Ireland and Hanna, 2002; Welkos et al., 2002). Macrophages incubated in medium or buffer with no serum can still phagocytose, but the intracellular spores do not germinate and thus cannot be killed (Hu et al., 2007; O'Brien D. and Friedlander A., unpublished data). Gentamicin is often added to macrophage cultures after the completion of spore uptake to inactivate germinated unphagocytosed spores. The antibiotic should be removed by 30 min of exposure; otherwise it can penetrate macrophages, possibly killing intracellular germinated spores and thus confounding bacterial viable count data (Dixon et al., 2000; Drevets et al., 1994; Hamrick et al., 2003; Hu et al., 2006; Pickering and Merkel, 2004; Pickering et al., 2004). However, residual adherent spores that are not engulfed or removed by washing and that fail to germinate during the antibiotic incubation will germinate upon viable count plating of macrophage lysates, yielding misleading data (Hu et al., 2006; Welkos et al., 2002). The inclusion of germinants in the culture medium, for example, during the gentamicin treatment, may enhance the proportion of unphagocytosed spores that germinate and are killed by the antibiotic (Heffernan et al., 2006). However, the germinants possibly could enter the cells and affect the progression of the intracellular infection (Hu et al., 2007; Welkos, unpublished data) in an unknown manner.

In reference to macrophage source, whereas macrophage cell lines, such as RAW264.7, were shown to phagocytose and inactivate spores in a manner similar to that of primary murine peritoneal cells (Guidi-Rontani et al., 2001; Welkos et al., 2002), others have noted differences. Primary bone marrow-derived macrophages took up spores of the Sterne strain significantly more efficiently than did the macrophage cell line, resulting in lower residual population of unphagocytosed spores (Hu et al., 2006, 2007). In some reports, cytochalasins (B or D) were used to synchronize the infection, to prevent continuing uptake of extracellular organisms after the phagocytosis period, and to measure the extent of residual unphagocytosed spores not removed by washing or germination/gentamicin treatment. As illustrated by Hu et al., each of these factors and manipulations can have important consequences on the data obtained and should be weighed when analyzing the outcome of any *in vitro* macrophage spore infection. A final macrophage-related variable to consider when interpreting these data is the animal species from which the cells were derived. For example, whereas macrophages from most, but not all, strains of mice that are susceptible to anthrax toxin *in vivo* exhibit lethal toxin-mediated susceptibility *in vitro* (Boyden and Dietrich, 2006; Friedlander et al., 1993a), lethal toxin has no effect on the viability of

alveolar macrophages from nonhuman primates (Ribot et al., 2006) and possibly humans (Boyden and Dietrich, 2006).

Finally, the strain of *B. anthracis*, the method for spore preparation, and the degree of spore purification all influence assay results. A majority of the macrophage studies have been done using Sterne or other pXO2$^-$ negative strain; others have used fully virulent strains, such as Ames. The influence of possible bacterial strain-related differences in gene expression associated with pXO2$^-$ or chromosomally encoded genes on macrophage infection is unknown. Various strain-related differences have been observed that might impact macrophage infection. Sterne strain spores have been observed to adhere especially tightly to macrophage cell surfaces and to be more difficult to remove than those of Ames (Hu et al., 2006; Welkos et al., 2002); variations in the presence or expression levels of specific spore proteins of Sterne and Ames have been observed (Mallozzi et al., 2008; Moody et al., 2010); the Vollum1B strain was shown to express lower levels of surface PA than the Sterne and Ames strains (Welkos et al., 2004); and the Sterne and Ames strains differ in the rate and synchronicity of sporulation (Welkos, unpublished data; Giorno et al., 2009; Welkos et al., 2004).

Several papers have attempted to identify and discuss the factors influencing the fate of the spore and of the host cell in experimental *in vitro* macrophage infections (Banks et al., 2005; Hu et al., 2006, 2007; Ireland and Hanna, 2002; Welkos et al., 2002). For example, in two recent studies, several assay variables were compared and the authors found that when using macrophages that phagocytose spores rapidly and efficiently (in this case, bone marrow-derived macrophages exposed to Sterne spores at a multiplicity of infection of five), those spores that germinate are effectively inactivated before they can replicate, and thus macrophages could play a critical host defense role *in vivo* (Hu et al., 2006, 2007). However, it is clear that host immunity can be overcome *in vivo* and *in vitro* by an overwhelming spore infection, as illustrated by the infection of macrophages at high multiplicity of infections with spores (Banks et al., 2005; Cote et al., 2008b). Ultimately, conclusions from *in vitro* macrophage assays must be confirmed by findings from appropriate whole animal models.

THE METABOLIC CONSEQUENCES OF INFECTION FOR *B. ANTHRACIS* AND THEIR HOST MACROPHAGES

Effects of the Macrophage Intracellular Environment on *B. anthracis* Metabolism

The survival of *B. anthracis* within a macrophage requires the expression of a number of bacterial genes. The acquisition of the nutrient iron is one important component of the survival strategy. Two siderophores are produced by *B. anthracis*, bacillibactin (or anthrabactin) and petrobactin (or anthrachelin) and the synthetic operons of each, *bac* and *asb*, respectively, were identified by Cendrowski et al. (2004). Deletion mutagenesis showed that the *asbA*-encoded product was required for growth *in vitro* in iron-depleted conditions, and an *asbA* mutant was significantly attenuated for growth in macrophages and for virulence in mice. These findings suggested that petrobactin (anthrachelin) plays a potential role in iron assimilation by *B. anthracis* during infection.

Gram-positive pathogens, including *B. anthracis*, express surface proteins attached by sortases to the peptidoglycan, which may play important roles during infection. These surface proteins contain an N-terminal signal peptide and a C-terminal sorting signal that

is recognized by the sortases. Sortases are transpeptidases that are anchored in the membrane via an N-terminal hydrophobic leader peptide. *B. anthracis* has four putative sortase genes (Budzik et al., 2008; Gaspar et al., 2005; Marraffini et al., 2006; Zink and Burns, 2005), as well as a number of putative sortase substrates as determined by the presence of the predicted C-terminal sequence (Read et al., 2003). Several studies have investigated the role of the sortase proteins in *B. anthracis* pathogenesis. For instance, Zink and Burns (Zink and Burns, 2005) showed that derivatives of Sterne strain 7702 harboring a mutation of the *srtA* or *srtB* gene were deficient in their ability to multiply intracellularly in the J774A.1 macrophage-like cell line in comparison to the parent strain. The roles of these genes in the interactions of *B. anthracis* with macrophages *in vivo* or in the outcome of infection are not known.

One group of important sortase substrates is surface proteins encoded by the *isd*-like (*i*ron-regulated *s*urface *d*eterminants) locus (Read et al., 2003). In other gram-positive pathogens, this locus encodes proteins involved in scavenging and transporting iron from heme (Mack et al., 2004; Maresso et al., 2006; Newton et al., 2005). Three additional *isd*-like genes of *B. anthracis* have been described (*isdG*, *isdK*, and *isdJ*). Although their gene products have been shown to be expressed *in vivo* and involved in iron acquisition, potential roles in virulence or in macrophage interactions have not been established (Gat et al., 2008; Skaar et al., 2006).

B. anthracis also produces four superoxide dismutases (SODs) enzymes that can detoxify oxygen radicals, such as those found in the phagolysosomes, which could potentially protect the organisms against reactive-oxygen species (ROS). The relative roles of these SODs *in vivo* are unknown; however, Passalacqua *et al*. showed that the deletion of one (SODA1) was associated with a reduction in mortality of mice compared to the wild-type strain (Passalacqua and Bergman, 2006). In a recent study by Cybulski and coworkers, a strain containing mutations in the genes encoding the two SODs located in the exosporium (SODA1 and SODA15) failed to exhibit a reduction in virulence. Furthermore, these results showed that whereas deleting each *sod* gene (*sod*15*, sod*A1*, sod*C, and *sod*A2) individually had little or no impact on virulence, a strain with quadruple mutations resulted in a total loss of SOD activity and a large reduction in virulence in an intranasal Sterne challenge murine model (Cybulski et al., 2008a). Thus, the SODs of *B. anthracis* exhibit functional redundancy, and the synthesis of these enzymes and their role in protection against oxidative stress likely have an important role in anthrax pathogenesis.

Various other functions have been identified that potentially fend off other antibacterial factors generated by host phagocytes. For instance, a *B. anthracis* arginase was characterized (Viator et al., 2008), and arginase activity, which can protect against effects of the ROS nitric oxide was detected in the spore exosporium (Weaver et al., 2007). However, its role in pathogenesis is unknown. Also, *B. anthracis* and other bacteria synthesize ribonucleotide reductase enzymes require for DNA synthesis, an especially important event in spore germination. The inhibition of one such reductase, NrdF, encoded by *B. anthracis,* increased sensitivity to antioxidants and thus could potentially impact infection. Finally, Fisher et al. (2006) characterized a D-alanine esterification system encoded by the *B. anthracis dltABCD* operon that is important in synthesis of the bacillary cell wall. Mutagenesis studies revealed that this system counteracts the adverse effects of phagolysosomal cationic peptides on vegetative growth and is required for virulence in mice (Fisher et al., 2006).

In addition to studies on specifically targeted genes which are potentially involved in macrophage survival and pathogenesis, other studies have focused on the global impact of macrophage infection on gene expression by *B. anthracis*. A whole-genome

transcriptional profile of *B. anthracis* (Sterne strain 34F2) isolated from within RAW264.7 macrophages at various times during infection was described by Bergman and colleagues (2007). From this study, a large number of *B. anthracis* genes were identified that are highly induced during growth within the host cell and appear to be virulence related (Bergman et al., 2007). During the period between 1 and 2 h postinfection, nearly 1100 genes were significantly altered for expression. Genes associated with sporulation/germination and prophage function were downregulated, whereas genes associated with energy metabolism were upregulated. Certain metabolic genes, such as those required for *de novo* purine biosynthesis and siderophore biosynthesis, were much more highly expressed during macrophage infection than they were during *in vitro* growth in medium. During the later stages of macrophage infection, many of the same genes expressed during early stages continued to be expressed. However, other metabolic genes were upregulated, such as the biosynthetic genes for amino acids, nicotinamide adenine dinucleotide (NAD), and biotin. Bergman et al. hypothesized that *B. anthracis* is oxygen-starved at this stage within this environment, as the genes necessary for alternate electron acceptors were upregulated. Also, in the later time points of macrophage infection, the virulence genes typically associated with *B. anthracis* pathogenesis were detected: the anthrax toxin genes (*lef*, *pagA*, and *cya*), regulatory loci (*atxA* and *pagR*), sortase B, and siderophore biosynthesis genes. Other *B. anthracis* genes that have homologues to macrophage- and virulence-associated genes in other pathogenic bacteria were detected, for example, catalase, hemolysins, phospholipases, and multidrug-resistance genes. Though this study presents a comprehensive analysis of mRNA expression by *B. anthracis* within macrophages, the strain used was an attenuated vaccine strain which lacks the pXO2 plasmid. Thus, the results could potentially differ from those obtained in macrophages infected with a fully virulent strain.

Effects of Spore Infection on the Host Cell Metabolism

In a study by Bergman and colleagues (2005), DNA microarrays were used to examine the transcriptional response of RAW264.7 macrophage-like cells during infection by the *B. anthracis* strain Sterne 34F2. Total RNA was isolated from the macrophages hourly for 6 h. From these studies, genes were identified that were part of the common immune response induced by many or all bacterial pathogens, as well as others not previously reported and may be unique to *B. anthracis*–macrophage interaction (Bergman et al., 2005). During the early stage of macrophage infection involving phagocytosis of the spores, spore germination, and the initiation of bacterial replication, 493 host cell genes were significant for differential expression: 213 genes were induced and 280 genes were repressed. The most prominent of these changes were shifts in genes involved in the induction of both the innate immune response and stress-related genes. Major shifts occurred in genes involved in cellular signaling, such as those encoding Toll-like and other receptor-linked signal transduction systems, ion transport, protein phosphorylation, and dephosphorylation, and guanosine-5′-triphosphate (GTP)-linked signaling cascades. Also, changes in genes involved in cell migration, cytoskeletal biogenesis and organization, and cell adhesion were detected early. The upregulation of these genes may be linked to the uptake of spores and the subsequent migration of macrophages or dendritic cells to the lymph nodes. During the later stages of macrophage infection (4–6 h), the bacteria escape from the phagolysosome into host cell cytoplasm. As reported by Bergman and coworkers, nearly 500 genes demonstrate alterations in expression later in infection, with 292 genes

induced and 188 genes repressed. Genes associated with the immune response and cytokine signaling exhibited enhanced expression. The immune response genes differentially expressed by macrophages during this later time resembled those considered to be typical of the general innate immune transcriptional response for a range of bacterial pathogens. The most significant upregulated functional gene family was that involved in regulating apoptosis. One of these genes is ornithine decarboxylase, which plays an important role in suppressing apoptosis in *B. anthracis*-infected cells. This observation agrees with the finding of membrane permeability changes in *B. anthracis*-infected macrophages reported by Popov et al. (2002a).

Mitochondria are known to be an important target of facultative and intracellular pathogens (Fischer et al., 2006; Tilney et al., 2001; Willhite and Blanke, 2004). Changes in the expression pattern of mitochondrial proteins of RAW 264.7 macrophage-like cells were measured after exposure to Sterne strain 34F2 spores. The affected proteins were identified through proteomic and MALDI-TOF mass spectrometric analyses (Seo et al., 2008), and 13 of the 200 mitochondrial proteins examined exhibited significant differences in their expression pattern. Those proteins with altered expression were ATP5b (encoding a subunit of mitochondrial ATP synthase), NIAP-5 (neuronal apoptosis inhibitory protein 5), Ras-related GTP-binding protein B isoform CRAa, and others. The ATP5b protein was downregulated in macrophages infected with the Sterne spores. Other studies of macrophages exposed to anthrax lethal toxin have reported conflicting results in regard to the up- (Kuhn et al., 2006; Sapra et al., 2006) or downregulation (Chandra et al., 2005) of the ATP5b protein. Further studies are needed on the influence of the infection and of the anthrax toxins on host cell mitochondrial function and immune responses.

As emphasized above, the results of these studies dissecting the anthrax-associated alterations in macrophages are relevant to the cell line and culture conditions used and thus might not be identical in cells infected with fully virulent strains or in primary mouse macrophages obtained from sites, such as bone marrow, lung, or peritoneum. Some of the important responses observed correlate well with observations of physiologic and histologic changes occurring *in vivo* during infection (Bergman et al., 2005); however, studies are needed that address the overall association between data acquired from *in vitro* macrophage models and the course and outcome of disease.

MACROPHAGES AS *IN VITRO* MODEL OF PATHOGENESIS AND IMMUNITY

Spore Opsonization and Phagocytosis

The nature of antibody-mediated opsonization of spores has been examined by several laboratories. Anti-PA antibodies interact with spores and increase phagocytic rates and also increase the efficiency of spore killing after phagocytosis (Cote et al., 2005; Sawada-Hirai et al., 2004; Welkos et al., 2002). These initial reports suggested that the antibodies generated from the anti-toxin PA vaccine also interacted with the spore form of the organism, but more importantly, could potentially affect pathogenesis (Cote et al., 2005; Kang et al., 2005; Welkos et al., 2002). Peterson et al. (2007) also observed that treating rabbits with a human monoclonal antibody to PA resulted in significantly decreased dissemination of the spores from the lungs of the infected rabbits. Similarly, Basu and colleagues (2007) reported that another human monoclonal anti-PA antibody protected mice against lethal

Sterne *B. anthracis* infection. These data again suggest that anti-PA antibodies play other roles besides preventing death by intoxication (Peterson et al., 2007). Other experiments revealed that antibodies directed against non-toxin antigens of *B. anthracis*, particularly spore epitopes, are also opsonic (Brahmbhatt et al., 2007; Cote et al., 2008a; Stepanov et al., 1996). The anti-spore antibodies have been shown to significantly increase the rate at which spores are phagocytosed by macrophages. This opsonic effect was observed when anti-whole spore antibodies were used but also when antibodies against specific spore antigens were examined (Brahmbhatt et al., 2007; Cote et al., 2008a; Cybulski et al., 2008b). Interestingly, the opsonic antibodies directed against whole inactivated spores also exhibited germination-inhibitory activity (Cybulski et al., 2008b; Enkhtuya et al., 2006; Stepanov et al., 1996; Welkos et al., 2004). Antibodies prepared against purified BclA (but not necessarily other identified spore antigens) additionally exhibited germination-inhibitory activity, although this activity appeared to be dependent on the assay and/or source of antigen used (Cybulski et al., 2008b; Welkos et al., 2004). Spores pretreated with these antibodies germinated significantly slower than untreated spores or spores that were treated with pre-immune IgG. The exact mechanisms responsible for the inhibition of germination remain unclear. While anti-PA antibodies resulted in an increased killing efficiency of spores by macrophages, anti-spore antibodies have only been shown to be opsonic without affecting spore-killing rates (Brahmbhatt et al., 2007; Cote et al., 2008a; Welkos et al., 2004).

Macrophage Models for Toxin-Mediated Apoptosis

Leukocytes exhibiting apoptotic cell death were observed in samples obtained from human victims of the Sverdlovsk anthrax exposure in the USSR (Grinberg et al., 2001). In these samples, Grinberg and colleagues noted apoptotic macrophages, particularly in the liver and spleens. The apoptotic mechanism of cell death was associated with lethal anthrax infection. *In vitro* studies have also indicated that anthrax lethal toxin can induce apoptosis or cytolytic death in macrophages (as discussed below). Popov et al. showed that RAW264.7 cells can undergo apoptosis in response to anthrax lethal toxin when the latter was given at sublytic doses. These macrophage-like cells demonstrated changes in membrane permeability, mitochondrial membrane potential, and DNA structure, all of which indicate apoptosis (Popov et al., 2002a). The authors proposed that sublytic levels of lethal toxin may better reflect macrophage death than larger lytic doses, as large amounts of lethal toxin are not observed *in vivo* until late in the infection process. The lethal toxin-induced apoptosis appeared to be initiated by Fas/FasL interactions on the cell surface resulting in the activation of caspases (i.e., caspase-3 and caspase-8) and ultimately disruption of mitochondrial activity (Popov et al., 2002a).

Park and colleagues further substantiated these findings (Park et al., 2002). Apoptosis was again triggered in LPS-activated macrophages by lower amounts of lethal toxin than those required to induce macrophage necrosis. Park et al. also demonstrated that, like LPS, lipoteichoic acids from gram-positive bacteria induce apoptosis in macrophages in the presence of anthrax lethal toxin. Interestingly, while only macrophages derived from certain mouse strains were shown to be susceptible to necrosis (Roberts et al., 1998), as discussed below, lethal toxin-induced apoptosis of activated macrophages was not restricted in this manner (Park et al., 2002). The apoptosis of the activated macrophages was shown to be dependent upon the inhibition of p38 activation. By inhibiting p38 MAPK kinases, *B. anthracis* can manipulate the activation state

of the macrophage, inducing apoptosis, and further paralyzing the host immune response.

Finally, Kassam et al. further expanded on this work by demonstrating that human monocytic cell lines were only susceptible to the effects of anthrax lethal toxin upon cellular differentiation into macrophage-like cells (Kassam et al., 2005). Although similar decreases in phosphorylation of p38 MAPK was observed in both the undifferentiated and macrophage-like cells of the human lines, the ultimate impact of lethal toxin on these cell states differed greatly. These data and that of others (Banks et al., 2006; Reig et al., 2008) demonstrate that the fate of the macrophage and other cells of the immune system in response to lethal toxin relies greatly on differentiation state.

Macrophage Models for Toxin-Mediated Cytotoxicity

The first demonstration of lethal toxin-mediated rapid cytotoxicity was reported in 1986, when Friedlander showed that murine macrophages lyse quickly (within approximately 2 h) in response to purified toxin (Friedlander, 1986). This report illustrated that macrophages, unlike other cell types, were sensitive to these rapid lethal effects of the lethal toxin under the conditions tested. Different macrophage-like cell lines (Singh et al., 1989) or primary macrophages obtained from different strains of mice (Friedlander et al., 1993a) were shown to exhibit differing sensitivities to anthrax lethal toxin. Primary macrophages obtained from C3H/HeN mice, for instance, were observed to be approximately 100,000 times more sensitive to lysis induced by anthrax lethal toxin as compared to primary macrophages harvested from AJ mice (Friedlander et al., 1993a). These differences in lethal toxin sensitivity of macrophages from different mouse strains were not due to alterations in the cell receptors for PA or in the internalization of the toxin but instead were due to posttranslocation cytosolic interactions of lethal factor on host function (Friedlander et al., 1993a).

Inbred strains of mice have long been recognized to differ in their susceptibility to anthrax infection and toxin. It was hypothesized more than 40 years ago that animals resistant to infection by *B. anthracis* are susceptible to challenge by its toxin, and that the inverse was true for infection-susceptible species (Lincoln et al., 1967). Using inbred and recombinant strains of mice, this apparent inverse correlation between the sensitivity of animals to challenge with purified lethal toxin and challenge with *B. anthracis* spores was substantiated and its genetic basis explored (Welkos, 1989; Welkos and Friedlander, 1988a; Welkos et al., 1986). It was found that mice that were relatively resistant to lethal infection with *B. anthracis* strain Sterne (such as BALB/c or C3H/HeN) died more quickly after intravenous injection of purified lethal toxin than did mice that were susceptible to lethal infection with low doses of Sterne spores (such as A/J). C57BL/6J displayed a moderate sensitivity to spore challenge and was resistant to lethal toxin. These *in vivo* data agreed with the observation that mice whose macrophages lyse rapidly in response to lethal toxin are more resistant to spore challenge than mice whose macrophages are lethal toxin-resistant (Friedlander et al., 1993a). The basis of the incremental differences in sensitivity to spore challenge between these different strains of mice is not fully known; and infection- and toxin-induced lethality in mice are clearly influenced by factors in addition to those that control macrophage lysis (Moayeri et al., 2003, 2004). However the mechanism(s) of the lethal toxin toxicity for macrophages continue to be the subject of intensive investigation.

Recent findings on the mechanisms of the lethal toxin-induced cytotoxicity can be summarized as follows. There are at least two lethal toxin-induced pathways of cell death

in macrophages and dendritic cells, a rapid death path leading to cell lysis and a slow pathway of apoptotic cell death. Recently, a single gene, *nalp1b*, which is a member of the nucleotide oligomerization domain (NOD)-like receptor (NLR) family of proteins, was shown to be involved in the caspase-1 production associated with the rapid death pathway (Boyden and Dietrich, 2006). The presence of the *nalp1b* sensitivity allele correlates with caspase-1 activation, release of inflammatory cytokines such as IL-1β and IL-18, and macrophage sensitivity to the *B. anthracis* lethal toxin (as demonstrated by BALB/c macrophages which harbor a *nalp1b* type 1 sensitivity allele). Macrophage lysis and release of IL-1β and IL-18 are predicted to induce inflammation and innate cellular immune responses to infectious diseases (Martinon, 2007; Tschopp, 1989).

In addition to the rapid death path that is induced by Nalp1b-dependent activation of caspase-1, there is a second lethal toxin-induced pathway of cell death in macrophages and dendritic cells: the slower caspase-1 independent apoptotic death pathway. Using a variety of mutant mice, researchers showed that, depending on the mouse strain, lethal toxin-associated death in macrophages and immature dendritic cells was mediated either by the fast Nalp1b- and caspase-1-dependent, or by the slow caspase-1-independent pathway that was triggered by the impairment of MEK1/2 (also known as MAPK kinase) pathways (Abrami et al., 2005; Duesbery et al., 1998; Reig et al., 2008). It has been observed in macrophages that lethal toxin can disrupt survival signals triggered by TLR4 and mediated by MAPK proteins due to lethal toxin cleavage of the upstream MAPK kinase. Activated p38 MAPK is required for the expression of the NF-κB-targeted anti-apoptotic genes that promote survival, and disruption of this pathway by lethal toxin leads to production of the caspase-3 enzyme and cellular apoptosis (Duesbery et al., 1998; Kim et al., 2005; Park et al., 2005). Cytokine production is suppressed and inflammatory responses are not observed during the ensuing apoptotic cell death. In addition to macrophages, lethal toxin also suppresses other cells of the innate and adaptive immune system by disrupting the MAPK signaling such as dendritic cells and lymphocytes (Baldari et al., 2006; Banks et al., 2006). However, it is clear that the MAPKK proteins are not the only proteolytic target of lethal toxin (Pellizzari et al., 2000). Also, while lymphocyte apoptosis appears to be a major histopathological response to infection with *B. anthracis* in humans (Grinberg et al., 2001), the precise roles and importance of immune cell responses, including macrophages, in the early stages of anthrax, especially the inhalational form, are unclear.

In Vitro Macrophage Correlate Assays of Immunity

In addition to the valuable contribution of macrophage-based *in vitro* models to the understanding of anthrax toxin function, these models have been critical in evaluating and understanding the protection afforded by anthrax vaccines. Most effective vaccination strategies against anthrax include PA (Friedlander et al., 1999, 2002). The antibody responses to the PA component of the vaccines are protective in animal models of anthrax (Friedlander et al., 1993b, 1999; Little and Knudson, 1986; Vietri et al., 2006; Welkos and Friedlander, 1988b). Anthrax vaccine adsorbed (AVA) consists of sterile *B. anthracis* culture filtrates. Both AVA and the next-generation anthrax vaccine consisting of solely recombinant PA (rPA) have both been well-tolerated in human trials (Pittman et al., 2002; Turnbull et al., 1986). However, due to the nature of the disease and the fact that infections with *B. anthracis* and the specific *B. anthracis*-induced immune responses to it are rarely documented in humans, vaccine efficacy data in humans are sparse at best (Madle-

Samardzija et al., 2002). Because of this and ethical considerations, an appropriate correlate of protective immunity is essential for current and future vaccine evaluation.

Anti-PA titers in response to vaccination with PA-based vaccine regimen have been extensively studied in several animal models (Friedlander et al., 1993b; Ivins et al., 1994; Kobiler et al., 2002; Phipps et al., 2004; Reuveny et al., 2001; Welkos and Friedlander, 1988b; Williamson et al., 1999, 2005). These titers, when determined by quantitative anti-PA IgG ELISA, have been correlated with survival (Fowler et al., 1999; Little et al., 2004a,b, 2007; Pitt et al., 2001). Additionally, anti-PA antibodies have been examined in a functional cell-based assay that utilizes the cultured murine macrophage-like J774A.1 cell line (Hering et al., 2004). Briefly, a toxin-neutralizing assay measures the ability of anti-PA antibodies to neutralize the cytotoxic effect of anthrax lethal toxin on cultured macrophages. This validated assay has been used as a correlate of protective immunity for the AVA vaccine (Pitt et al., 2001) as well as for the next-generation rPA vaccine (Little et al., 2004a; Marcus et al., 2004; Peachman et al., 2006; Reuveny et al., 2001; Williamson et al., 2005). When anti-PA antibody titers are combined with toxin-neutralization titers, the correlation between the immune response and animal survival are statistically significant, and the assays themselves have a high level of repeatability and reproducibility. The toxin-neutralization assay, generally perceived as the current gold standard for evaluating the protection afforded by PA-based anthrax vaccines, emphasizes the significance of macrophages in establishing *in vitro* correlates of protective immunity.

In conclusion, macrophages are exceedingly important in the pathogenesis of and host immune response to *B. anthracis*. While much has been discerned on the interactions between *B. anthracis* and macrophages, there are still substantial gaps in our basic knowledge of these interactions. A better understanding of the relationships between the macrophage, bacteria, and host will likely promote novel hypotheses regarding pathogenesis as well as novel therapeutic and prophylactic approaches required to combat engineered and emerging threats.

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Chapter 11

Bacillus anthracis and Dendritic Cells: A Complicated Battle

Jean-Nicolas Tournier and Anne Quesnel-Hellmann

INTRODUCTION

Bacillus anthracis is a nonmotile, facultative anaerobe that occasionally infects humans. However, it more frequently causes disease in animals, including field-grazed herbivores in particular (Mock and Fouet, 2001). The ecological cycle of this gram-positive bacterium involves the proliferation of vegetative bacilli in the host, leading to the generation of dormant spores, which may persist in the soil in a viable state for decades. Spores ingested by grazing herbivores typically constitute the infectious form of this pathogen. *B. anthracis* has two main virulence factors, which have been described in detail in previous studies: the poly-γ-glutamate (PGA) capsule (encoded by pXO2; Candela and Fouet, 2006) and protein toxins (encoded by a temperature-sensitive pXO1 plasmid; Young and Collier, 2007). The capsule plays an important role in establishing disease, by protecting the bacillus against complement fixation and phagocytes. Toxins essentially provide the *coup de grace* in infected hosts, killing the host and triggering the sporulation of the bacilli responsible for perpetuating this deadly cycle. The combination of an enzymatically active component—lethal factor (LF) or edema factor (EF)—with protective antigen (PA) as a cell surface-binding component results in the production of lethal toxin (LT) and edema toxin (ET), respectively (Young and Collier, 2007).

There are three main clinical presentations of anthrax disease in humans (Inglesby et al., 2002): a cutaneous form, the most frequent in natural conditions and often benign, and the more aggressive intestinal and pneumonic forms, involving sepsis and resulting in death. Interest in the pneumonic form was fuelled by the bioterrorists' attacks in 2001 in the United States. Cutaneous anthrax has been shown to occur after the deposition of spores on the skin. Cuts or abrasion of the skin may favor infection. The areas of skin exposed to the air, as on the arms, hands, face, and neck, are the most frequently affected. The spores are thought to germinate in the skin and to proliferate locally, giving rise to a black eschar surrounded by edema (Inglesby et al., 2002). The intestinal form of the disease is linked to the ingestion of contaminated meat from the carcasses of animals that died from anthrax. The lesions of intestinal anthrax occur mostly in the terminal ileum and cecum, in the lower intestinal tract. A recent study with a bioluminescent capsulated

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non-toxicogenic strain in the gastrointestinal model showed that this strain proliferated in the Peyer's patches, consistent with a role for DC capture at this crucial site within the intestine (Glomski et al., 2007c).

The pulmonary form is caused by the inhalation of spores. In a "historical model" based on analyses of the gross anatomy of experimentally infected animals, it was suggested that alveolar phagocytes, mostly macrophages (MΦs), captured and transported spores to the draining thoracic lymph nodes (LNs) where proliferation occurred, causing sepsis (Ross, 1955; Tournier et al., 2007). Studies on rhesus monkeys (Henderson et al., 1956; Klein et al., 1962) and chimpanzees (Albrink and Goodlow, 1959) infected with spore aerosols have confirmed that *B. anthracis* is taken up by alveolar MΦs and is carried to regional LNs within 6–18 h. Macroscopic and pathological analysis of natural (Albrink and Goodlow, 1959) and provoked (Abramova et al., 1993; Grinberg et al., 2001; Guarner et al., 2003) pneumonic anthrax in humans has shown that spores are carried by phagocytes accumulating in the capsular sinus of the thoracic LNs. Spores germinate in the LNs and the resulting bacilli proliferate, producing distinctive chains, which then diffuse throughout the circulatory system. Lung dendritic cells (DCs) have recently been implicated in the transport of spores to thoracic LNs (Cleret et al., 2007). Nasal-associated lymphoid tissue (NALT) may also constitute a crucial port of disease entry and proliferation after spore inhalation (Glomski et al., 2007c). In the three clinical forms of anthrax, DCs, the main cell type involved in the control and regulation of the immune response, are suspected or have been shown to be involved in various aspects of the disease. DCs are present throughout the peripheral tissues in the skin and mucosa and are responsible for sampling environmental antigens. They are therefore among the first phagocytes to be encountered by *B. anthracis* during its infectious cycle, regardless of the port of entry. Interactions between *B. anthracis* and DCs are therefore thought to be crucial, particularly during the very early stages of disease.

We will begin by presenting what is currently known of DCs and their subsets in the tissues potentially in contact with anthrax spores during the course of infection. We will then consider the way in which DCs handle the pathogen and the ways in which the pathogen deeply undermines DC functions and thrives in the host.

DCS: SUBSET DIVERSITY, TOPOLOGY, AND FUNCTIONS

Global Overview of the Biology of DCs

DCs behave as frontline sentinels at various entry portals throughout the body. They play a crucial role in controlling infections and modulating the innate and adaptive immune response. The origin, functions, and phenotypic diversity of DCs have been discussed in many extensive reviews (for selected papers, see Banchereau et al., 2000; Banchereau and Steinman, 1998; Steinman and Banchereau, 2007). We will therefore present here only the information required to understand *B. anthracis*–DC interactions.

DCs are veiled, stellate cells, their shape being highly suited to their functions. DCs are professional antigen-presenting cells (APC) that capture and present antigens to T lymphocytes, thereby activating these cells. Antigens must be processed before their presentation, as T cell receptors (TCR) recognize only fragments of antigen bound to the major histocompatibility complex (MHC) on the surface of APCs. The peptide-binding proteins are of two types—MHC class I and MHC class II—responsible for stimulating cytotoxic T cells and helper T cells, respectively. DCs display a highly specialized type

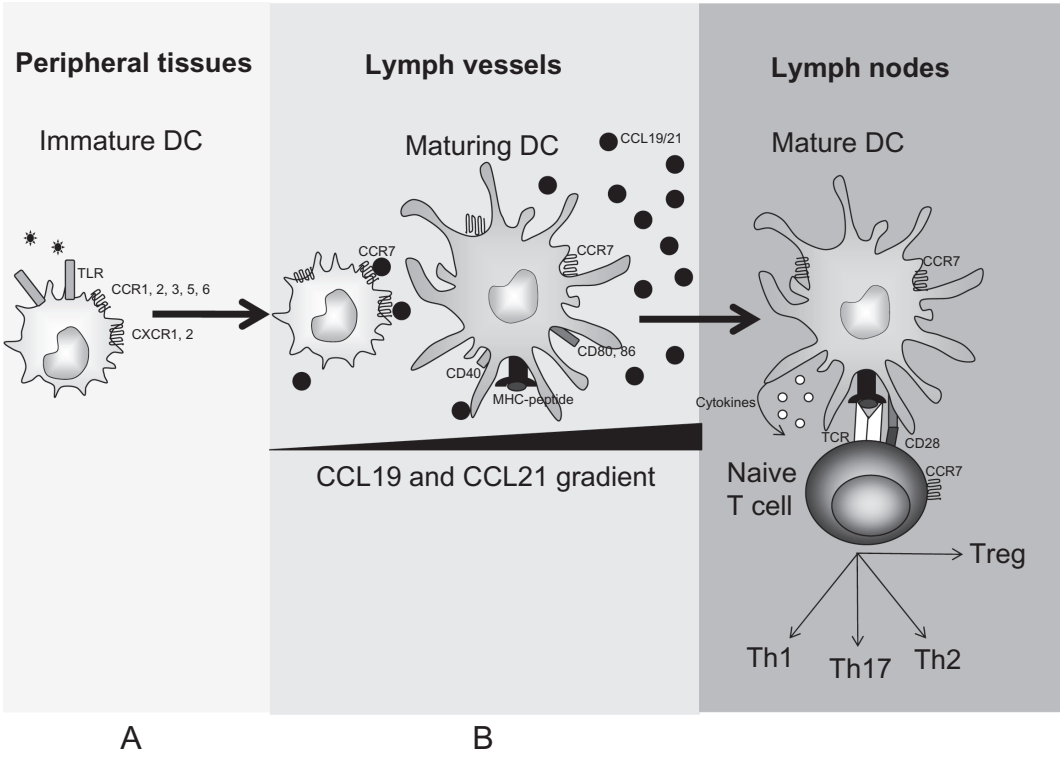

Figure 11.1 Simplified overview of the role of DCs during maturation. DCs provide an important bridge between innate and adaptive immunity, performing two different functions at different sites: (a) in peripheral tissues, immature DCs are adapted for the capture and accumulation of antigens; (b) in LNs, mature DCs that have completed terminal differentiation acquire a potent capacity to present antigens and to prime T cell responses efficiently, thereby initiating polarization of the emerging T cell response to a T helper (Th)1 (interferon [IFN]-γ producing), Th2 (interleukin [IL]-4-, IL-5-, and IL-13-producing), Th17 (IL-17- and IL-22-producing), or Treg response.

of endocytosis, facilitating the receptor-based delivery of captured pathogens and antigens to processing compartments (Trombetta and Mellman, 2005).

DCs provide an important bridge between innate and adaptive immunity, performing two different functions at different locations (Banchereau and Steinman, 1998) (Figure 11.1): (a) in peripheral tissues, immature DCs capture and accumulate antigens; (b) in LNs, after their terminal differentiation, mature DCs acquire considerable potency for antigen presentation and efficient priming of the T cell response, and are involved in initiating the polarization of the emerging T cell response toward T helper (Th)1 (interferon (IFN)-γ producing), Th2 (interleukin (IL)-4-, IL-5-, and IL-13-producing), or Th17 (IL-17- and IL-22-producing) responses (Bettelli et al., 2008; Dong, 2008). Specific stimuli initiate the generation of tolerogenic DCs, which induce Tr1 (IL-10-producing) and $FOXP3^+$ T regulatory (Treg) cells (Sakaguchi and Powrie, 2007). Thus, DCs are not only key mediators in the delivery of antigen-specific signals to T cells, they also provide T cells with packets of information that must be decoded before an appropriate immune response can be mounted (Lanzavecchia and Sallusto, 2001). DC maturation is a key step in the initiation of immunity and has major consequences for the quality of the immune

response. Many pathogens affect the ability of DCs to migrate, to mature, and to infect their hosts (Sansonetti and Di Santo, 2007).

The last feature of DCs that we will deal with here is the diversity of DC subsets in peripheral tissues and lymphoid organs. The various subsets of DCs express a large repertoire of phagocytic receptors and have different cytokine-secretion capacities (Naik, 2008; Shortman and Naik, 2007). They communicate with each other to coordinate the immune response. Conventional DCs have dendrites and a myeloid phenotype, characterized in mice by expression of the integrin CD11c and MHC class II, and a large repertoire of phagocytic receptors. Plasmacytoid DCs (PDCs) derive their name from the cytological similarities to antibody-producing plasma cells they display. These cells express CD123 and produce large amounts of type I IFN following the recognition of viral motifs. PDCs are involved in defense against viral infections and also play a role in tolerance. The complexity of DC subsets can be accounted for in terms of ontogeny, as the same lineage may be present in different tissues with different phenotypes as a function of the stage of differentiation (Shortman and Naik, 2007; Wu and Liu, 2007). We will focus below on the various subsets of conventional DCs in the skin, gut, and pulmonary mucosa at risk of exposure to *B. anthracis* infection.

Cutaneous DCs

Langerhans cells (LCs), discovered by Paul Langerhans, are the only DC subset present in healthy skin, in which they are found in the epidermis immediately above the basal layer of proliferating keratinocytes. LCs specifically express a lectin called langerin or CD207, CD1a, CD11c and CD11b, and MHC class II (for a review, see Merad et al., 2008). Recent studies have identified another subset of DCs in the dermis; these cells express langerin, CD11c, and CD103 (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). The dermis also contains a more classical population of langerin- and CD103-negative DCs expressing CD11c and CD11b.

The functions of the various subsets of DCs in the skin remain a matter of debate (Merad et al., 2008), and nothing is yet known about the role of skin DC subsets in the capture and transport of spores from the skin to the LNs. Further investigations are clearly required.

Mucosal DCs: Pulmonary and Gastrointestinal Tract DCs

The gut and lungs are both mucosal, but are organized differently in terms of the local immune system and DC subsets (reviewed in detail by Iwasaki, 2007; Kunisawa et al., 2008). Most of the studies carried out have focused on the mouse. We will therefore restrict our discussion to the conventional DC subsets of mouse organs.

Gastrointestinal Tract DCs

The gastrointestinal tract represents a large area of contact with pathogens and antigens in nutrients and fluids. Gut DCs have been intensively studied in recent years (reviewed in Coombes and Powrie, 2008; Niess and Reinecker, 2006). Various subpopulations of DCs are present in organized lymphoid structures of the intestinal immune system, including Peyer's patches and mesenteric LNs. In Peyer's patches, conventional DCs are predominantly of the $CD11c^{hi}CD11b^{+}CD8\alpha^{-}$, $CD11c^{hi}CD11b^{-}CD8\alpha^{+}$, and

$CD11c^{hi}CD11b^{-}CD8\alpha^{-}$ subtypes (Coombes and Powrie, 2008). Small intestinal lamina propia DCs have been reported to have a subset composition similar to that of Peyer's patches DCs. A subset of the lamina propria DCs expressing CD103 has been found to drive the expression of CCR9 and $\alpha4\beta7$ on $CD8^{+}$ T cells in the mesenteric LNs and to be critical for the induction of $FOXP3^{+}$ Treg cells (Annacker et al., 2005; Coombes et al., 2007; Johansson-Lindbom et al., 2005). The DCs in the colon seem to be concentrated in isolated lymphoid follicles, with very few present in the lamina propria. Antigens from the intestinal lumen are taken up through various routes (Coombes and Powrie, 2008). First, specialized M cells (microfold cells) present in the follicle-associated epithelium of the Peyer's patches may capture luminal antigens and transfer them to DCs via the basolateral membrane. Second, luminal antigens may be transported by neonatal Fc receptors for IgG. Third, lamina propria DCs may capture pathogens through transepithelial extensions (Chieppa et al., 2006; Niess et al., 2005). Once they have captured antigens, intestinal DCs migrate efficiently to the mesenteric LNs.

Pulmonary DCs

Unlike the gut, the lungs have no lymphoid tissue associated with the mucosa in the absence of inflammation. Furthermore, the lower part of the respiratory tract is sterile. However, DCs are nonetheless highly heterogeneous in the lung, as in the gut (GeurtsvanKessel and Lambrecht, 2008; Hammad and Lambrecht, 2008; Holt et al., 2008; Tournier and Mohamadzadeh, 2008). DCs are found in most tissue compartments, including the conducting airways, the lung parenchyma and the alveoli. The conventional DCs of the lung may be $CD11b^{+}$ or $CD11b^{-}$. The DCs present in the airways form a fine web of intraepithelial $CD11c^{+}CD11b^{-}CD103^{+}Langerin^{+}$ cells, whereas the submucosal DCs express mainly CD11c and CD11b, but not CD103. The lung parenchyma also contains these two subtypes of DCs, which may reach the alveoli. Functionally, $CD103^{+}$ DCs may have specialized functions for cross-priming innocuous antigens for presentation to $CD8^{+}$ T cells under tolerogenic conditions (del Rio et al., 2007). Properties of $CD103^{+}$ DCs in the lung similar to those of the cells triggering differentiation into Treg cells in the gut have not been observed (Coombes et al., 2007). Lung DCs have been shown to capture and transport spores in a murine model of anthrax respiratory infection (Cleret et al., 2007). We will discuss these results further below.

DCs form a heterogeneous population located in an ideal position for detecting and interacting with pathogens in peripheral tissues. We will now examine these interactions in *B. anthracis* spore infection.

DCS AND *B. ANTHRACIS*: A COMPLICATED BATTLE

Molecular and Cellular Interactions between *B. anthracis* and DCs

Some of the dynamic molecular interactions between spores and phagocytes have recently been deciphered. These interactions are complex, due largely to the presence of two forms of *B. anthracis* during infection: as a spore in the initial phase of infection and subsequently proliferating and spreading as an encapsulated bacterium. Spores begin to germinate within phagocytes, which simultaneously activate pattern recognition receptor (PRR) pathways, triggering bactericidal and sporicidal activities with complex kinetics. The spore

consists of a complex overlay of layers: the cortex, coat, and exosporium, protecting the genome confined to the core (Moberly et al., 1966). The exosporium is the outermost layer of the spore. It is loose-fitting, balloon-like, and hairy, and is the part of the spore most exposed to the environment. Analyses of *B. anthracis* and *B. cereus* spores have identified 20 exosporium-associated proteins and glycoproteins (Boydston et al., 2005; Redmond et al., 2004; Steichen et al., 2003, 2005; Sylvestre et al., 2002, 2005; Todd et al., 2003). The major immunodominant component of the hair-like fibers is the BclA (*Bacillus* collagen-like protein of anthrax) glycoprotein, the conformation of which mimics that of the C1q component of complement (Daubenspeck et al., 2004; Rety et al., 2005; Steichen et al., 2003). BclA (21 kD) has a collagen-like region (CLR), a proline-rich sequence, and is a member of the tumor necrosis factor family, which is characterized by a trimeric, jelly-roll fold. The CLR domain is highly polymorphic and its length determines the length of the exosporium filaments (Sylvestre et al., 2003). The deletion of the gene encoding BclA from spores of various genetic backgrounds seems to have no overall effect on pathogen virulence in mice (Bozue et al., 2007a; Brahmbhatt et al., 2007; Sylvestre et al., 2002), although one study reported conflicting findings (Oliva et al., 2008).

Recent studies have established that human DCs, like those of mice, are able to take up spores by phagocytosis *in vitro* (Brittingham et al., 2005; Cleret et al., 2006; Tournier et al., 2005). Molecular interactions of the exosporium with various cell subsets and the role of BclA have been investigated in more detail recently, highlighting the very specific nature of interactions between professional phagocytes and spores. The exosporium shields *B. anthracis* against killing by nitric oxide in the phagosome, due to its intrinsic arginase activity (Weaver et al., 2007). BclA is also probably involved in protection, as spores lacking this protein germinate more rapidly (Brahmbhatt et al., 2007). An analysis of the adhesive properties of BclA-deficient spores showed that these spores bound more efficiently to fibronectin and laminin (Brahmbhatt et al., 2007). These results suggest that BclA weakens the adhesion of spores to the conjunctive reticulum, facilitating cell target. Other studies have shown that BclA specifically targets spores toward professional phagocytes, such as MΦs (Bozue et al., 2007b). Spores lacking BclA adhere much more strongly than wild-type spores to various epithelial cells (including bronchial epithelial cells), whereas the adhesion of spores to MΦs is not affected by gene deletion (Bozue et al., 2007b). The adhesion of BclA-defective spores to DCs was not assessed in these studies, but it can be inferred that BclA may also target adhesion to DCs. Cell-type selectivity may be important for the pathophysiology of the disease, as phagocytes (MΦs and DCs) have been identified as a possible target for spores during infection (Tournier et al., 2007). Another recent report identified the complement receptor (CR)3, also known as CD11b/CD18 (or Mac-1), as the specific receptor for anthrax spores (Oliva et al., 2008). The authors also claimed that there is an alternative uptake mechanism independent of Mac-1. Thus, *B. anthracis* spores seem to use several different methods to enter different cell types. As CD11b is expressed by all DC subsets in the respiratory tract, gastrointestinal tract, and skin, it is possible that BclA targets spores to CD11b-dependent DC phagocytosis throughout infection. Rhamnose, a carbohydrate component of the exosporium, seems to play a role in adhesion (Bozue et al., 2005).

The exosporium plays a role in targeting spores to the appropriate cells, whereas the host PRRs present on DCs detect the presence of pathogen-associated molecular patterns (PAMPs) (Figure 11.2). The exosporium is not inert and is recognized by PRRs via the myeloid differentiation (MyD)88 pathway (Basu et al., 2007; Glomski et al., 2007b; Hughes et al., 2005). It remains unclear whether MyD88 signals spore motif recognition through direct interaction, or whether it requires receptors from the Toll-like receptor

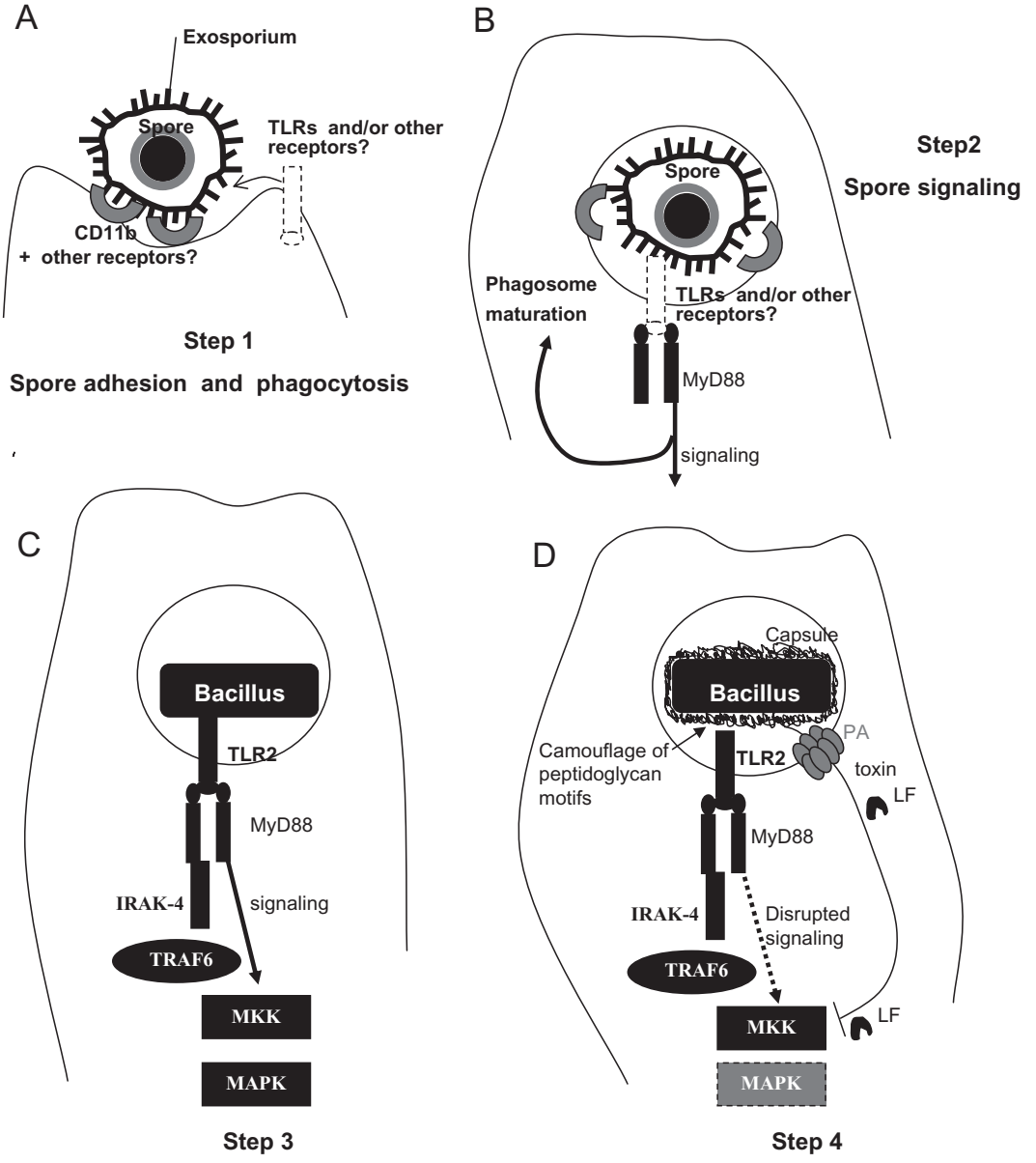

Figure 11.2 Molecular interactions between *B. anthracis* and DCs early in infection. In panel (a), the adhesion of *B. anthracis* spores to DCs is targeted through CD11b and other unknown receptors. In a second step (b), spore molecular motifs are recognized in a MyD88-dependent manner, triggering phagosome maturation and DC activation. Simultaneously, the spore germinates (c) and new pathogen-associated molecular patterns are recognized in a TLR2-dependent manner. (d) Germination is associated with the production of virulence factors, such as the capsule, which may decrease TLR recognition, and toxins, which may shut down the MAPK pathways, providing the DCs with a negative signal.

(TLR) family. It also remains unclear whether spore motifs are recognized on contact with the membrane or within phagosomes.

It has been shown that several germination loci are required for intracellular germination *in vitro* (Ireland and Hanna, 2002; Weiner and Hanna, 2003). After germination, bacteria harbor PAMPs different from those of spores, which can be recognized by other host PRRs. One of these PAMPs, anthrolysin O, a cholesterol-dependent cytolysin, is recognized via TLR4 (Park et al., 2004), whereas heat-killed bacteria activate TLR2 (Hughes et al., 2005). These TLRs are MyD88-dependent. A recent study showed that peptidoglycan purified from *B. anthracis*, in which it is a major cell wall component, activates strong, p38α-dependent TNF-α production by human monocytes (Langer et al., 2008). Peptidoglycan has been shown to bind to TLR2 and TLR6 (Akira and Takeda, 2004), suggesting that TLR2 may play a key role in the recognition of vegetative bacilli by phagocytes.

The molecular interactions between host and pathogen are highly dynamic. The exosporium plays a critical role in targeting phagocytes (MΦs and presumably DCs), possibly accounting for spores being the only infective form. The molecular motifs of the spore are rapidly recognized after phagocytosis, in an MyD88-dependent manner, triggering the host phagosomal degradation machinery and the production of proinflammatory chemokines and cytokines. At the same time, the spores germinate and begin to produce virulence factors. It remains unclear whether DCs have sporicidal and bactericidal capacities, and whether the pathogen can escape the phagosomal degradation pathway and proliferate within DCs or elsewhere.

Anthrax Virulence Factors and DCs

Toxins as Model of Inhibitory Effectors of the Immune System

The anthrax toxins provide a useful model of the induced suppression by a bacterium of both the innate and adaptive immune systems (Baldari et al., 2006). The crystal structures of the LF and EF components of the LTs and ETs, respectively, have, like that of PA, been informative (Drum et al., 2002; Pannifer et al., 2001; Petosa et al., 1997). The mechanisms by which anthrax toxins enter cells have been described in previous detailed reviews (Abrami et al., 2005; Puhar and Montecucco, 2007). Briefly, PA binds to at least two cellular receptors—ANTXR1/TEM-8 and ANTXR2/CMG-2—and to a putative co-receptor identified tentatively as LDL receptor-related protein (LRP)6 (Bradley et al., 2001; Ryan and Young, 2008; Scobie et al., 2003; Wei et al., 2006; Young et al., 2007) (Figure 11.3). Following the cleavage of PA (83 kDa) by a furin-like protease, releasing a 20-kDa peptide from the N-terminus, PA63 self-assembles to form a heptameric prepore. Endocytosis is both lipid raft-dependent and clathrin-mediated, and results in prepore-receptor transport through the early endosome in intraluminal vesicles called multivesicular bodies (MVB) and delivery to the late endosome. Under acidic endosome conditions, the pore is formed by a PA heptamer and the LF/EF moieties are translocated in MVBs. These MVBs can fuse with the endosomal membrane, delivering LF/EF into the cytosol, where these compounds exert their toxic effects.We will restrict our discussion here to the principal effects of these toxin components on host cells and signaling systems. After translocation from an acidified endosome, EF remains bound to the late endosomal membrane, whereas LF is released into the cytosol (Dal Molin et al., 2006, 2008). EF is a calcium- and calmodulin-dependent adenylate cyclase that increases intracellular cAMP levels (Leppla, 1982). The restriction of EF to the perinuclear induces protein kinase A (PKA) signaling, culminating

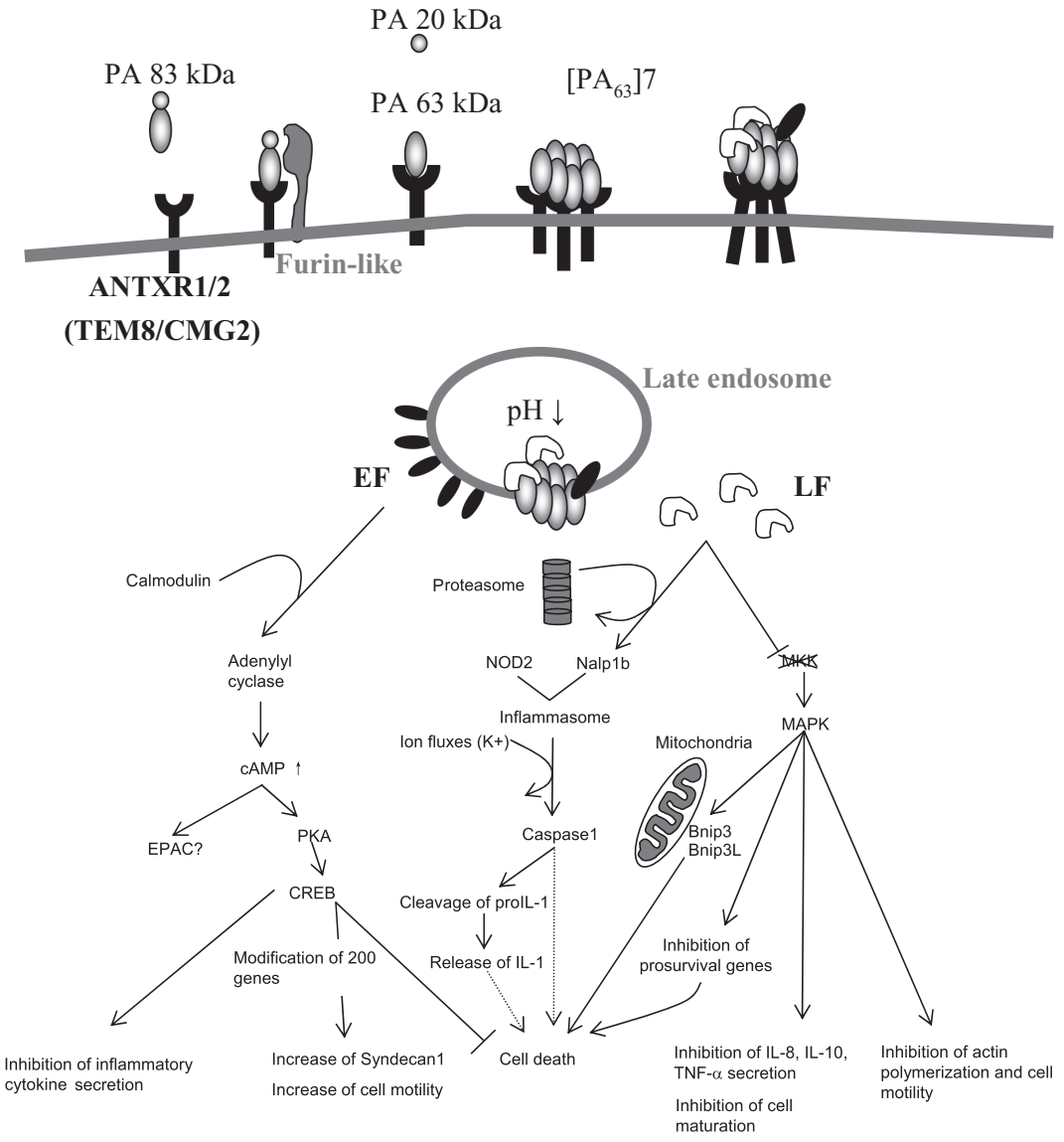

Figure 11.3 Model of the intracellular effects of lethal and edema toxins. After PA83–ANTXR interactions, PA83 is cleaved by a cell-surface protease (furin or furin-like), and PA20 is subsequently released from the complex. PA63 associates with lipid rafts and forms homoheptamers that associate with three molecules of EF and/or LF. LF and EF are translocated with PA into the late endosome. LF is released into the cytosol, whereas EF remains associated with the late endosomal membrane. EF is a Ca^{2+}-calmodulin-dependent adenylate cyclase that increases cAMP levels and induces cellular dysfunction through protein kinase A (PKA) and cAMP response-element binding protein (CREB). LF cleaves all mitogen-activated protein kinase kinases (MKKs), and MKK5 induces major cell dysfunction and death through MAPK pathway deregulation. NALP1b is another critical target for the initiation of cell death through caspase-1 and IL-1 secretion.

in cAMP response-element binding protein (CREB) phosphorylation and the activation of gene transcription (Puhar et al., 2008). By contrast, LF is a zinc metalloprotease that cleaves most isoforms of mitogen-activated protein kinase kinases (MAPKKs or MEKs) close to the N-terminus (Duesbery et al., 1998; Vitale et al., 1998, 2000). LF may also have other, less well-defined roles in *B. anthracis* pathogenesis. LF inhibits the activation of many cells of the innate and adaptive immune systems, including polymorphonuclear neutrophils (PMNs) (O'Brien et al., 1985), monocytes (Kassam et al., 2005), MΦs (Erwin et al., 2001; Pellizzari et al., 1999), T cells (Paccani et al., 2005), and B cells (Fang et al., 2006). The effects of LF on MΦ survival have revealed differences in susceptibility between mouse strains (Friedlander et al., 1993) independently of MEK cleavage (Alileche et al., 2005): some mouse strains (such as BALB/c) are highly susceptible to LT-induced MΦ death, whereas others (e.g. C57BL/6) are resistant. A recent study identified the locus associated with susceptibility to cell death as the gene encoding the inflammasome component NALP1b, as a potential target of LF (Boyden and Dietrich, 2006). This locus is highly polymorphic among mouse strains (Boyden and Dietrich, 2006). Inflammasome formation involves the association of NALP1 with NOD2, leading to caspase-1 activation (Fink et al., 2008). LF may induce the apoptosis of MΦs through p38α-dependent and p38α-independent pathways (Park et al., 2002). Very recent studies have revealed roles for the proteasome, potassium efflux, and caspase-1 activation in LF-induced cell death (Fink et al., 2008; Wickliffe et al., 2008a). Several mitochondrial proteins and members of the inhibitors of apoptosis (IAP) family are involved in controlling LF-induced cell death (Ha et al., 2007; Wickliffe et al., 2008b).

Several studies have analyzed the effects of toxins on DC functions in more detail (Agrawal et al., 2003; Tournier et al., 2005). A first study on spleen-derived DCs demonstrated highly inhibitory effects of LT on proinflammatory cytokine secretion, resulting in the impairment of T and B cell-dependent immune responses to ovalbumin (Agrawal et al., 2003). We and others confirmed these results with bone marrow-derived DCs (BMDCs) from two different LT-sensitive mouse strains (BALB/c and C57BL/6). We have also reported a cooperative disruptive effect following the addition of EF (Tournier et al., 2005). Similar results were obtained with DCs purified from mouse lung (Cleret et al., 2006). DCs from mouse strain with MΦs susceptible to LT killing are also susceptible to cell death (Alileche et al., 2005; Reig et al., 2008). The susceptibility of DCs to LT killing is due to the existence of a rapid, NALP1B-dependent pathway in susceptible mouse strains (Reig et al., 2008). In resistant mice, LT can kill DCs in a slow caspase-1-independent pathway triggered by the impairment of ERK1/2 pathways. DC maturation renders these cells resistant to LT killing early in this process and independently of the type of stimulus triggering maturation (Reig et al., 2008). Immature DCs in peripheral tissues can be killed by LT, whereas mature DCs in the LNs are unaffected. Human monocyte-derived DCs (MoDCs) have a similar susceptibility to LT-induced cell death to DCs from the resistant mouse strain C57BL/6 (Alileche et al., 2005). Two independent studies have also shown that LT and ET impair cytokine secretion by human MoDCs or primary blood DCs (Brittingham et al., 2005; Hahn et al., 2008). EF also disrupts monocyte (Hoover et al., 1994) and DC (Cleret et al., 2006; Tournier et al., 2005) functions, alone or in cooperation with LF. EF specifically inhibits IL-12p70 and TNF-α, a Th1 and a pleiotropic proinflammatory cytokine, respectively, but has no effect on production of the anti-inflammatory cytokine IL-10 (Tournier et al., 2005). Thus, anthrax toxins may fine-tune the balance of signals passing through DCs, inducing the terminal differentiation of T cells toward a Th2, rather than a Th1 immune response. ET strengthens the humoral immune response when administered subcutaneously or intranasally, suggesting that EF

favors the Th2 response (Duverger et al., 2006; Quesnel-Hellmann et al., 2006). Conversely, IFN-γ producing $CD4^+$ T cells play a critical role in vaccine-induced protection against encapsulated strains that do not produce toxins (Glomski et al., 2007a). The Th1 immune response may thus be crucial for host survival, and its inhibition by toxins may play a key role in pathogenesis. Analyses of Th1/Th2 imbalance, and of the potential role of toxins in the overinduction of Treg or impairment of the Th17 lineage may also constitute promising lines of research. The IL-10 induced by ET is indeed a critical cytokine for peripheral Treg induction. Finally, the anthrax toxins act in a complementary manner to abolish cytokine production by DCs—the critical function linking innate and adaptive immunity. The toxins then generate negative signals within the DC signaling network (Tournier and Quesnel-Hellmann, 2006).

The effects of anthrax toxins begin inside *B. anthracis*-containing phagosomes and extend distally throughout the host. The edema and LTs affect disease progression, from early to late stages. Three main phases can be described: (1) an invasion phase at the site of entry, where toxins have short-distance effects on the host; (2) a phase of bacterial proliferation in the secondary lymphoid organs, with deleterious effects mostly restricted to immune cells; and (3) a terminal diffusion phase consisting of the circulation of high toxin levels in the bloodstream, with long-distance effects on many organs, ultimately leading to death (Tournier et al., 2007). During the early stages of anthrax, toxins have a principally anti-inflammatory effect, blocking the recruitment of proinflammatory cells, and inhibiting both innate and adaptive immune cell functions critical for maintaining host health (Baldari et al., 2006; Tournier et al., 2007). Large amounts of *B. anthracis* toxins are subsequently released into the bloodstream, and these proteins disrupt endothelial cell function and cause shock plus cardiac dysfunction, resulting in significant morbidity and mortality (Watson et al., 2007).

Capsule and Phagocytosis

The PGA capsule is known to be a critical virulence factor, as the nonencapsulated Sterne strain is avirulent and has been used for more than 65 years as a live vaccine for veterinary medicine (Turnbull, 1991). The biochemical properties of PGA and details of its synthesis have been thoroughly covered elsewhere (see Candela and Fouet, 2006). The products of the four genes of the *CapBCAD* operon are sufficient for capsule production. In the inhalational model of infection, the *CapBCAD*-deleted mutant strain is much less virulent than the wild-type parental strain (Drysdale et al., 2005). CapD, a capsule depolymerase, also mediates the anchoring of the capsule to the bacterial cell wall (Candela and Fouet, 2005). The *CapD* mutant strain is less virulent than the wild type, suggesting that the capsule affords no protection if not anchored (Candela and Fouet, 2005). Little is known about the effects of the capsule on DC function. The capsule is believed to inhibit phagocytosis, although the mechanisms by which it does so are unknown (Keppie et al., 1963). Recent studies have shown that the capsule enhances the phagocytosis and killing of encapsulated bacteria after vaccination, suggesting that this structure is a critical target for phagocytes (Chabot et al., 2004; Schneerson et al., 2003). One elegant study showed that the treatment of bacilli with CapD decreases their phagocytosis by MΦ cell lines and human PMNs, suggesting that the capsule is essential for the avoidance of phagocytosis (Scorpio et al., 2007). The same team subsequently showed that the administration of CapD *in vivo* to mice infected with bacilli or spores protected these animals against infection, providing further evidence of the role of the capsule in host immune system evasion (Scorpio et al., 2008).

Recent data for human MoDCs have indicated that encapsulated strains induce lower levels of cytokine secretion than nonencapsulated bacteria, suggesting that the capsule may affect the recognition of PAMPs by the DC PRRs (Hahn et al., 2008). The capsule may provide "camouflage" against PRR recognition, disrupting the coordinated DC response. The capsule thus disrupts both the phagocytosis of encapsulated bacteria and PAMP recognition, impairing DC activation.

Capsule molecules shed into fluids *in vivo* rapidly accumulate in the splenic MΦs, the hepatic sinusoidal endothelial cells, and the Kupffer cells of the liver (Sutherland et al., 2008). These results also suggest that the capsule may favor the attachment of bacteria in the liver and spleen, which then serve as the primary target organs for proliferation. The role of the PGA released during infection remains unknown, although this molecule has been reported to inhibit host defense throughout the body (Uchida et al., 1993). It has recently been shown that LT is associated with PGA in the blood of moribund infected animals, suggesting that toxins and capsule may have a physically and functionally more complicated role (Ezzell et al., 2008).

DCs and Transport of *B. anthracis*: A "Trojan Horse" for Inhalational Anthrax?

Many studies have tried to identify the cell subsets responsible for transporting *B. anthracis* from the alveoli to the thoracic LNs. Several studies have highlighted the key role played by alveolar MΦs in the early events immediately following infection, including scavenging for spores (Cleret et al., 2007) and sporicidal activity (Ribot et al., 2006).

We recently showed, with spores of the nonencapsulated *B. anthracis* Sterne strain, that lung DCs play a much more important role than alveolar MΦs in capturing spores and transporting them to the thoracic LNs (Cleret et al., 2006, 2007). These results also raise questions about how DCs sample the alveoli. Does the entire cell migrate into the lumen or do DCs send extensions through the epithelium, as in the gut? Early reports on bronchial DCs in rats described dendritic extensions between epithelial cells (Holt and Schon-Hegrad, 1987; Sertl et al., 1986). It subsequently became clear that the dendritic extensions of these cells provided a very rapid mechanism for sampling airway and alveolar contents much more efficiently than could be achieved by the entire cell body crossing into the lumen, as shown in the gut. A recent study showed that such extensions into the trachea are triggered by killed bacteria (Jahnsen et al., 2006). We have shown that these extensions into the alveoli very rapidly and efficiently sample the environment within minutes of the inhalation of anthrax spores, immediately transporting any cargo they obtain to the thoracic LNs (Cleret et al., 2007). These data are consistent with very rapid kinetics of airway sampling by the pseudopod extensions of DCs into the airway lumen.

On the basis of these reports, DCs may serve as a "Trojan horse" for spore incubation and subsequent dissemination throughout the body (Cleret et al., 2007). These findings must be viewed in the more complex context of lung interactions with an "atypical" pathogen such as *B. anthracis*. Alveolar MΦs, DCs, and lung epithelial cells play highly coordinated roles, and further dissection of these roles is required to improve our understanding of anthrax pathogenesis (Tournier et al., 2007). For example, it remains unclear whether the uptake and transport of *B. anthracis* throughout the host are amplified by the bacterium, to increase its chances of survival. ET may, for example, increase the capacity of phagocytic cells to migrate, by upregulating syndecan-1 and several genes downstream from that encoding the CREB (Kim et al., 2008). We have also shown, with MoDCs, that

ET increases the migration of DCs toward CCL19/CCL21 and CXCL12, through the upregulation of CCR7 and CXCR4, respectively (Anne Quesnel-Hellmann, submitted).

Clearly, improvements in our understanding of *B. anthracis* pathogenesis and the virulence factors it uses are essential if we are to make anthrax control more effective.

CONCLUDING REMARKS

Kinetic interactions between DC subsets and *B. anthracis* during the course of infection are complex and partly account for the clinical features of the disease. Identification of the DC subsets involved in the capture and transport of spores and bacilli, susceptibility to the toxin, and the mechanism of evasion from DCs may increase our understanding of the disease and open up new possibilities for its treatment. Many aspects of the interactions between *B. anthracis* and DCs remain unclear. Future studies may shed light on new aspects of these critical interactions.

B. anthracis may also serve as a broader model of host–pathogen interactions, for investigations of the physiopathology of many other pathogens.

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Chapter 12

Bacillus anthracis Dissemination through Hosts

Ian J. Glomski

INTRODUCTION

Over a century ago, during the "Golden Age" of Microbiology, Robert Koch first developed and fulfilled his eponymous Koch's Postulates with *Bacillus anthracis* (Koch, 1876), thereby proving *B. anthracis* to be the etiological agent of the disease anthrax. *B. anthracis* is a spore-forming facultative anaerobic gram-positive bacterium that is immotile and forms characteristic "boxcar" chains under many growth conditions. *B. anthracis'* ability to form extremely durable dispersible spores and cause fatal disease quickly singled it out for development as a bioweapon and has been used as such since the early twentieth century by both governmental agencies and nongovernmental organizations (Abramova et al., 1993; Keim et al., 2001). Its use as a bioweapon in 2001 in the attacks emanating though the United States postal system has brought this pathogen to the forefront of the public's awareness of biological threats. These events greatly increased interest in eliminating the gaps in our scientific knowledge regarding basic aspects of its ability to infect and ultimately overwhelm a host.

Anthrax Pathology

Historically, anthrax has been primarily associated with herbivorous livestock and game. Indeed, some have proposed that the 5th Plague described in the Old Testament (Exodus 9:3), which killed all of the cattle of Egypt, was anthrax (Holmes, 2003). However, humans and most other mammals can also contract anthrax via alimentary, cutaneous, and inhalational routes of exposure to *B. anthracis* spores (Abramova et al., 1993; Inglesby et al.; 2002; Mock and Fouet, 2001). Most commonly, human infections initiate in those who work closely with animals or animal products, such as wool sorters or hide processors (CDC, 2008; WHO, 2008). Each of these three routes of infection manifests anthrax differently at early stages of infection, but unfolds similar to the other forms once the bacteria have spread systemically.

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

Cutaneous anthrax is the most common form of the disease in humans and accounts for greater than 95% of the cases worldwide (WHO, 2008). Fortunately, cutaneous anthrax is also the most treatable, where mortality drops below 1% with antibiotic treatment, but is approximately 20% in the absence of treatment. Cutaneous infections generally initiate when *B. anthracis* spores are introduced into preexisting lesions (Bischof et al., 2007). In humans, these cutaneous lesions begin as small painless boils that eventually become surrounded by a ring of vesicles followed by ulceration that ultimately develops into the characteristic "eschar," which is a black central crust that forms over the lesion (Spencer, 2003). Indeed, this black crust is the impetus for the name *anthracis*, which is derived from the Greek word for coal *anthrax*, although the eschar is rarely seen on animals besides humans.

The oral ingestion of spores can lead to infections of the alimentary tract and is believed to be the primary route of infection of grazing herbivores, where the plants being consumed are contaminated with *B. anthracis* spores. Similarly, humans may contract gastrointestinal anthrax by eating contaminated meat derived from infected animals (Beatty et al., 2003; Berthier et al., 1996). This version of anthrax is the most underreported since consuming contaminated meat is most likely to occur in unregulated rural areas where *B. anthracis* is endemic and technologies for positive diagnosis are absent. In some community-based studies, the incidence of anthrax of the alimentary tract even exceeded cutaneous infections (Sirisanthana and Brown, 2002), while also representing a more lethal form of the disease. Oropharyngeal infections in humans are characterized by fever and ulcers in the posterior oropharynx associated with lymphoadenopathy and neck swelling. Gastrointestinal infections occur most frequently in the stomach or bowel wall and are associated with nausea, vomiting, fever, and severe abdominal pain, and can progress to include bloody stool (Amidi et al., 1974; Dutz et al., 1970). Relative to cutaneous and inhalational anthrax, these forms are associated with a moderate mortality rate of approximately 40% with antibiotic treatment and 97% in the absence of treatment (Beatty et al., 2003).

Inhalation of *B. anthracis* spores causes the most lethal form of the disease and is the version that is of greatest concern when spores are weaponized, since broad dispersion of spores over a large area is relatively easily achieved. Previous to the deliberate contamination of the U.S. mail, the mortality rate for inhalational anthrax exceeded 95% even with antibiotic treatment, which is partly attributed to the difficulty of diagnosing the disease. Early symptoms are insidiously described as "flu-like," but later suddenly develop to include cyanosis, fever, and difficulty breathing attributed to pleural effusions (Spencer, 2003). If exposure is known and the case history is well established, such as with the treatment associated with many of the individuals involved in the U.S. mail incident, early intervention can reduce the mortality rate to approximately 45% (Brookmeyer and Blades, 2002).

Virulence Factors

B. anthracis is primarily differentiated from other members of the *Bacillus cereus* group through the presence of two plasmids that carry the structural genes for a tripartite toxin (pXO1) and capsule biosynthetic machinery (pXO2) (Ivanova et al., 2003). Loss of either of these two plasmids greatly reduces virulence in most animal model systems and is the basis of the earliest live-vaccine strains developed for animal usage (Turnbull, 1991). However, there is also substantial evidence that chromosomal and other unidentified

plasmid encoded genes likely have an effect on virulence, though perhaps not as profound as the toxins and capsule (Welkos, 1991).

Spores

A vital component in *B. anthracis* pathogenesis is the bacterium's ability to form spores. Though not specifically defined as a "virulence factor," the spore structure and its associated properties have an immense influence on *B. anthracis* pathogenesis. Spore formation is a complex multistep process that exists in both the genera *Bacillus* and *Clostridia*. Much of what is known about *B. anthracis* sporulation and germination has been established through comparison with the more extensively studied *Bacillus subtilis,* though significant differences between the two exist (Giorno et al., 2007). Most of the inner structures of *B. anthracis* are similar to *B. subtilis*, including the following: the core, which is highly dehydrated and contains the DNA and all the biochemical machinery necessary for establishing the nascent vegetative cell; the cortex, which is a thick peptidoglycan layer that gives the spore much of its durability against physical stress; and the proteinaceous coat, which has sieving properties that exclude some chemicals and enzymes from passing deeper into the spore (Nishihara et al., 1989). Unlike *B. subtilis*, *B. anthracis* has an additional layer beyond the coat called the exosporium, which is a loose-fitting sac-like structure that is the outermost layer of the spore (Gerhardt, 1967).

Toxins

B. anthracis produces a tripartite A-B-type toxin that consists of protective antigen (PA), lethal factor (LF) and edema factor (EF). PA in complex with LF is referred to as lethal toxin (LT) and PA complexed with EF is called edema toxin (ET), though it is likely that at times all three units are in a single ternary complex (Young and Collier, 2007). PA is the receptor-binding (B) unit that binds anthrax toxin receptors (ANTXR) 1 or 2 (Bradley et al., 2001; Scobie et al., 2003). Upon binding ANTXR1 or 2 on a wide variety of cells, PA is cleaved by cell surface proteases, which induces heptamerization and allows the binding of LF and/or EF (Young and Collier, 2007). This complex is then endocytosed and upon acidification of the endosome, the PA heptamer forms a pore that gives the enzymatically active EF and LF (A) units access to the host cell cytosol (Melnyk and Collier, 2006).

Within the host cell cytosol, the LF metalloprotease specifically cleaves most MEKs and inhibits the production of cytokines in a number of different cell types (Agrawal et al., 2003; Duesbery et al., 1998; Glomski et al., 2007b; Paccani et al., 2005). Cytosolic activity of LF can also cause cell death, to which macrophages are particularly sensitive. The exact mechanisms of LF-induced cell death have not been completely elucidated but appear to involve the NOD-like pattern recognition receptor Nalp1b that is a component of the inflammasome (Boyden and Dietrich, 2006). It is unclear whether there is any direct connection between MEK cleavage and cell death, for cells that are resistant to killing still undergo MEK cleavage (Pellizzari et al., 1999; Watters et al., 2001). The ability of LT to cause cell death has also led to the suggestion that host death is primarily attributed to LT. This is supported by the observations that despite effective elimination of bacteria with antibiotics, hosts often die nonetheless (WHO, 2008), purified LT can be lethal (Moayeri et al., 2003), and neutralizing antibodies protect immunized hosts from lethal infection (Pitt et al., 1999). However, it is difficult to conceive that the bacterial load at the time of

death (ranging from 10^5 to 10^9 CFU/mL tissue depending on the host species) can also be readily supported by the host.

Within the host cytosol EF associates with host cell calcium and calmodulin to become an activated adenylate cyclase with enzymatic activity that converts ATP to cAMP to levels 1000-fold higher than those achieved by endogenous adenylate cylases (Barth et al., 2004; Leppla, 1982). As its name suggests ET induces edema in the host tissues, yet the mechanisms by which this phenomenon occurs remains undetermined (Tessier et al., 2007). Furthermore, the role of ET in anthrax pathogenesis is less well defined than LT; however, it is clear that elimination of EF reduces *B. anthracis* virulence (Pezard et al., 1991). Like LT, ET has been shown to modulate cytokine secretion by immune cells to suppress the immune response (Hoover et al., 1994; Tournier et al., 2005). Recently, it has been reported that purified ET is lethal in the mouse model of intoxication (Firoved et al., 2005). Mice injected with purified ET had extensive lesions in tissues including adrenal glands, lymphoid organs, bone, bone marrow, gastrointestinal mucosa, heart, and kidneys, which likely led to death by multiorgan failure. The lethality of ET remained unknown for years because of the technological difficulties of producing sufficient quantities of highly pure EF.

Capsule

A vital component for full virulence of *B. anthracis* is a capsule. The *B. anthracis* capsule consists of gamma-linked poly-glutamic acid covalently linked to the cell wall (Candela and Fouet, 2005; Richter et al., 2008), which is a rarity among bacteria. The poly-γ-glutamic acid capsule is formed soon after spore germination within a host and inhibits phagocytosis by host phagocytes, functions as a nonimmunogenic surface, and may inhibit complement activation when the bacteria are exposed to serum components (Makino et al., 1989, 2002; Schneerson et al., 2003). Bacteria that lack capsule are particularly sensitive to complement-mediated clearance, since C5 depletion sensitizes mice to infection with avirulent non-capsulated strains (Harvill et al., 2005). Reports from the mid-twentieth century introduced the concept that capsule liberated from the bacteria may also have independent effects on the host, referring to this as "aggressin" activity (Zwartouw and Smith, 1956), but relatively little data since have supported this concept beyond some indication that free poly-γ-glutamic acid may act as a "decoy" for complement activation at a distant site from the vegetative bacteria (Makino et al., 2002). The decoy activity of released capsule consists of activation of the complement cascade at a site that is not immediately adjacent to the bacterial body, and thus the lytic and chemotactic effects of complement activation are ineffective.

Other Virulence Factors

Toxins and capsule are the best characterized virulence factors utilized by *B. anthracis*, yet there are a growing number of factors being reported that fit the National Library of Medicine's definition of a virulence factor: "Those components of an organism that determine its capacity to cause disease but are not required for its viability per se." However, many of these factors do not have as dramatic an effect on virulence as the traditionally recognized virulence factors, but may play more nuanced roles that additively help define *B. anthracis* pathogenesis.

A broad class of virulence factors includes those that perform their function through enzymatic activity. In *B. anthracis*, phospholipase Cs (PLC) and proteases have both been

proposed to act as virulence factors. There are at least three PLCs encoded in the *B. anthracis* genome that redundantly contribute to virulence and downregulate immune cell function (Heffernan et al., 2006; Zenewicz et al., 2005). Over 11 families of proteases are represented in the genome, but few have been analyzed with regard to their role in pathogenesis, since most attention has been focused on the metaloprotease activity of LF (Popov et al., 2005). Nevertheless, expression of some of these proteases is controlled by the virulence plasmid pXO1 and a few have been shown to contribute to virulence (Aronson et al., 2005; Popov et al., 2005).

For many years, it was thought that the only exotoxins produced by *B. anthracis* were LT and ET; however, more recently, anthrolysin O (ALO), was identified on the *B. anthracis* chromosome (Mignot et al., 2001). ALO is a member of the cholesterol-dependant cytolysin (CDC) family that forms large oligomeric pores in cholesterol-containing membranes. Functions of other CDCs include mediating escape of *Listeria monocytogenes* from phagosomes to the cytosol via the activity of listeriolysin O and effector translocation for *Streptococcus pyogenes* via the activity of streptolysin O (Madden et al., 2001; Portnoy et al., 2002). Determining the role of ALO in *B. anthracis* has been more elusive since deletion of the gene *alo* did not significantly affect the LD_{50} (Heffernan et al., 2007). Yet concomitant deletion of three PLCs along with *alo* showed a virulence defect that was eliminated if *alo* was complimented *in trans* (Heffernan et al., 2007). Furthermore, exploration of the importance of ALO to *B. anthracis* pathogenesis via immunization with ALO yielded mixed results. Immunization with a non-pore forming ALO toxoid protected mice against challenge with purified toxin, but offered no protection against challenge from intraperitoneal *B. anthracis* spores (Cowan et al., 2007). Alternatively, some monoclonal antibodies raised against ALO appear to prolong the survival time of mice intravenously infected with *B. anthracis* spores (Nakouzi et al., 2008), yet it remains unclear whether these monoclonal antibodies are inhibiting ALO function or acting to opsonize ALO-expressing bacteria, leading to greater bacteria clearance.

ENTRY

The durable spores of *B. anthracis* are considered the infectious form of the organism, since introduction of spores cause disease in animal models, and there is little evidence of long-term growth of vegetative bacteria in the environment (Titball et al., 1991). As described above, the distinct forms of anthrax (cutaneous, alimentary, or inhalational) largely depend on the route by which spores are introduced into a potential host. Many factors come into play in the process of a quiescent environmental spore entering a host and establishing an infection. Some factors include environmental conditions; livestock tend to be infected after a hot and dry period, while it is also clear that the health status of the host is important (Abramova et al., 1993; Gleiser et al., 1963; Glomski et al., 2007c, WHO, 2008).

Predispositions

A common factor that increases the likelihood of infection to all forms of anthrax is damage to epithelial barriers, though infections also appear to occur in the absence of known epithelial breaches. Indeed, most cutaneous infections are thought to initiate in breaks in the skin, whether it is due to insect bites or physical trauma (Bischof et al., 2007; Davies, 1983). In retrospective necropsies on 42 humans that died from the accidental

release of *B. anthracis* spores from the military weapons facility near Sverdlovsk in the former USSR, 11 individuals had a single primary focus of *B. anthracis* infection associated with a Ghon focus lesion caused by *Mycobacterium tuberculosis* infections (Abramova et al., 1993). The increased susceptibility of infection caused by epithelial damage has also been observed in experimental settings. In their studies of inhalational anthrax in *Macaca mulatta* (Rhesus monkey), Gleiser et al. (1963) noted an association of *B. anthracis* infections with lung mite lesions, which was absent when lung mites were eliminated (Fritz et al., 1995). Likewise, in her seminal manuscript, Joan Ross noted that in her experimental guinea pigs there was vegetative *B. anthracis* growth associated with damage caused by her catheterization of the trachea during spore delivery while bacteria remained as spores in undamaged areas (Ross, 1957). In a similar vein, abrasions caused in the larynx by the intubation of the trachea for instillation of spores into the lungs of mice gave rise to infections of the larynx at a rate much more rapid than otherwise seen in noninvasive methods of introducing spores (Glomski et al., 2007c). Furthermore, is has been proposed that the association of anthrax in livestock with hot and dry weather may be caused by the ingestion of dried vegetation that are more likely to cause internal abrasions to the alimentary tract and pharynx, thereby promoting infection (Friedlander, 1997).

Germination

A vital step in the infective process is the conversion of a dormant spore into a virulent vegetative bacillus. Germination can be measured through a number of parameters; the most common include sensitivity to killing by heat, transitioning from phase-bright to phase-dark oblong bodies in a light microscope, and loss of optical density at 600 nm. Five different germination pathways have been identified in *B. anthracis*. The alanine pathway only requires L-alanine or at a greater concentration L-proline to induce germination, but requires concentrations that far exceed those predicted to be present within in a host (Fisher and Hanna, 2005). There is also a second amino acid sensing pathway that is responsive to aromatic amino acids, including L-histidine, L-tyrosine, or L-tryptophan. The threshold for amino acid-based germination induction can be reduced through the addition of co-germinants, in particular purine ribonucleosides which do not substantially induce germination alone, but synergize with amino acids (Ireland and Hanna, 2002). Interestingly, the stereoisomer of L-alanine, D-alanine, can act as a germination inhibitor (Hu et al., 2006; McKevitt et al., 2007).

Six genetic loci have been identified within the *B. anthracis* chromosome (*gerA, gerH, gerK, gerL, gerS,* and *gerY*), and one is carried upon the pXO1 virulence plasmid (*gerX)* (Read et al., 2003). The *gerA* and *gerY* loci contain frameshift mutations, and thus are not predicted to influence germination. The remaining five loci interact in a complex network of interdependence that defines the spore's sensitivity to the *in vitro* germinants defined above. The specific germinants responsible for germination within a host remain unidentified, though tissue culture assays suggest that macrophages condition media to promote germination through the secretion of inosine and amino acids (Weiner and Hanna, 2003).

Eliminating the function of either *gerH* or *gerX* reduce spore germination induced in tissue culture with macrophages and protects the bacteria from killing (Hu et al., 2007), suggesting that spores must germinate to be destroyed by host defenses. Indeed, a spore-associated alanine racemase that can convert L-alanine to D-alanine acts as a spore density-dependant control on germination (McKevitt et al., 2007). High densities of spores convert L-alanine into D-alanine to prevent germination and increase virulence, presumably by

delaying germination to prevent destruction by macrophages until the spores have dispersed and disseminated to favorable anatomical locations.

Germination of spores clearly occurs within the host, but when, where, and by what means remain unclear. A substantial amount of evidence suggested that spores remain in a dormant state for long periods of time and do not grow when situated within the lumen of the lung (Abramova et al., 1993; Brookmeyer and Blades, 2002; Cleret et al., 2007; Cote et al., 2006; Friedlander et al., 1993; Henderson et al., 1956). However, it is not yet known whether the particularly low rate of germination in the lung is due to the lack of germinants or the active repression of germination through host or bacterial factors. The fact that vegetative bacteria have been observed in the lung in close association with lesions (Gleiser et al., 1963; Ross, 1957), which may allow the leakage of serum components into the airspace, support the conclusion that the cause of extended dormancy is a lack of germinants and/or nutrients. The role of some types of serum as a germinant has been established in cell culture (Hu et al., 2007) and appears to be sufficient for germination in abrasion models of cutaneous anthrax in mice (Bischof et al., 2007). Less data is available regarding the germination of spores in the alimentary tract except that a small percentage of spores injected intragastrically germinate precociously in the mouse gut to become heat-sensitive in fecal pellets (Glomski et al., 2007c). The influence of engulfment by a phagocyte on the relative rate and efficacy of germination versus extracellular germination within a host remains unclear.

Aerosol Size

Entering a host through both cutaneous and oral inoculation of *B. anthracis* spores is likely dependant on local circumstances; the potential host must either have spores introduced into a break in the epithelial barrier or eat food contaminated with an infectious dose of spores. There is little information regarding whether the vehicle of their delivery affects the likelihood of the establishment of infection. On the other hand, there is significant evidence that the particle size of spore aerosols greatly affect the location of spore deposition in the airways and that there is a correlation between particle size and infectivity for inhalational anthrax. In a classic study, Druett et al. found that airborne spore particles less than 5 μm were approximately 17 and 14 times and more infective than 12 μm particles in guinea pigs and Rhesus monkeys, respectively (Druett et al., 1953). These researchers attributed much of this difference to the observations of their colleagues that large particles are primarily deposited in the upper respiratory tract, and they proposed that this location may be more refractory to infection than the lung (Harper and Morton, 1953). Similar findings on the connection between particle size and location of spore deposition have withstood the test of time (Thomas et al., 2008), but it should be noted that monkeys that had large particles delivered to the upper respiratory tract also developed fulminant lethal infections that included massive edema of the face and head that persisted for a day or two before they succumbed to death (Harper and Morton, 1953). Upper respiratory tract infections have also been observed in mouse models of inhalational anthrax (Glomski et al., 2007c, 2008; Loving et al., 2009), suggesting that this phenomenon is not restricted to primates. Like oral inoculation of spores, which can give rise to infections in the upper or lower alimentary tract with distinct characteristics, it appears a similar situation exists for inhalational anthrax.

We must then ask ourselves, what is the most likely size of spore particle that is expected to be inhaled under natural or man-made spore aerosolization settings? The

answer to this question will define which of these two locations is most relevant to the prevention of inhalational anthrax in humans. Lacking the ability to experimentally determine this answer, the best way to establish the likelihood of one form of infection over the other would be to review the data from known human exposures. Unfortunately, two of the most well-documented incidents of human anthrax, Sverdlovsk in the former USSR and the attacks propagated through the United States postal system in 2001, likely occurred with highly refined weaponized spores that were conscientiously milled to maximize infectivity and thus might not be representative of the range of spore-aerosol particle sizes produced by non-intentional means or poorly deployed/produced weaponized spores (Dance, 2008; Guillemin, 1999). Thus, it seems both portals of entry merit greater analysis when pursuing a better understanding of *B. anthracis* uptake and invasion in inhalational anthrax.

Adhesion

Once *B. anthracis* has entered a host, it must then adhere to tissues to ultimately allow invasion. Presumably, these interactions first take place between the outermost portion of the spore, the exosporium, and the host. The exosporium is a loose-fitting sac-like structure composed of proteins, lipids, and carbohydrates that has a fine layer of hair-like fibers pointing outward from its most exterior surface (Gerhardt and Ribi, 1964; Matz et al., 1970). Over a dozen proteins have been identified in the exosporium by proteomic and traditional approaches (Liu et al., 2004; Redmond et al., 2004; Steichen et al., 2003), but the immunodominant protein BclA has garnered the most attention.

BclA, *Bacillus* collagen-like protein A, is a major constituent of the hair-like "nap" on the outside of the exosporium and defines the length of the hairs (Sylvestre et al., 2002, 2003). A number of studies suggest that BclA has no significant influence on *B. anthracis* virulence (Bozue et al., 2007a; Sylvestre et al., 2002), yet to the contrary, others have found that mutant bacteria that do not express BclA are more virulent and cause shorter mean times to death (Brahmbhatt et al., 2007; Oliva et al., 2008). Interestingly, this increase in virulence was attributed to BclA functioning as an anti-adhesin. In the absence of BclA, *B. anthracis* spores promiscuously bind most cell-types (Bozue et al., 2007b), whereas the wild-type spores only interact with phagocytes (Oliva et al., 2008). By these means, it was hypothesized that *B. anthracis* specifically promotes interactions with phagocytes, which may ultimately promote entry into the host in a manner that is evolutionarily beneficial. An additional function for BclA is its barrier function. Antibodies that target structures further toward the interior of the spore than BclA are normally blocked from interacting with their target, but the elimination of BclA allows them access to their antigen (Cybulski et al., 2009). It has not yet been demonstrated that this function plays a role in the progression of the disease, but one might propose that exclusion of antibodies may decrease the deposition of complement components and therefore reduce the targeting of spores for elimination by the innate immune system.

Depending on the timing and location of germination, proteins associated with the vegetative bacterium may also play a role in adhesion to host tissues, since the exosporium is quickly lost after germination (Steichen et al., 2007). *B. anthracis* S-layer protein A (BslA) was recently identified as an adhesin coded for by a gene within the pXO1 virulence plasmid (Kern and Schneewind, 2008). BslA is expressed under host-like conditions and binds to the S-layer covering vegetative bacteria to mediate adhesion to a variety of human cells. The S-layer is a paracrystalline surface structure formed on the exterior of the bacte-

rial cell wall, but beneath the capsule (Mock and Fouet, 2001). The binding partner(s) of BslA have not yet been identified. Determining if and when BslA has the opportunity to interact with host cells during infection is complicated by the envelopment of the vegetative bacteria by a capsule that is covalently linked to the cell wall (Candela and Fouet, 2005). Indeed, there is evidence that suggests that the presence of the capsule can greatly affect the dissemination of *B. anthracis* in mouse models of infection (Drysdale et al., 2005, Glomski et al., 2007a,c), yet at this point in time, data have not been produced that establishes whether the capsule alters dissemination through activity of its own or by blocking adhesins on the surface of the bacterial body. Furthermore, it is not clear that the capsule is expressed at all times during infection (Mesnage et al., 1998), which may allow modulation of access to BslA based on the presence or absence of capsule.

A number of bacterial pathogens produce proteins that bind components of the mammalian extracellular matrix. Among others, these microbial surface component recognizing adhesive matrix molecules (MSCRAMM) include *Staphylococcus aureus* CNA (Patti et al., 1992), *Yersinia enterocolitica* YadA (Skurnik et al., 1994), and *Enterococcus faecalis* ACE (Rich et al., 1999). Similarly, two cell-wall anchored MSCRAMMs (BA0871 and BA5258) were identified within the genome of *B. anthracis* (Xu et al., 2004). Like BslA, BA0871 and BA5258 are expressed by vegetative bacteria, and their ligand has been identified as collagen by biochemical analysis with surface plasmon resonance. Additionally, they mediate bacteria-to-cell adhesion when expressed in a heterologous system with *Staphylococcus carnosus*. Since collagen is a major component of the skin, the authors of this study hypothesize that BA0871 and BA5258 promote the retention of *B. anthracis* at the site of cutaneous infection. However, much like BslA, the presence of the capsule complicates the interpretation of how these cell-wall linked proteins traverse the thick capsule to gain access to their ligands located on host tissues.

INVASION

B. anthracis is generally considered an extracellular pathogen once an infection has been established. However, at early stages, *B. anthracis* are at least transiently intracellular, which would justify the claim that *B. anthracis* can be called an invasive organism, albeit at a relatively low level. The earliest evidence for intracellular bacteria was obtained through the study of macrophages (Dixon et al., 2000, Guidi-Rontani et al., 1999; Ross, 1957), but likely can be generalized to include dendritic cells (Brittingham et al., 2005, Cleret et al., 2007). Ungerminated spores are phagocytosed by macrophages, germinate and grow intracellularly, and then escape from the cell (Ruthel et al., 2004). Whether bacterial toxins are necessary for these events to take place and whether these events are phagocyte-type dependent have been debated with no clear resolution (Dixon et al., 2000; Guidi-Rontani et al., 2001; Ruthel et al., 2004).

The Trojan Horse Model

From the coupling of the observations of phagocytes carrying a spore through the lymphatics that drain the lung toward the tracheo-bronchial lymph nodes (Cleret et al., 2007; Ross, 1957), the germination and outgrowth of spores within macrophages (Ruthel et al., 2004), and clinical manifestations of inhalational anthrax including mediastinitis (Friedlander, 1997), the most widely accepted “Trojan horse” model was born (Guidi-Rontani, 2002). In this model, inhaled dormant spores enter the alveoli and are phagocytosed by alveolar

macrophages. In response to bacterially derived signals, these macrophages then migrate to the mediastinal lymph nodes that drain the lung in their typical response designed to promote their function as antigen presenting cells to the adaptive immune system. While in transit or upon arrival in the lymph node, the spores germinate intracellularly, begin to grow and express virulence factors, and ultimately kill the "Trojan horse" host-cell that transported the bacteria through the epithelial barrier and introduced them into the lymphatics. Once extracellular and producing its full array of virulence factors, the bacteria then rapidly multiply, protected from future phagocytosis by the capsule and the immunocrippling effects of LT and ET. After the lymph node is overwhelmed, the bacteria then gain access to the circulatory system through the return of lymph to the blood via the thoracic or right lymphatic duct.

Revisiting the Trojan Horse Model

Though not necessarily named as such, models similar to the Trojan horse model of inhalational *B. anthracis* infection have existed since the 1950s. More modern studies have begun to suggest that aspects of this model should be revisited, with particular emphasis on invasion of host cells. Most significantly, a number of studies now demonstrate that normally non-phagocytic cells, such as endothelia, epithelia, and fibroblasts can engulf spores and vegetative bacteria in an actin-dependant manner (Russell et al., 2007; van Sorge et al., 2008). In cell culture, spores are engulfed by primary human lung epithelial cells and can transcytose through the A549 lung epithelial cell line without disrupting the barrier function of the cells (Russell et al., 2008). Spore uptake by non-phagocytic cells occurs at a rate lower than that seen for professional phagocytes. Like spores within macrophages, spores within epithelial cells also germinate and begin to grow, representing a majority of the bacteria found on the basolateral side of the epithelial later after transcytosis. The concept of invasion of epithelia is particularly attractive since macrophages have been demonstrated to be very effective at killing spores (Hu et al., 2006; Kang et al., 2005); thus, by avoiding exposure to macrophages, the bacteria may be able to emerge on the opposite side of an epithelial barrier in a form that is primed and ready to deal with immune defenses through the active production of virulence factors. Further supporting a model that excludes spore transport by phagocytes is that inhalational infections can occur in the absence of alveolar macrophages (Cote et al., 2004, 2006).

Modes of initiating inhalational anthrax, like the upper respiratory tract infections in Rhesus monkeys described above, which do not include an initiation of infection in the thorax and thus initiate through mechanisms not described by the classic Trojan horse model, clearly occur. In mouse models of inhalational infection that introduce spores in an aerosol, by intratracheal intubation, or by intranasal instillation, the first site of bacterial growth occurs in the nasal passages, more specifically, the nasal-associated lymphoid tissues (NALT) (Glomski et al., 2007c, 2008; Loving et al., 2009). NALT are similar in structure to gut associated lymphoid tissues (GALT), such as the Peyer's patch, which has also been implicated as a portal of entry for gastrointestinal anthrax (Glomski et al., 2007c). Interestingly, at a time point when there is rapid multiplication of bacteria in the NALT, bacteria can be found in the lungs and mediastinal lymph nodes, yet these bacteria are still in the dormant spore state and thus have not even begun to grow (Glomski et al., 2007c, 2008; Loving et al., 2009). These observations were made possible through the development of imaging technologies (called *in vivo* Bioluminescent Imaging—BLI) that allow the detection of light produced by metabolically active bacteria (not spores) through-

out the entire body of the mouse in an unbiased manner. A particular targeted tissue does not need to be removed from the host at a particular time to obtain the data; thus, the entire body of a single animal can be observed noninvasively from the initiation of infection until death or resolution. BLI also allows researchers to detect when infections initiate in an unintended manner, such as in undetected abrasions in the airways caused by experimental technique (Glomski et al., 2007c).

As proposed by the Trojan horse model of inhalational anthrax, spores only germinate upon exiting the lung lumen within a phagocyte. However, using a *B. anthracis* construct that expresses light under the control of a promoter that activates upon germination (*sspBp*) and BLI, Sanz et al. (2008) were able to detect spore germination within the lungs of mice. Complicating interpretation of these data is that germination does not always lead to growth, for germination makes *B. anthracis* susceptible to destruction (Hu et al., 2006). It is unclear then, whether those recently germinated spores represent bacteria that will soon be destroyed or those that are now capable of growth and disseminated disease. In a similar fashion, bacterial constructs that produced light throughout *in vivo* growth examined with BLI demonstrated that not only do spores germinate but also actively grow within the lung tissues (Glomski et al., 2008), albeit only when very high numbers of spores are used. In both studies, microscopic analysis demonstrated vegetative bacteria within the alveoli, either free or within phagocytes. What remains uncertain is whether isolated *in situ* germination and growth leads to host death or whether a portion of these bacteria are transported to the draining lymph nodes as described by the Trojan horse model, or for that matter, whether the large numbers of spores used to inoculate the lungs are in any way representative of likely real-world scenarios.

Conceivably, infections that initiate in the NALT or lumen of the lung could also lead to the diagnostic human clinical manifestation of mediastinitis. This relation is easily envisioned for bacterial growth in the lumen of the lung: bacteria and/or toxins could drain into the mediastinal lymph nodes from the lung upon breakdown of epithelial barriers after significant bacterial growth. Growth in the distant NALT could lead to mediastinitis via its connection to the lymphatics. Bacteria in the NALT soon spread to the submandibular lymph nodes giving access of both bacteria and secreted toxins to the lymph system (Glomski et al., 2007c). From the submandibular lymph node the lymph drains into the right lymphatic duct, enters the blood circulation, passes through the right side of the heart to then have its first encounter with a fine capillary bed, the lungs. With such a great surface area and narrow diameter of vessels, the lung is the most likely place for bacteria to lodge or toxins to bind receptors after exiting from the lymph nodes. Numerous studies have reported the hematogenous spread of *B. anthracis* to the lungs (Abramova et al., 1993; Drysdale et al., 2005; Glomski et al., 2007c, Loving et al., 2009). The exotoxic activity of ET to induce edema and the ability of LT to alter endothelial cell permeability could both contribute to the mediastinal widening associated with human inhalational anthrax even in the absence of closely associated bacteria (Warfel et al., 2005). This sequence of events is supported by recent data reported by Loving et al. that demonstrate that inhaled toxin-producing non-capsulated bacteria primarily remain as dormant spores within the lungs and mediastinal lymph nodes of mice until late stages of infection when vegetative bacteria are detected, likely arriving hematogenously. Since this scenario would require a significant amount of bacterial growth and invasion to take place before mediastinal widening occurs, this may help explain why it is so difficult to prevent the death of a human who has progressed as far as mediastinal widening. Indeed, Fritz et al. (1995) noted that mediastinitis is more common in animals that survive longer. The widening only manifests late in the infection long after large amounts of toxin have been secreted

and bacteria have covertly multiplied in a distant site, a time point at which antibiotics can have less of a beneficial impact on outcome.

Ultimately, the question of which of these newfound and classic portals of inhalational entry is the most relevant to infection depends on a number of factors, including: (1) what are the size of the inhaled spore particles and thus, where are most of them deposited; (2) how many total spores are deposited in a particular location; (3) despite the presence of *B. anthracis* in the alveoli, the NALT, and mediastinal lymph nodes, which population overcomes local host defenses and in due course disseminate systemically; and (4) whether the invasion of NALT and germination of spores in the alveoli is applicable to other host species beyond the mouse. Only through future research can the relative contribution of each of these factors be determined.

DISSEMINATION

Dissemination of *B. anthracis* throughout a host depends on the bacterium's ability avoid destruction by host defenses, to acquire nutrients and grow, and transit to new tissues. Lack of the ability to perform any one of these functions would drastically alter the outcome of infection. As one would expect, *B. anthracis* has evolved means by which to address each of these obstacles to such a degree that that a majority of inhalational and gastrointestinal infections are lethal, and if *B. anthracis* can overcome the spatial limitations of cutaneous infections, death is also highly likely.

Early Survival

Coping with the Oxidative Burst

The earliest events that promote *B. anthracis* survival within a host largely depend on spore functions. In part, survival can be attributed to the durability of the spore; while dormant, it resists most antimicrobial defenses, whether those defenses are extracellular or intracellular. However, there are also active spore defense mechanisms that include enzymes to counter antimicrobial oxidative stresses that could negatively affect nascent vegetative bacteria. Two superoxide dismutases (SOD) are found in the exosporium (SODA1 and SOD15) and another two (SODA2 and SODC) can become spore-surface associated if the previous two are deleted (Cybulski et al., 2009; Steichen et al., 2003). Functionally, these SODs are redundant, with some indication that SODA1 has dominant function (Passalacqua et al., 2006), but together act to protect vegetative bacteria from oxidative stress, protect the bacteria when spores are phagocytosed by macrophages, and promote virulence in mouse models of cutaneous and inhalational infection (Cybulski et al., 2009). In a similar manner, an exosporium-associated arginase scavenges arginine from macrophages to competitively reduce the enzymatic production of reactive nitrogen species from arginine by host NOS2, and thereby protect nascent vegetative bacteria from the macrophage's oxidative defenses (Weaver et al., 2007). Additionally, lethal toxin has been demonstrated to inhibit the oxidative burst of human neutrophils (Crawford et al., 2006).

Interestingly, oxygen radicals stimulate spore germination (Baillie et al., 2005), perhaps acting as a signal to indicate that the spore has entered a phagocyte and is now in an environment that is favorable to growth. These observations highlight the balance *B. anthracis* must maintain between remaining in a dormant spore, and resisting host defenses, and germinating, potentially within the very cell that is capable of killing the

bacterium. It is likely that this interplay is highly dependent on the timing of the interaction and the activation status of the engulfing phagocyte, since once macrophages become activated, they are more anthracidal (Kang et al., 2005).

Role of Toxins

B. anthracis toxin expression is quickly induced, on the order of 15 min, soon after germination within a virulence-factor inducing environment (Cote et al., 2005). Significant cell culture data suggest that LT and ET can modulate the function of a wide variety of immune effector cells, including macrophages, dendritic cells, T and B cells, and neutrophils, to exert a generalized effect of suppressing cytokine production (Baldari et al., 2006). However, it should be noted that a number of studies report that the toxins have immunostimulating functions as well (Kim et al., 2008; Warfel et al., 2005; Xu et al., 2008). *In vivo* the data are more scant. Some studies have shown that serum cytokine levels increase upon infection with spores after the first 3 days of a murine infection (Pickering et al., 2004), but the cytokine response has been analyzed primarily at the systemic level rather than examining activity on specific cell types. If cell culture data are taken to be representative of what is occurring inside a host, one would predict that newly germinated spores would inhibit the activation of local innate immune cells and prevent the chemotaxis-signaling cascade from recruiting greater numbers of immune effector cells to the site of infection. This process would decrease both the cidality of the immune effectors and reduce their effective numbers, perhaps allowing *B. anthracis* to grow in a less restrictive environment. In support of this notion, a recent study by Cote et al. (2008) found that supplementing the murine immune system with macrophages that were insensitive to both *B. anthracis* toxins increased their resistance to infection. This suggests that the toxins directly intoxicate macrophages to prevent them from countering *B. anthracis* growth. Future studies will need to focus on other effector cells to determine if this aspect of toxin function is broadly applicable.

Nutrient Acquisition and Growth

The tissues of vertebrates are generally considered nutrient-rich environments for microbial growth. Reflecting this property, the genome of *B. anthracis* reveals its essentially obligate pathogen lifestyle in that its metabolism is particularly well tuned for taking advantage of a protein-rich environment (Read et al., 2003). Bacteria, including most pathogens, including *Mycobacterium tuberculosis* (De Voss et al., 2000), *Yersinia pestis* (Bearden et al., 1997), and *Klebsiella pneumoniae* (Nassif and Sansonetti, 1986), also have an acute need to acquire iron from their surroundings to maintain numerous basic processes necessary for life. The microbial requirement for iron is taken advantage of by vertebrate innate defenses in the form of extensive iron sequestration that limits the growth of bacteria that do not have specific countermeasures to deal with the resultant iron deprivation (Bullen et al., 2006). Extracellular iron is primarily bound by transferrin, lactoferin, and heme within vertebrates. *B. anthracis* can acquire iron from these molecules through the use of siderophores (Garner et al., 2004), small secreted chelating molecules with extremely high binding affinities for iron, or heme oxygenases that degrade heme to release the bound iron (Skaar et al., 2004).

Once bound to iron, a siderophore binds a bacterial receptor and is internalized in an ATP-dependant manner to deliver the iron to the bacterial cytosol. The most relevant

siderophore for promoting infection by *B. anthracis* is petrobactin. The gene cluster *asbABCDEF* codes for petrobactin synthesis and function. Deletion of asbA eliminates the ability of *B. anthracis* to grow in iron-limiting conditions or within macrophages, and reduces the bacteria's virulence by approximately three logs (Cendrowski et al., 2004). Interestingly, *B. anthracis* is the only pathogen known to produce petrobactin, and it is not bound by the mammalian "anti-siderophore" defense protein siderocalin (Abergel et al., 2006).

Iron bound by heme represents the vast majority of the total iron found in vertebrates (Skaar et al., 2006), and thus is an abundant resource of iron for pathogens that are able to extract it from heme proteins and eventually release it from the heme porphyrin ring. Such a system has been identified within *B. anthracis* through homology to the *S. aureus* iron-regulated surface determinant (*isd*) locus. All the proteins coded for within the *isd* locus have not been fully characterized in *B. anthracis*, but a few of the major players have been identified. IsdC is a cell wall-anchored protein that is necessary for heme binding and utilization (Maresso et al., 2006). IsdG is the heme oxygenase that cleaves hemin to liberate iron and is vital for both the bacteria's ability to grow on heme substrates and for virulence (Skaar et al., 2006). More recently, IsdX1 and IsdX2 were found to perform overlapping functions consisting of extracting the heme group from hemoglobin, and thus freeing heme and ultimately Fe(III) for bacterial uptake (Maresso et al., 2008). Future studies will be necessary to characterize the remaining proteins in the *isd* locus and will likely contain a number of proteins dedicated to iron transport across the bacterial membrane, much like the *S. aureus isd* system.

Bacterial Dissemination

Once *B. anthracis* spreads from the initial site of infection, the prognosis of host survival decreases drastically. Unfortunately, very little is known about the earliest bacterial–host interactions that define whether the bacteria are sequestered or freed to disseminate. However, the events leading to disseminated infection depend both on the initial site of infection and the host species. For example, neither the characteristic black eschar that is associated with cutaneous anthrax in humans nor their greater resistance to disseminated disease via a cutaneous infection has been adequately modeled in animals. Documenting the diversity of differences noted between dissimilar animal models is beyond the scope of this chapter, and thus will primarily concentrate on information derived from nonhuman primates, rabbits, and mouse models of infection linked to clinical observations and autopsies of humans that died of anthrax. From these data, a generalized model can be generated and will be presented at the end of the section.

Dissemination of B. anthracis *in Animal Models*

In all animal models and all routes of infection (except intravenous), the lymphatics play a major role in bacterial dissemination. When the thoracic and/or right lymphatic ducts of Rhesus monkeys were cannulated, to allow kinetic sample gathering from a single animal over a long time period, bacteria were consistently detected in the lymph before the blood after inhalational, intradermal, and intraperitoneal infection (Lincoln et al., 1965), suggesting that lymphatic infection precludes hemotogenous spread. A notable difference between the different routes of infection is that the number of bacteria in the lymph of monkeys that inhaled spores slowly increased over time, whereas intradermal or intraperitoneal

infections suddenly increased over a 1-hour period, suggesting that local lymph nodes associated with cutaneous or peritoneal infections are suddenly compromised to allow a bolus of bacteria to be released into the lymph in an instant.

Similar data have been obtained in dynamic mouse models of infection. BLI analysis of light-producing *B. anthracis* within a mouse showed that bacteria move into the draining lymph nodes associated with infection and then later colonize distal sites, such as the spleen and lungs, subsequent to lymph node colonization (Glomski et al., 2007a,c, 2008). If infection initiates in the NALT, the submaxillary lymph nodes are the next major source of luminescence (Glomski et al., 2007c, 2008); if the infection initiates by intradermal injection of spores in the ear, the superficial parotid lymph nodes are the next location to indicate the presence of bacteria (Glomski et al., 2007a,c), and gastrointestinal infections spread to the jejunal lymph nodes before becoming systemic (Glomski et al., 2007c).

Determining *B. anthracis* dissemination characteristics through the use of histology and determination of bacterial counts in specific organs from single animals at a single time point have made interpretation of the data difficult. This is primarily due to the fact that the relative stage of infection is hard to establish if only single samples can be derived from a single animal. Once the infection has progressed beyond the associated draining lymph node, the bacteria are dispersed through the body in the blood circulation, making it difficult to establish whether the bacteria identified in one particular location are new arrivals or that this is a site of early replication. As noted previously, it is unclear whether the mediastinitis that is associated with late-stage infections is an indicator of the initial site of infection or a result of bacteria lodged in the lungs that arrived via the blood (Glomski et al., 2007c). In particular, mediastinitis is observed even when systemic disease is initiated through cutaneous infections in both rabbits and Rhesus monkeys (Gleiser et al., 1963, Zaucha et al., 1998), and not all Rhesus monkeys killed by inhalational anthrax have mediastinitis (Fritz et al., 1995), raising doubts about the linkage of causation between inhalational infections and mediastinitis.

One of the defining characteristics of *B. anthracis* is that it is nonmotile (WHO, 2008), thus dissemination is completely dependent upon host bulk fluid transport or taxis within a host cell. Which of these two modes of transport is dominant is partly dependent on the state of the tissues in which the bacteria reside. In livestock, humans, and animal infection models, hemorrhaging is frequently associated with bacterial lesions (Abramova et al., 1993; Friedlander, 1997; Fritz et al., 1995; Glomski et al., 2007c). The escape of blood from the circulatory system is indicative of a breakdown in the endothelial barrier and may indicate that *B. anthracis* has means to promote the entry of bacilli into the circulation as free bacteria. As noted earlier, there is ample evidence of hematogenous spread of bacteria, but whether the spread takes place via the bulk transport of free bacteria in the plasma or within circulating cells is unknown.

Dissemination-Promoting Factors

Both LT and ET can cause hemorrhaging and endothelial barrier breakdown when administered as purified proteins (Firoved et al., 2005; Moayeri et al., 2003; Warfel et al., 2005). Thus, it is reasonable to propose that one of the functions of the exotoxins is to promote the dissemination of bacteria. Indeed, a recent study by Loving et al. reports precisely that bacteria that do not produce toxins remain trapped in the draining lymph nodes (Loving et al., 2009). The mechanism of barrier disruption may simply be explained by the ability of LT and ET to cause necrotic cell death, but in addition

to killing cells, lower concentrations of both LT and ET alter the barrier function of endothelial cells (Tessier et al., 2007; Warfel et al., 2005). The absence of toxin production alters the dissemination characteristics of bacteria, so that the major site of colonization after the draining lymph node is first the spleen, followed by the lungs (Glomski et al., 2007c).

Secreted bacterial proteases are generally recognized as factors that can promote the breakdown of host tissues to encourage bacterial spread (Miyoshi and Shinoda, 2000). On zymographic gels of bacterial supernatants that have LT and ET genetically eliminated, at least four different protease species that digest gelatin, casein, or collagen are detected (Popov et al., 2005). These same supernatants cause hemorrhaging within hours of administration subcutaneously in mice and are lethal when introduced intratracheally (Popov et al., 2005). Both InhA and Npr599 were identified as active proteases within these supernatants that cleave fibronectin, collagen, and laminin, as well as a number of serum protease inhibitors (Chung et al., 2006). To date, the role of InhA and Npr599 in promoting *B. anthracis* virulence, through enhancing dissemination or otherwise, has not been defined, but the precedent set by *Vibrio vulnificus, Pseudomonas aeruginosa,* and *S. aureus* make their further characterization an attractive goal of future research.

The *B. anthracis* capsule also promotes dissemination through the host. Capsulated bacteria progress to the draining lymph nodes and ultimately to distal organs much more quickly than non-capsulated bacteria (Drysdale et al., 2005; Glomski et al., 2007c), but how the capsule mediates this dissemination is unclear. A primary function of the capsule is to inhibit phagocytosis (Makino et al., 1989), thus if phagocytes are responsible for disseminating after germination, as proposed to occur for spores in the Trojan horse model, it would seem that capsule would inhibit this process. Instead, the necessity of capsule for rapid dissemination would appear to support a scenario in which bacteria multiply at the initial site of infection, are unable to be phagocytosed, and thus reach higher concentrations than non-capsulated counterparts, leading to greater secretion of toxins and/or proteases to break down physical barriers to ultimately promote greater dissemination of bacteria into the lymphatics by bulk flow. After entering the lymphatics and spreading to the blood, a similar situation would be expected to occur. As observed, the absence of capsule alters the dissemination pattern of bacteria so that the major sites of bacterial colonization after the draining lymph nodes are the kidneys, lungs, and stomach (Glomski et al., 2007a; Loving et al., 2009), which is markedly different from capsulated bacteria that initially predominantly colonize the spleen. Presumably, non-capsulated bacteria are quickly eliminated by the large number of phagocytes located in the spleen if the capsule is not present to protect them.

The Proposed Model for *B. anthracis* Dissemination

As depicted in Figure 12.1, there are three distinct locations and means of entry into a host. Cutaneous infections (Step 1A) require that the integrity of the skin is compromised in some way. The cutaneous damage may be due to abrasions, insect bites, chemical irritants, or other physical insults. If spores are introduced into damaged skin they will germinate, acquire nutrients from their surroundings with the aid of siderophores and hemeoxygenases, begin secreting toxins, reduce oxidative damage with SODs, and become encapsulated. Germination in tissues does not necessarily require phagocytosis. Often infection is limited to the skin, and may tend to remain in the skin through the adhesive activity of MSCRAMMs or BslA, but has the potential to progress into the draining lymph

node (Step 2). The means of transit from the cutaneous lesion to the lymph node is likely to occur as free extracellular bacteria carried by bulk fluid flow, since the bacteria are encapsulated to inhibit phagocytosis, are secreting LT and ET to cripple the inflammatory response, and are secreting proteases to break down tissue barriers.

The second means of entry is through epithelial barriers in the nasal or gastric mucosa (Step 1B). The mechanism of uptake in these locations is unknown, but may include epithelial transcytosis, phagocytosis by dendritic cells that extend their processes into the lumen, or M-cell engulfment. Unlike cutaneous infections, particularly in the alimentary tract, spores may germinate within the lumen and thereby alter the surface with which they interact with the mucosal epithelia. In this scenario, BslA and MSCRAMMs would be expressed and could promote adhesion to the mucosa. Once within the tissues, bacteria promote their growth by similar means as cutaneous infections (protected by capsule and toxin secretion), and ultimately enter the draining lymph node (Step 2).

Entrance through the lung alveoli (Step 1C) requires movement of spores from the lumen to the opposite side of the epithelium. This may occur via spore transcytosis through the squamous epithelial cells, phagocytosis by dendritic cells that extend their processes into the lumen, or movement of spore-containing alveolar macrophages across the barrier in transit to the lymph node. Though in some circumstances spores can germinate and bacteria can grow in the lumen of the alveoli, this does not appear to be typical of lung-based entry, since vegetative bacteria are normally not seen at early stages within lung tissue in the absence of preexisting epithelial damage. Spores are transported to the draining lymph node within phagocytes (Step 2), but whether *in vivo* these spores are competent to germinate, escape from the host cell, and cause disseminated infection remains to be determined.

Regardless of the portal of entry, once vegetative bacteria have entered the draining lymph node, dissemination occurs in the same manner (Step 2). Within the lymph node, bacteria rapidly divide and reach large numbers. At high numbers, there is an associated high concentration of toxins and proteases that allow the physical breakdown of the node structure allowing escape of bacteria via the efferent lymph duct. From the efferent lymph duct, the lymph then enters the blood circulation, primarily through the thoracic or right lymphatic ducts into the subclavian vein (Step 3). Encapsulation decreases the transit time of bacteria, probably because the capsule prevents phagocytosis and destruction by phagocytes and therefore effectively increases the bacterial burden over a shorter time interval than non-capsulated bacteria. Likewise, the toxins promote bacterial growth in the lymph node by crippling the immune response, and at these high concentrations, may induce death of immune effector cells.

After entering the blood circulation (Step 3) via the subclavian vein, the bacteria pass through the wide passages of the right heart and then encounter their first fine capillary bed within the lungs. If blood-borne bacteria are in the long chains often observed in host tissues, a portion of these bacteria will lodge in the fine capillaries of the lungs and another portion will pass through the lungs, return through the wide passages of the left heart, and then be distributed to the rest of the body to lodge where constriction or adhesion ultimately trap the bacteria. The adhesion process may be aided by BslA or MSCRAMMs. Those bacteria lodged in the parenchyma of the lung contribute to the mediastinitis that is associated with anthrax via the secretion of LT and ET and potentially the drainage of bacteria from the lungs into the mediastinal lymph nodes. Ultimately, the bacteria that have disseminated via the blood rapidly grow both in the blood and within the organs in which they have lodged, causing damage via toxins and an enormous bacterial load to cause the death of the host.

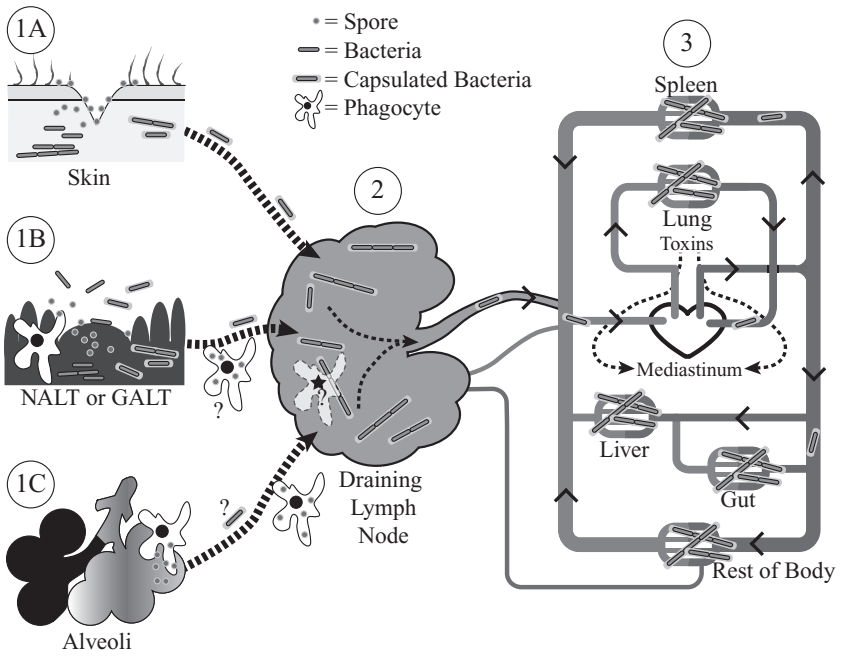

Figure 12.1 A model for *B. anthracis* dissemination through a host. See color insert.

A verbal description of the details of the model depicted above can be found at the end of the main text. The diagram depicts the entry of *B. anthracis* through the skin (1A), NALT, or GALT (1B), or lung alveoli (1C). Evidence suggests that *B. anthracis* can be trafficked from the alveoli to the draining lymph node within a phagocyte, but the movement of extracellular bacteria to the draining lymph node remains a possibility for all routes of entry depicted. Once within the draining lymph node (2), bacteria overcome local defenses through the function of their virulence factors and multiply extracellularly. Growth and toxins break down endothelial barriers and give bacteria access to the efferent lymph flow, thus introducing bacteria to the blood circulation via the reentry of lymph by the thoracic or right lymphatic ducts. Once within the blood circulatory system (3), many bacteria are trapped in the first fine capillary bed they encounter in the lung. The remaining bacteria disseminate and colonize the other major organs. Growth in the blood and other vital organs ultimately lead to death of the host.

CONCLUDING REMARKS

Over a century after *B. anthracis* was identified as the causative agent of anthrax, very basic aspects of its pathogenesis remain unknown. Much of the lack of scientific progress can be attributed to the establishment of a very effective veterinary vaccine that eliminated anthrax from the herds of developed countries, thus greatly reducing its prevalence in those regions and promoting its perception as a "beaten" disease. However, the disease still commonly occurs in less developed nations. A resurgence of interest in *B. anthracis* has occurred, especially since the dissemination of *B. anthracis* spores through the U.S. Postal System, because of its recognition as an easily weaponized pathogen. This renewed inter-

est has led to a veritable explosion of *B. anthracis* research since 2001 that has rapidly altered the ways in which its pathogensis is viewed. Much of the recent progress in understanding how *B. anthracis* causes disease can be attributed to the application of newly developed technologies, but the lion's share of credit must be given to the commitment of an increasing number of dedicated researchers and their financial support by governmental and nongovernmental agencies. With continued interest and support, we can look forward to filling some of the gaps in knowledge of *B. anthracis* pathogenesis in the years to come.

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Chapter 13

Pathology, Diagnosis, and Treatment of Anthrax in Humans

Jeannette Guarner and Carlos del Rio

Anthrax is primarily a disease of domestic and wild herbivores to which humans and other carnivores are susceptible as incidental hosts (Hugh-Jones and Vos, 2002). The majority of human cases occur when infected animal or animal products come in contact with humans; however, unusual exposures can also cause human disease such as injection drug use in a fatal case of cutaneous anthrax in Norway (Ringertz et al., 2000), residing in certain locations in 1979 when there was accidental release of spores by a military facility in Sverdlovsk, Russia (Abramova et al., 1993), and contaminated letters during the 2001 bioterrorist attack in the United States (Heller et al., 2002; Jernigan et al., 2002; Schmid and Kaufmann, 2002). The latter two outbreaks demonstrated that it is possible to weaponize anthrax to cause disease and is the reason why anthrax is considered a class A bioterrorism agent. In this chapter we will review the surveillance and control activities that the World Health Organization (WHO) has proposed to decrease anthrax in animals and consequently sporadic human cases, the diagnostic clinical and laboratory characteristics of patients with anthrax, the pathology that is observed in human cases, and the treatment for this disease in humans.

WHO ACTIVITIES ON ANTHRAX SURVEILLANCE AND CONTROL

Today, sporadic cases of anthrax occur in all continents of the world (Hugh-Jones and Vos, 2002). For example, in Europe, anthrax animal and human cases remain relatively common in countries adjoining the Mediterranean Sea, including Turkey, Greece, the Balkan countries, Italy, and Spain. Anthrax is also endemic in the Russian Federation, particularly in the Caucasus region. Other European countries have reported to have eliminated anthrax (Sweden, Austria, the Czech Republic, Denmark, Finland, and Luxemburg) (Schmid and Kaufmann, 2002). In North America, anthrax has been reported in the past two decades in bison, moose, and bears in Alberta and the Northwest Territories of Canada, and in cattle in Texas and Montana (Hugh-Jones and Vos, 2002). In Africa, Asia, and South America, animal and human cases of anthrax occur; however, specific diagnosis of

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the disease is challenging in these continents due to the difficulties in reaching the areas where infected animals or patients are located and the diagnostic capabilities available in those countries (Turnbull et al., 1999a).

The drastic decline of anthrax in Europe and North America during the twentieth century was due to the development of the Sterne vaccine for livestock in 1937, the successful use of penicillin treatment, and the implementation of quarantine regulations (Hugh-Jones and Vos, 2002). In an effort to decrease anthrax in other continents, WHO has published guidelines for surveillance and control of anthrax and has initiated a model program in Zambia (Turnbull et al., 1999a,b). The guidelines center in the following activities:

1 detection, diagnosis, and identification;

2 surveillance, reporting, and epidemiology;

3 disposal, disinfection, and decontamination;

4 treatment and prophylaxis, and

5 education, training, and information.

All activities in these guidelines have faced implementation challenges. Efforts to detect, diagnose, and identify cases have been hampered because of lack of resources to transport officials to the site where cases occur and problems with collection, transport, and testing of specimens. Meat is a valuable commodity in many countries in Africa, South America, and Asia; thus, reporting and disposing of infected carcasses has encountered resistance by the people who own the animals. In addition, proper disposal of infected dead animals can be very expensive for certain communities. Treatment of diagnosed human cases takes place and prompts retrospective vaccination of animals; unfortunately, already infected animals continue to die, and there is loss of confidence in the vaccine. In order to encourage implementing the anthrax surveillance and control activities, WHO has organized workshops in multiple countries where anthrax is still endemic.

DIAGNOSTIC CLINICAL CHARACTERISTICS OF PATIENTS WITH ANTHRAX

In a patient, the portal of entry of anthrax spores determines certain key clinico-pathological features that serve to classify anthrax into three types or forms: cutaneous, gastrointestinal, or inhalational (Dixon et al., 1999). Even though none of the clinical features is specific or pathognomonic for any of the types of anthrax, it is imperative for health-care providers to recognize these features so as to be able to obtain adequate specimens and order diagnostic laboratory tests specific for anthrax. High level of suspicion of a diagnosis of anthrax in cases with unusual exposure histories can make the difference in establishing prompt treatment. Of the three types of anthrax, the cutaneous form is the most frequently encountered in humans and has the lowest mortality rate (approximately 14%), while inhalational and gastrointestinal anthrax occur very rarely but have higher mortality rates (around 60%) (Bravata et al., 2007).

Cutaneous Anthrax

In the past 10 years, seven case-series from the Caribbean, South America, and the Middle East describe in detail the clinical features of cutaneous anthrax associated with exposure

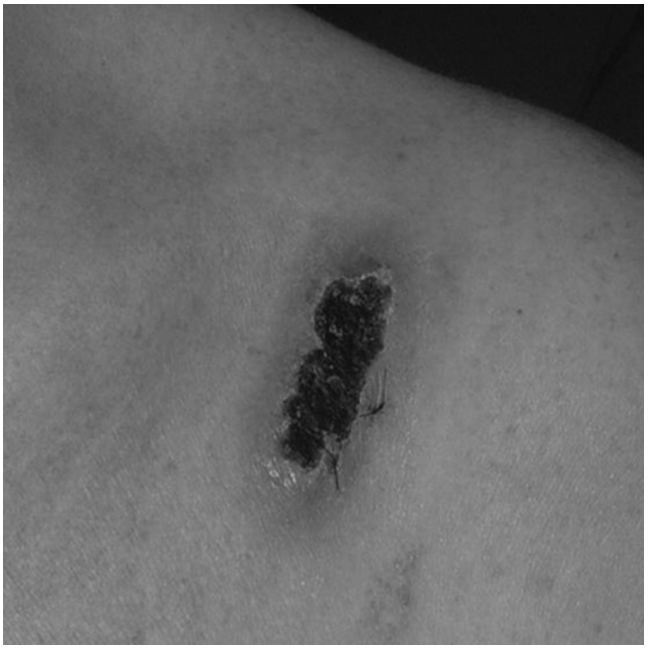

Figure 13.1 Cutaneous anthrax lesion in shoulder. See color insert.

to animals or animal products (Irmak et al., 2003; Karahocagil et al., 2008; Kaya et al., 2002; Maguiña et al., 2005; Oncül et al., 2002; Ozkurt et al., 2005; Smego et al., 1998). The majority of cases have a history of slaughtering or caring for animals, meat processing, or performing crafts with animal products. Not surprisingly, and depending on the series, between 60% and 90% of the lesions occur in the hands, fingers, and arms, while 6–30% occur in the face or neck. Typically, the first manifestation is a painless, pruritic erythema, which evolves within 48 to 72 h to a papule or multiple vesicles accompanied by local inflamed edema. By the seventh day, the papule or vesicles break, leaving a painless ulcer with a black center, eschar (Figure 13.1). This is the lesion that has given anthrax its name (*anthrax* meant coal in ancient Greek). At this time, the patient presents fever, general malaise, toxemia, severe edema, and painful regional lymphadenitis.

Around 80% of cutaneous anthrax cases cure spontaneously; however, resolution of the eschar may take several weeks depending on the size of the lesion (Maguiña et al., 2005). The most frequent local complications include secondary bacterial infections, scars in eyelids that require plastic surgery (Irmak et al., 2003), and severe edema of the face or neck which can be so extensive to cause airway obstruction and is referred to as malignant edema (Karahocagil et al., 2008; Kaya et al., 2002; Maguiña et al., 2005; Ozkurt et al., 2005). Palsy of the facial nerve has also been described as a local complication (Faghihi and Siadat, 2003). Systemic complications include sepsis, meningoencephalitis, and death (Kaya et al., 2002; Maguiña et al., 2005; Ozkurt et al., 2005). Microangiopathic hemolytic anemia is another systemic complication of cutaneous anthrax that has also been described (Freedman et al., 2002).

The differential diagnosis of cutaneous anthrax includes staphylococcal infection (erysipelas), gangrenous ecthyma, glanders, cat-scratch disease, diphtheria, typhus and other rickettsiosis, rat bite fever, syphilitic chancre, cutaneous plague, tularemia, cutaneous

tuberculosis, leprosy, blastomycosis, sporotrichosis, cutaneous zygomycosis, orf, cowpox, insect bites, and vasculitis (Freedman et al., 2002; Irmak et al., 2003; Karahocagil et al., 2008; Kaya et al., 2002; Maguiña et al., 2005; Oncül et al., 2002; Smego et al., 1998). However, the most important clue to the diagnosis of anthrax is the development of a painless ulcer surrounded by a zone of edema. Although the exposure history is very important in animal-associated cases, another bioterrorist attack could deliver anthrax spores in an unusual fashion, thus making the diagnosis of the index or initial cases difficult.

Inhalational Anthrax

The term "inhalational anthrax" indicates that spores are inhaled and has replaced the use of "pulmonary anthrax" since active infection occurs in the mediastinal lymph nodes, rather than the lung itself. Inhalational anthrax is a biphasic disease with a prodromal phase that consists of a benign, nonspecific, flu-like illness followed by a fulminant phase that consists of severe, acute respiratory distress, shock, and culminates with death. A systematic review of published inhalational anthrax cases that occurred between 1900 and 2005 shows that cases were highly heterogeneous with respect to year of disease onset, nationality, patient's age, treatment regimen received, and survival (Holty et al., 2006). The mentioned review excluded the 74 patients that died during the 1979 outbreak in Sverdlovsk because there was no information about symptoms, disease progression, or treatment. A total of 82 patients were reviewed, including 11 patients with inhalational anthrax that occurred during the 2001 bioterrorist attack in the United States. The prodromal phase in the 82 patients lasted 4.1 days while the fulminant phase lasted 1.1 days. The most common symptoms included fever (81%), abnormal lung findings (80%), chills (67%), tachycardia (66%), fatigue or malaise (64%), cough (62%), or dyspnea (52%). Thirty-one patients (38%) developed anthrax meningoencephalitis.

Comparisons of previously published cases of anthrax and those that occurred during the bioterrorism attack have shown interesting differences that may be due to the *Bacillus anthracis* strain, the amount of the innoculum, or the use of adequate treatment during the prodromal phase. The patients of the U.S. bioterrorism attack tended to be older (Holty et al., 2006). Dyspnea was the single most discriminating symptom followed by presence of nausea and vomiting in inhalational anthrax cases that occurred during the bioterrorist attack (Bell et al., 2002; Hupert et al., 2003). Profound sweating was also documented as a prominent feature in these patients (Jernigan et al., 2001).

Comparing inhalational anthrax cases with other respiratory diseases, primarily bacterial pneumonia and influenza, showed that chest discomfort or pleuritic pain were more common in patients with anthrax while headache, sore throat, and rhinorrhea were seen more frequently in patients with influenza (Cosgrove et al., 2005; Kuehnert et al., 2003). Laboratory features that were seen in patients with inhalational anthrax during the bioterrorist attack included leukocytosis, elevated transaminases (AST and ALT), and low sodium (Kuehnert et al., 2003).

Descriptions of chest radiographs in patients with inhalational anthrax are available in 26 patients, and abnormal findings include pleural effusions (69%) or widened mediastinum (54%) (Figure 13.2) (Bell et al., 2002; Holty et al., 2006). The few patients that have been studied using CT scan of the chest have shown widening of central silhouette, enhancement of peribronchial adenopathy that with contrast demonstrates high attenuation, and pleural effusions (Mayer et al., 2001; Wood et al., 2003).

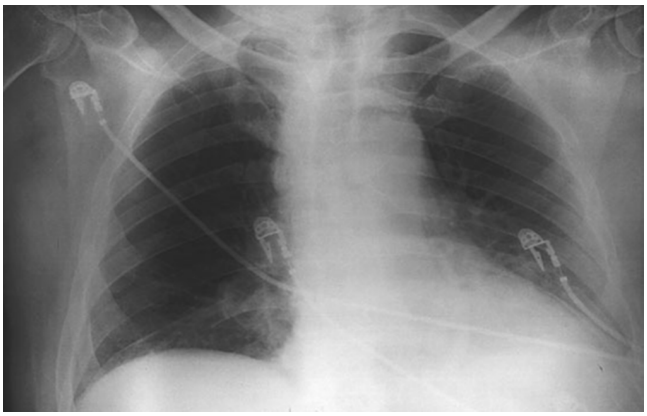

Figure 13.2 Inhalational anthrax chest X-ray showing widened mediastinum.

The 11 cases of inhalational anthrax associated with the bioterrorist attack in the United States in 2001 challenged the ability of the health-care system to provide adequate triage, diagnosis, and treatment to the overwhelming amount of patients who came to emergency rooms with possible inhalational and cutaneous anthrax. Two approaches have been proposed as pivotal for bioterrorism preparedness: a triage algorithm for screening for inhalational anthrax and education of first-line health-care providers (Cosgrove et al., 2005; Hupert et al., 2003; Sox, 2003; Temte and Zinkel, 2004). The first step in the proposed algorithm asks if neurologic symptoms excluding headache are present, step 2 uses the presence of temperature abnormalities, step 3 asks if dyspnea, nausea, vomiting, or abnormal lung examination are present, and step 4 asks if there is presence of rhinorrhea or sore throat (Hupert et al., 2003; Sox, 2003). Affirmative answers to the first 3 steps suggest inhalational anthrax, and the patient should be sent to a health-care facility for definitive diagnostic testing and treatment. Affirmative answer to the last question (step 4) suggests the patient has a viral illness but considers sending the patient home on prophylactic antibiotics. Although the algorithm has not been tested with inhalational anthrax patients, it provides a guideline that could be useful for triage of patients in situations such as the one lived October through December 2001 in the United States. Education of health-care providers about rare diseases that could be part of a bioterrorist attack is important for early recognition of the disease, obtaining adequate specimens to make definitive diagnosis, instituting prompt adequate treatment, and gathering the necessary information for health and federal and state law enforcement authorities to investigate possible bioterrorism cases.

Gastrointestinal Anthrax

Published reports of the clinical manifestations of gastrointestinal anthrax are scarce and are poorly detailed because this entity can be difficult to document (Kanafani et al., 2003). In general, the patients have consumed contaminated meat that has been poorly cooked. The disease progresses through three different stages (Babamahmoodi et al., 2006; Kanafani et al., 2003; Sujatha et al., 2002): The first phase appears as a viral-like infection with fainting spells, asthenia, low-grade fever, and headache. Within 24 to 72 h, the patients present abdominal pain of variable intensity, fever, nausea, anorexia, vomiting,

and diarrhea. During the last stage, the patients' abdomen is distended with ascites; there may be a palpable mass which corresponds to hemorrhagic mesenteric lymph nodes, there are paroxysms of abdominal pain, there may be gastrointestinal bleeding due to mucosal ulcerations, and shock can be present.

A variant of gastrointestinal anthrax, the oropharyngeal form, has been documented in Thailand and Zimbabwe (Sirisanthana and Brown, 2002; Sirisanthana et al., 1984). These patients present with sore throat, dysphagia, and hoarseness. Neck lymph nodes swell and there is soft tissue edema. Ulcerative lesions are observed in the tonsils, posterior pharyngeal wall, or hard palate. After 1 week, the mouth lesions develop a pseudomembrane.

The differential diagnosis of gastrointestinal anthrax will depend on the stage of disease. For example, in the early stages of intestinal anthrax, food poisoning should be considered, while in the later stages, acute abdomen and hemorrhagic gastroenteritis caused by other microorganisms, particularly necrotizing enteritis caused by *Clostridium perfringens* and amebic dysentery, should be included. The differential diagnosis in cases of oropharyngeal anthrax include diphtheria, complicated tonsillitis, streptococcal pharyngitis, Vincent angina, Ludwig angina, parapharyngeal abscess, and deep-tissue infection of the neck.

In any form of anthrax, sepsis and meningitis could be a potential complication. The patient with anthrax meningitis presents with nonspecific meningeal symptoms such as nuchal rigidity in addition to fever, fatigue, myalgias, nausea, seizures, and delirium. There is rapid neurologic deterioration and death. The cerebrospinal fluid is often bloody and numerous gram-positive bacilli can be observed on Gram stain. Anthrax meningitis is almost always fatal, with death occurring approximately 6 days after the onset of illness despite appropriate therapy (Dixon et al., 1999).

LABORATORY DIAGNOSIS OF ANTHRAX

Specimens

The specimens to be submitted for testing will depend on the form of anthrax. For cutaneous anthrax, fluid from vesicles, scraping from a papule, fluid or scraping from underneath an eschar, or drainage from the lesion can be used for Gram staining and culture. A punch biopsy from the edge of the lesion can be divided (or 2 biopsies can be obtained), one half should be sent for culture while the other is formalin-fixed so it can be studied using histopathology, immunohistochemistry, and PCR (Carucci et al., 2002).

In cases of inhalational and gastrointestinal anthrax, blood cultures should be obtained. In addition, the following samples can be obtained and sent for Gram staining, culture, and polymerase chain reaction (PCR):

1. Pleural effusions
2. Broncho alveolar lavage fluid
3. Stool
4. Ascitis, and
5. Cerebrospinal fluid (if there are signs of meningitis).

Tissue specimens, including scrapings or biopsies of oropharyngeal lesions, bronchial biopsies, or gastrointestinal surgical specimens, should be cultured and studied

using histopathology, Gram and Silver impregnation staining, immunohistochemistry, and PCR.

Ultimately, a variety of specimens can be obtained from autopsies of deceased patients for study. Performance of autopsies in patients diagnosed with anthrax who do not have usual animal or animal product exposure is indispensable because the case could be part of a bioterrorist attack. These autopsies should be performed to secure legal evidence of homicide and to determine the portal of entry of anthrax, which will help define potential sources of anthrax spores and will help institute public health measures to contain other possible casualties (Guarner et al., 2003; Nolte et al., 2004). Differentiation between inhalational and gastrointestinal anthrax at autopsy may be difficult because hemorrhages can occur in the gastrointestinal tract wall secondary to generalized spread of the infection. Thus, the decision as to how the disease was contracted may have to be based, at least in part, on the patient's clinical history.

Pathologists performing autopsies of patients with anthrax should be aware of the precautions necessary to perform the autopsy and of disposing of the cadaver (Guarner et al., 2003; Nolte et al., 2004). In anthrax patients, most of the bacteria present in tissues during the first hours after death are in the vegetative stage; thus, the risk to personnel performing autopsies is through splashes and splatters to mucous membranes and percutaneous injuries. Procedures such as opening the skull and embalming should be avoided if possible since they can create aerosols with microscopic droplets containing bacilli, which may eventually sporulate. To prevent persistence of *B. anthracis* (in either vegetative or spore forms) in the environment, the autopsy instruments and suite should be decontaminated with 0.5% hypochlorite solution.

Testing Performed in Hospitals

The tests that should be performed by the microbiology laboratory in any hospital include culture and Gram staining (Winn et al., 2006; CDC, ASM, & APHL, 2002). For those specimens plated directly into agar, such as fluid from skin lesions or biopsies, growth may be evident as early as 8 h after plating on sheep blood and chocolate agar. If present in blood or cerebrospinal fluid, *B. anthracis* usually grows within the first 24 h of incubation of the specimen. Gram stain from the blood culture bottle will show large, gram-positive rods in short chains of 2 to 4 cells. Spores are usually not present. The positive aerobic blood culture bottle needs to be subcultured in sheep blood and chocolate agar.

In sheep blood agar incubated at 35°C for 18–24 h, *B. anthracis* grow as flat or slightly raised, gray to white colonies that have a ground glass surface. The characteristic "Medusa head" appearance is given by an undulate, curling edge. When the colonies are picked, they are sticky or tenacious. *B. anthracis* are nonhemolytic, nonmotile, catalase-positive organisms. Motility can be tested using a microscopic wet preparation or motility media. An India ink preparation can reveal a capsule. As the culture ages, a Gram stain may demonstrate oval, central to subterminal spores.

After the bioterrorism anthrax attack in the United States, a telephone-based, gram-positive rod surveillance study to detect inhalational anthrax was performed in Connecticut for 10 months (Begier et al., 2005). Hospitals were asked to report gram-positive rods isolated form cerebrospinal fluid or blood within 72 h of culture inoculation. To select for patients with respiratory symptoms, the authors contacted the patient's physicians. Of 623 gram-positive rods isolated, almost half corresponded to *Corynebacterium* spp. (293) followed by *Bacillus* spp. (193). Because of hemolysis and motility characteristics, only 23

of the 193 *Bacillus* isolates were forwarded to the Connecticut state laboratory for gamma-phage lysis study. During this study period, *B. anthracis* was not identified.

Testing Performed by the Laboratory Response Network (LRN)

Any rapidly growing, nonmotile, large, gram-positive rod showing flat, ground glass, nonhemolytic colonies in sheep blood agar plates should trigger the thought of possible anthrax and should be reported as soon as it is found to the local Laboratory Response Network (LRN). The LRN was established by the Department of Health and Human Services and the Centers for Disease Control and Prevention to ensure an effective laboratory response to bioterrorism attacks (CDC, 2005). This network became operational in August of 1999. Today, more than 100 state and local public health laboratories, veterinary, agriculture, military, and water- and food-testing laboratories are linked through the LRN. In addition, laboratory facilities located in Australia, Canada, and the United Kingdom also participate in the network.

In order to rule out the diagnosis of *B. anthracis*, LRN reference laboratories perform the following tests on cultures form gram-positive rods that are referred to them (CDC, 2001a):

1. Sporulation: Spores will appear in a growing culture after 18–24 h incubation at 35–37°C in a non-CO_2 atmosphere. Oval, central to subterminal spores that do not appreciably swell may be observed by Gram stain (can be performed in Level A laboratories), wet mount (in Level B), or malachite green stain (in Level B).
2. Lysis by gamma-phage: This test is highly specific for *B. anthracis*, and when demonstrated concomitantly with the presence of a capsule, provides confirmatory identification (Abshire et al., 2005; Schuch et al., 2002).
3. Assays detecting *B. anthracis* proteins: Several proteins specific for *B. anthracis*, such as galactose/*N*-acetylglucosamaine cell wall-associated polysaccharide, capsule antigen, and EA1 can be detected using antibodies or the C-terminal region of gamma-phage lysin protein (Fujinami et al., 2007; Love et al., 2008). Detection can be performed using either direct fluorescence or enzymatic labels. If these methods are used, confirmation of *B. anthracis* requires detection of at least two antigens.
4. Antimicrobial susceptibility testing: An array of selected antimicrobics is used to determine their respective minimum inhibitory concentrations using standardized methods against *B. anthracis*.

In addition, several methods which detect *B. anthracis* nucleic acids from isolates, blood, or nasal swabs have been published and may have future applications within the LRN laboratories (Hoffmaster et al., 2002; Oggioni et al., 2002; Sacchi et al., 2002; Tomioka et al., 2005; Uhl et al., 2002).Some of the PCR-based detection methods have been validated in formalin-fixed, paraffin-embedded samples, providing the possibility of using tissue samples that are noninfectious (Levine et al., 2002; Tatti et al., 2006).

There are several publications of serologic tests for potential exposure to *B. anthracis* (Biagini et al., 2004a; Quinn et al., 2002). The assays detect immunoglobulin G antibodies to anthrax toxin protective antigen. The clinical utility of serologic assays is not known, but they have been used to determine exposure to *B. anthracis* in workers that performed environmental sampling after the 2001 bioterrrorist attack (Biagini et al., 2004b).

PATHOLOGY OF ANTHRAX IN HUMAN CASES

Cutaneous Anthrax

Even though cutaneous anthrax is more frequent than other forms of anthrax, descriptions of the histopathology are primarily case reports with few series of cases (Godyn et al., 2004; Lebowich et al., 1943; Mallon and McKee, 1997; Shieh et al., 2003). The largest and most recently published series describes the histopathologic features of 11 cutaneous anthrax cases related to the bioterrorist attack in the United States in 2001.

Histopathologically, several features are commonly observed in the epidermis and dermis, including edema of various degrees (spongiosis in the epidermis and separation of collagen bundles in the dermis), coagulation necrosis, and hemorrhage (Figure 13.3). In addition, the epidermis shows acantholysis or bullous formation, and various degrees of ulceration. Examination of the dermis frequently shows vasculitis. The degree of inflammation varies from case to case and can be composed of polymorphonuclear infiltrate in the spongiotic epidermis or mononuclear perivascular infiltrate.

If the patient has received antibiotic treatment, the tissue may or may not demonstrate gram-positive bacilli with the Gram stain (Shieh et al., 2003). Silver stains such as Steiner silver stain may demonstrate more bacilli than Gram stains. Immunohistochemical assays using antibodies against the cell wall and capsule were very useful for the diagnosis of the bioterrorism-related cutaneous anthrax cases in the United States since they highlighted bacilliform and granular antigens primarily in the dermis in all cases even after 16 days of treatment. The amount of antigens present varied from case to case and depended on the amount of treatment received.

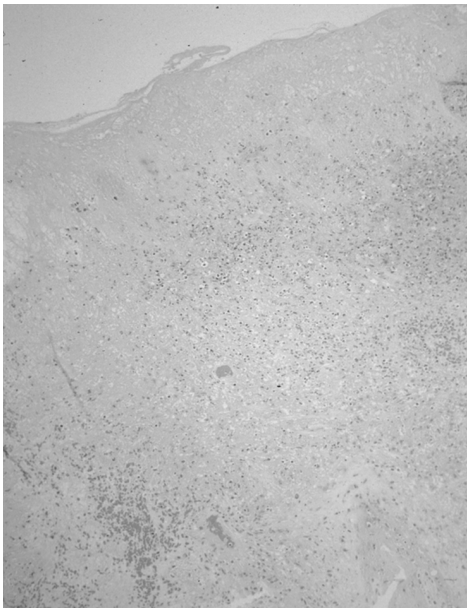

Figure 13.3 Histopathology of cutaneous anthrax showing necrosis of epidermis, inflammation in upper dermis and focal hemorrhage (hematoxylin and eosin stain, original magnification 25×). See color insert.

Inhalational Anthrax

The largest series of cases describing the pathology of inhalational anthrax is the outbreak that occurred in Sverdlovsk in 1979 (42 cases) followed by the cases that occurred during the bioterrorist attack in the United States (8 cases) (Abramova et al., 1993; Grinberg et al., 2001; Guarner et al., 2003; Walker et al., 1994). All the Russian cases were fatal while the cases in the United States included three survivors. There are differences in the pathology encountered in these two outbreaks which can be explained by the advances in the therapies patients received in 1979 and 2001 and the *B. anthracis* stain that was released in both instances. Over half the individuals who died in Sverdlovsk and had postmortem examinations showed the "Cardinal's cap sign" which corresponds to extensive hemorrhage, edema, and inflammatory infiltrates of the leptomeninges (Abramova et al., 1993). In other organs, the Russian cases showed fibrin deposition in capillaries and vessel walls surrounded by various amounts of neutrophilic infiltrate which lead to high- and low-pressure hemorrhages (Grinberg et al., 2001). The cases from the U.S. bioterrorist attack showed less prominent inflammatory lesions around vessels and meningeal involvement was documented clinically in only one patient (Guarner et al., 2003).

The two most prominent findings in inhalational anthrax are pleural effusions and edema and hemorrhage of mediastinal lymph nodes. During autopsies of fatal cases, large amounts of serosanguinous fluid are present when the chest cavity is opened (Grinberg et al., 2001; Guarner et al., 2003). Bacilli are abundantly present on the pleural surface. In nonfatal cases of inhalational anthrax, pleural effusions of more than 500 cc can persist several days even after treatment has been instituted (Guarner et al., 2003). Cytologic study of the pleural fluid show reactive mesothelial cells admixed with inflammatory elements and bacilli. Cell block preparations from the pleural fluid show bacilli and granular antigen staining by using immunohistochemistry (Figure 13.4). Pleural effusions of a recent case of naturally acquired inhalational anthrax (Walsh et al., 2007) showed that cell wall and capsular antigens persisted inside pleural fluid cells up to 10 days after treatment was initiated (Figure 13.5).

The second most prominent gross autopsy finding is edema and hemorrhage of mediastinal lymph nodes and surrounding soft tissues (Figure 13.6). Histopathologically, the mediastinal lymph nodes show hemorrhage, various amounts of necrosis, lymphocytolysis, and infiltration by neutrophils and immunoblasts (Grinberg et al., 2001; Guarner et al., 2003; Perl and Dooley, 1976). The amount of bacilli that can be observed with special stains depends on the amount of time the patient received antibiotics. Gram stains tend to be negative 48–55 h after antibiotic treatment has been instituted while silver impregnation stains can demonstrate bacilli several days after treatment has been received, although the shape of the bacteria may appear "ghost-like," folded, or knobbed (Figure 13.7). Immunohistochemical assays demonstrate bacilli and granular antigen staining in mediastinal lymph nodes (Guarner et al., 2003).

In general, lungs may show focal hemorrhage and edema of pleura in the interhilar septa which may be accompanied by various degrees of inflammation around capillaries. Other findings in the lungs have included variable intra-alveolar edema, focal areas of hyaline membrane formation, hemorrhages, and interstitial inflammation mainly due to capillaritis and vasculitis. Half of the patients in Russia had acute bronchopneumonia; however, this was not encountered in the U.S. patients and may relate to treatment advances. In survivors, transbronchial biopsies have shown soft tissue edema, various amounts of hemorrhage, and variable inflammatory infiltrate around capillaries.

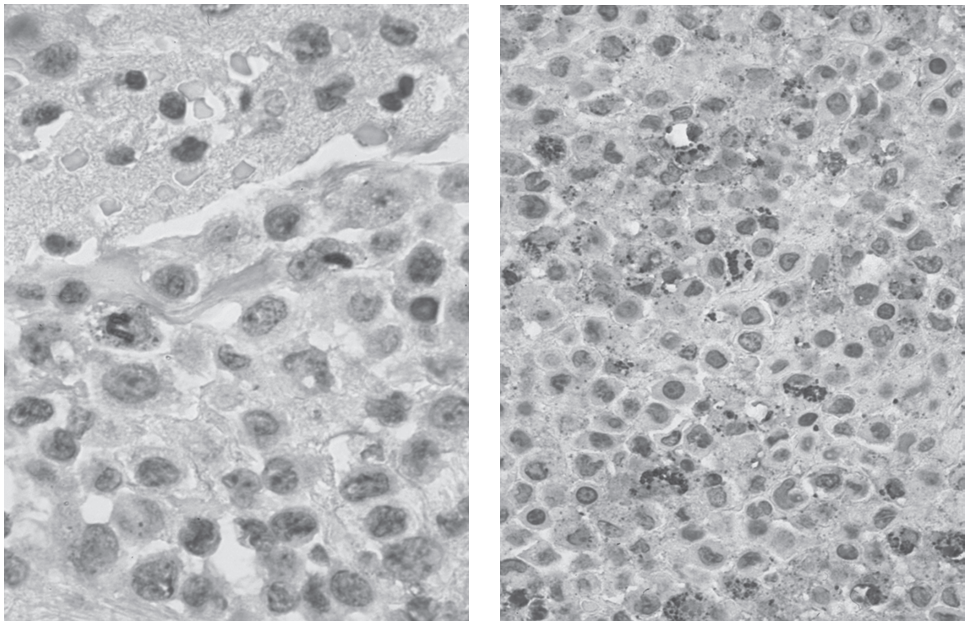

Figure 13.4 Cell block prepared from pleural fluid of a patient with inhalational anthrax showing bacilli and granular antigen staining (immunohistochemical assay using monoclonal antibodies against cell wall (a) and capsule (b), original magnifications 250× (a) and 157.5× (b). See color insert.

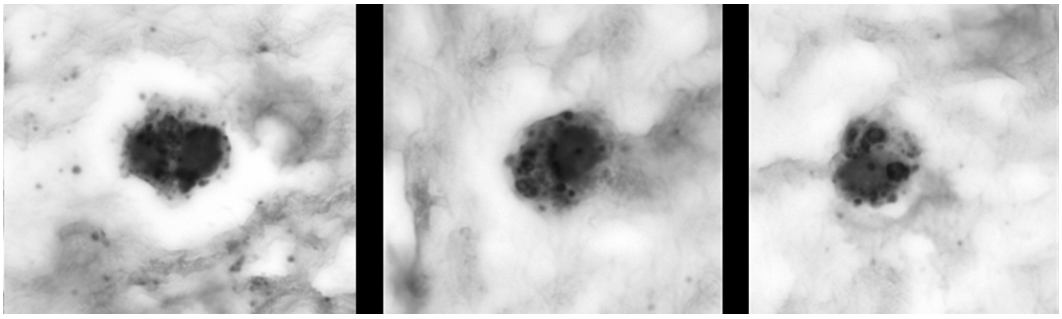

Figure 13.5 Cells in pleural fluid 10 days after initial hospitalization showing granular antigen staining for *B. anthracis* (immunohistochemical assay using a monoclonal antibody against the capsule, original magnification 250×). See color insert.

The most frequent changes in the abdominal cavity were present in the spleen, where congestion, presence of immunoblasts and neutrophils, and necrosis including lymphocytolysis were observed (Grinberg et al., 2001; Guarner et al., 2003). The intestines show submucosal hemorrhages. In the central nervous system, the meninges show hemorrhage and fibrin while the parenchyma shows vasculitis (Grinberg et al., 2001).

In the Russian series, the highest burden of *B. anthracis* was in the brain followed by the intestines, which may indicate that these patients had already progressed to more advanced stages of disease. In the U.S. series, all patients showed bacilli and granular

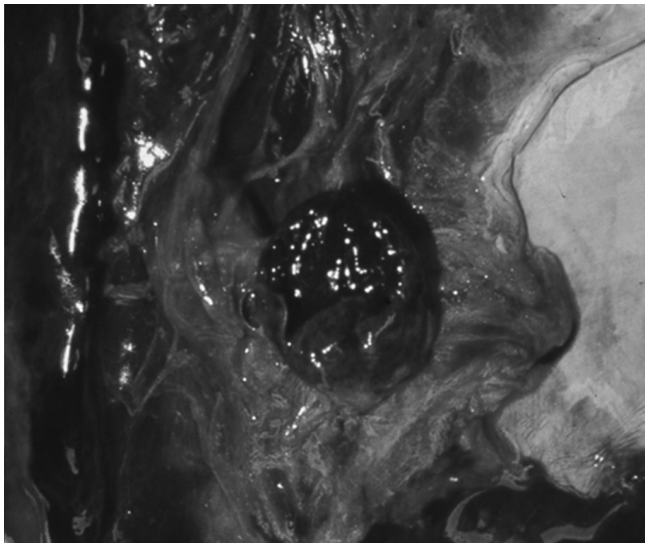

Figure 13.6 Edematous and hemorrhagic mediastinal lymph node. See color insert.

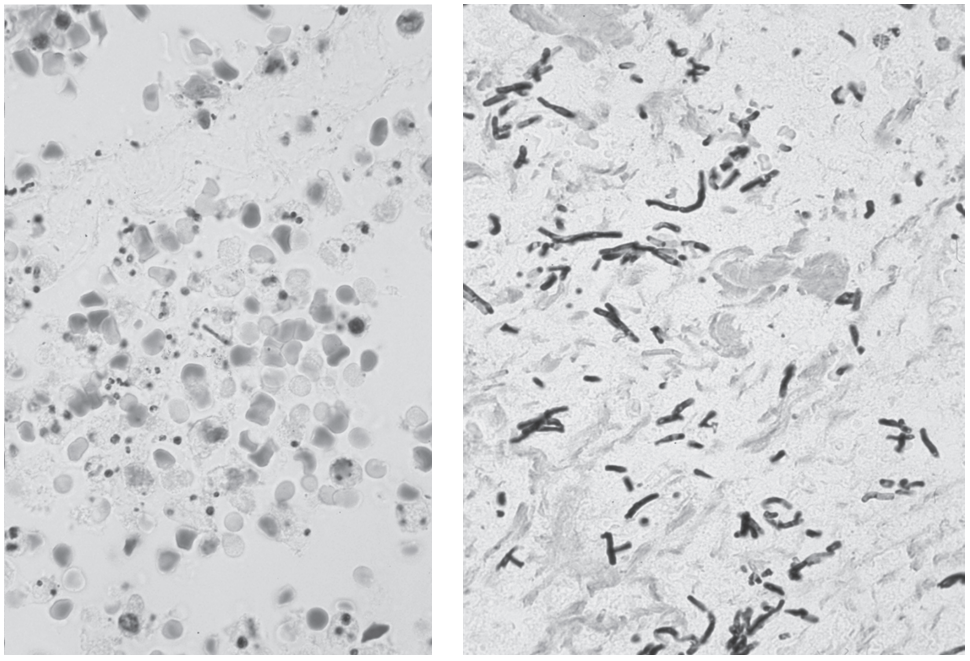

Figure 13.7 Gram stain and Steiner silver impregnation stain of same area of lymph node showing sparse gram-positive bacilli (a) and abundant silver staining organisms (b) (original magnification 250×). See color insert.

antigen staining using immunohistochemistry in the mediastinal lymph nodes and pleura while bacilli and antigens were not consistently present in other tissues, suggesting that treatment contained the disease.

Gastrointestinal Anthrax

Gastrointestinal anthrax is a rare disease and cases from outbreaks in Thailand and other countries have not been studied using histopathology. Thus, single case reports constitute the knowledge of pathology about this form of anthrax. A patient with gastrointestinal anthrax who survived showed semipurulent turbid brown fluid in the peritoneal cavity, firm, enlarged mesenteric lymph nodes, and confluent petechiae in an erythematous mesentery when a laparotomy was performed (Nalin et al., 1977). The lymph node showed edema, vessel dilatation, and proliferation of reticulum cells and lymphocytes. No organisms were observed in the fluid or lymph node that was biopsied.

The rare cases of gastrointestinal anthrax that have been described at autopsy have usually shown the ileum and cecum to be the portal of entry with hemorrhagic massively edematous ulcers; however, lesions may occur anywhere within the gastrointestinal tract, including the esophagus, stomach, duodenum, or jejunum (Pantanowitz and Balogh, 2003; Perl and Dooley, 1976). Microscopically, the ulcers have extensive acute hemorrhage, edema, and necrosis. Lymphatic vessels in the gastrointestinal wall show gram-positive bacilli. Regional lymph nodes are usually hemorrhagic, edematous, and necrotic, similar to what occurs in mediastinal lymph nodes of patients with inhalational anthrax. The hepatic sinusoids show acute inflammatory infiltrate and rare bacilli phagocytosed by Kupffer cells. Intestinal obstruction, perforation, and massive ascities are reported as complications.

TREATMENT OF ANTHRAX IN HUMAN PATIENTS

Cutaneous Anthrax

Penicillin G, 4–6 million units intravenously every 6 h or doxycycline 100 mg intravenously or orally for 7–10 days was the recommended treatment prior to the bioterrorism outbreak in the United States in 2001 (CDC, 2001b). After the events of 2001, the treatment recommendations were revised (Inglesby et al., 1999), and penicillin monotherapy was no longer recommended by the CDC because the strain implicated in the outbreak contained an inducible β-lactamase. For patients with severe cutaneous anthrax (those with systemic involvement, extensive edema, lesions in the head and neck) or when the patient is under the age of 2 years, the recommendations are the same as those for inhalational anthrax. In patients with localized disease, the current recommendation is ciprofloxacin 500 mg orally twice per day or doxycycline 100 mg orally twice daily for at least 60 days. Amoxicillin is an alternative treatment for those with contraindications to ciprofloxacin or doxycycline such as children, pregnant and/or lactating women.

Apart from antibiotic therapy, routine local wound care with sterile moist dressings changed regularly and elevation of the affected region to drain edema are recommended (Binkley et al., 2002). Surgical debridement is contraindicated because of risk of systemic dissemination of bacilli. However, in case of compartment syndrome and vascular compromise of extremities with massive edema, fasciotomy should be performed, but the primary lesion should be avoided. Excision of the eschar with skin grafting may be needed in cases with large lesions once the lesion has been sterilized.

Inhalational Anthrax

In cases of severe anthrax including inhalational anthrax and anthrax with sepsis or meningitis, prompt initiation of antibiotic therapy may improve survival. The CDC recommends combination therapy with either ciprofloxacin 400 mg intravenously twice daily or doxycycline 100 mg intravenously twice daily plus one to two additional antibiotics that are known to be active against *B. anthracis* (CDC, 2001c). These include clindamycin, vancomycin, imipenem, meropenem, chloramphenicol, penicillin, ampicillin, rifampin, or clarythromycin. The decision of which one of these antibiotics to use is arbitrary and is not based on clinical trials, but many experts favor clindamycin on the basis of its ability to inhibit toxin production. In patients with meningitis, an antibiotic that penetrates the blood–brain barrier is preferred, such as high-dose penicillin. The duration of antibiotic therapy is controversial because spores persisted in lung tissue of monkeys challenged with anthrax after 100 days of therapy. Thus, clinicians can give a 60-day course of antibiotics followed by close clinical observation, extend the course of antibiotics for 100 days, or administer a 100-day course of antibiotics combined with administration of anthrax vaccine.

In addition to antibiotic therapy, corticosteroids should be considered for patients with meningitis or significant mediastinal edema (Bartlett et al., 2002). Supportive care including drainage of pleural effusions is also important in the management of patients with inhalational anthrax.

Gastrointestinal Anthrax

In addition to the antibiotic management described above for inhalational anthrax, patients with gastrointestinal anthrax may require surgical resection of the affected areas of the gastrointestinal tract (Binkley et al., 2002).

Immune-Based Therapies

Antibiotics are only effective against anthrax if administered early in the course of the infection as toxin production and its effects are the most important pathogenic mechanisms leading to death in cases of anthrax. For this reason, there have been efforts to develop therapies that target the toxin and its effect. Hyperimmunoglobulin obtained from persons immunized with the anthrax vaccine and human monoclonal anti-PA antibodies are examples of such an approach, but the value of such therapies in clinical cases remains to be proven (Walsh et al., 2007).

Antibiotic Prophylaxis

The administration of prophylactic antibiotics is recommended for persons exposed to aerosolized *B. anthracis*. The current recommendation for adults is for the administration of ciprofloxacin 500 mg orally twice daily or doxycycline 100 mg orally twice daily for 60 days (CDC, 2001d). In a pregnant woman exposed to anthrax, prophylaxis with amoxicillin 500 mg three times a day for 60 days may be considered (CDC, 2001e). These recommendations are based on the fact that incubation period longer than 40 days were noted during the Sverdlovsk accident in 1979 and some primate studies.

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Chapter 14

Anthrax Vaccines

Elke Saile and Conrad P. Quinn

INTRODUCTION

The development of anthrax vaccines from the preindustrial era to the present reflects our changing perception of the threat from infectious diseases.* Tied to the progress of bacteriology as a scientific discipline and the maturation of germ theory as a conceptual framework for the rational explanation of infectious disease, the nineteenth-century effort to protect domestic farm animals by vaccination had an early focus on anthrax as one of the oldest livestock and human diseases known to man. In the course of the Industrial Revolution, the risk of anthrax migrated from agricultural, rural settings to population centers and factories in which animal-derived raw materials from distant locations were processed. The exposure of large numbers of factory workers in England, continental Europe, and North America to *Bacillus anthracis*-contaminated animal materials resulted in systematic investigations and early epidemiological analyses of human anthrax cases by physicians with a great deal of interest in the science of bacteriology (Jones and Teigen, 2008). Thus, the perception of anthrax changed from that of an agricultural disease to one affecting increasing numbers of industrial workers. Due to the seminal efforts of John Henry Bell in promoting decontamination guidelines for wool materials in 1884, occupational anthrax became a cornerstone in the movement to protect factory workers by implementing specific improvements in their working conditions (Fee and Brown, 2002). However, the development, evaluation, and deployment of vaccines for human anthrax were eventually driven by concerns in WWI, WWII, during the Cold War, and after the Gulf War in 1991 that *Bacillus anthracis* spores may be used as a biological weapon. More obvious than in many other examples, the history of anthrax vaccines attests to how biomedical science is influenced by society's perception of the threat posed by infectious diseases and vice versa.

*Required Disclaimer: The findings and conclusions in this chapter are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

DEVELOPMENT OF CELLULAR VACCINES

Following the pioneering efforts of Edward Jenner in 1796 on immunization against smallpox and of Koch in 1877 on the etiology of anthrax, the development of vaccines against disease had become an empirical science. Toward the end of the nineteenth century, improved handling conditions and decontamination procedures had dramatically reduced the incidence of anthrax among industrial workers by creating a safer working environment. However, the disease was still enzootic in European livestock, and both human and animal cases were still being reported in the United Kingdom (Ezzell et al., 1985; LaForce, 1978), continental Europe, and the United States (Evans and Brachman, 1991; Swiderski, 2004). In 1880, William Smith Greenfield preempted Louis Pasteur's technique of heat-attenuation by publishing in the Lancet that the anthrax "virus" (*sic*)—named by Cohn as *Bacillus anthracis*—could be attenuated by prolonged cultivation at elevated temperature to prevent sporulation (Tigertt, 1980). In a series of published lectures, Greenfield also described the inoculation of cattle with attenuated strains of *B. anthracis* and that, following mild symptoms, the animals were protected against "more severe attacks" (Tigertt, 1980). Nonetheless, it is Pasteur who is credited with production of the first attenuated bacterial vaccine for use in livestock (Ezzell et al., 1985). As with Greenfield, Pasteur noted that prolonged subculture (15–20 days) at temperatures of 42–43°C prevented sporulation and reduced virulence. Pasteur's vaccine, which was tested in a public experiment in 1881, was a duplex of attenuated *B. anthracis* cultures designated "Type I" and "Type II" (Hambleton et al., 1984). The Type I culture administered in the first inoculation had been attenuated for 15–20 days; the Type II culture in the second inoculation was less attenuated, having been cultivated for only 10–12 days (Ezzell et al., 1985). Pasteur's acclaimed experiment at Pouilly-le-Fort in 1881 involved inoculation of cattle, sheep, and goats. Stemming from this success, the attenuated anthrax vaccine was adopted for use throughout Western Europe, America, and Australia (Hambleton et al., 1984; Rountree, 1983). The degree of attenuation in these vaccines was, however, unpredictable (Ivins and Welkos, 1988), and there were occasional vaccine-associated outbreaks (Sterne et al., 1942; Turnbull, 2000). To address this variability, the vaccine was modified to use only the Type II inoculation to which saponin had been added to reduce its virulence (Hambleton et al., 1984). The saponin may also have had an adjuvant effect (Cameron, 1969). Pasteur-type vaccines were the basis of veterinary prophylaxis until 1935 and Whitford (1983) reported the use of such vaccines as recently as 1974 in a large anthrax outbreak in Texas (Whitford, 1983). The use of a double vaccine for anthrax and "blackleg" was reported in 1974. The vaccine formulation contained an attenuated capsule producing *B. anthracis* strain $A_4$3 to protect against anthrax and β-propiolactone-killed *Clostridium chauvoei* to protect against "blackleg" (Blancou, 1974).

Following elucidation of plasmid-mediated toxin (pXO1) and capsule (pXO2) production in *B. anthracis* (Green et al., 1985; Mikesell et al., 1983), the basis of the Pasteur vaccine was deduced by Mikesell et al. who reported that the pXO1 toxin plasmid could be cured selectively by prolonged subculture at 42.5°C (Mikesell et al., 1983). Thus, it may be surmised that in any heat-attenuated culture of *B. anthracis*, a mixed population of $pXO1^-$, $pXO2^+$, and $pXO1^+$, $pXO2^+$ bacteria might exist. Inoculation of an animal with this consortium would, in most cases, produce a sublethal infection resulting in full anamnestic immunity (Ezzell et al., 1985; Mikesell et al., 1983).

The current veterinary vaccine of use in the western hemisphere is derived from a non-capsulated, toxigenic strain isolated by Sterne in 1936 (Hambleton et al., 1984; Ivins

and Welkos, 1988; Sterne, 1937). This strain was isolated as a non-capsulated variant from extended culture on 50% horse serum agar in a 20% CO_2 atmosphere. The Sterne live-spore vaccine proved safer than heat-attenuated cellular vaccines and inoculation reactions were less frequent and less severe (Sterne, 1939; Sterne and Robinson, 1939). The Sterne live spore vaccine in use today is essentially unchanged and is the veterinary vaccine of choice. This vaccine formulation still contains 0.5% saponin, mainly for its adjuvant effect (http://www.coloradoserum.com). It has proved to be very safe for most animal genera, is effective, and is therefore of considerable economic importance in bringing the disease under control throughout the world (Cameron, 1969; Sterne, 1967). Alternative live spore vaccines have been proposed; for example, Ivins and coworkers generated attenuated live spore vaccine derivatives of *B. anthracis* UM23C-1 (Vollum) by insertional deactivation of aromatic amino acid synthesis pathways (Aro-) using the transposable element Tn*916*. The resultant strains were auxotrophic for phenylalanine, tyrosine, and tryptophan (Ivins et al., 1988). When used as live vaccines in animal models, these strains conferred significant levels of protection in guinea pigs and mice. However, because Tn*916* carries a tetracycline resistance gene, requires constant selection pressure to maintain insertion, self-excises precisely, and is of broad host range, those particular *B. anthracis* constructs were unsuitable for further vaccine development (Ivins et al., 1990).

Live spore preparations derived from the *B. anthracis* strains phenotypically similar to that of *B. anthracis* Sterne strain are used in eastern Europe and China as both veterinary and human vaccines (Turnbull, 2009). Ivins and Welkos (1988) reported that Sterne-type vaccines may cause necrosis at the inoculation site and occasionally kill laboratory animals. Although reports of livestock deaths due to the vaccine are rare and anecdotal, the live spore vaccine is not considered suitable in Western Europe and the United States for human use (Ivins and Welkos, 1988; Ivins et al., 1986; Turnbull, 1991).

DEVELOPMENT OF ACELLULAR VACCINES

In 1954, Smith and Keppie demonstrated that anthrax was a toxin-mediated disease (Smith and Keppie, 1954). However, the potential of cell-free anthrax vaccines—immunizing preparations that did not contain live or killed organisms—had been shown as early as 1904 by Bail, using sterile edema fluid from *B. anthracis* infected animals (Bail, 1904; Cromartie et al., 1947; Hambleton et al., 1984; Salsbery, 1926; Watson et al., 1947). Watson et al. (1947) also demonstrated that the toxic side effects associated with the edema fluid vaccinations could be eliminated following adsorption of the material onto calcium phosphate, and this group coined the term "protective antigen," proposing that it was probably a protein.

By 1948, Gladstone (1946, 1948) had shown that immunizing antigen could be produced *in vitro* and detected in cell-free culture filtrates. The culture medium contained large amounts of serum proteins in comparison to antigen, however, but the protection was similar to that afforded by edema fluid. The importance of hydrogen carbonate anions (HCO_3^-) for improved toxin yield was also noted although the underlying genetic basis for this was not resolved until 1989 (Bartkus and Leppla, 1989). The first effective cell-free vaccine intended for eventual use in humans was produced in 1954 when Wright and coworkers succeeded in producing protective antigens in a protein-free, chemically defined medium. Although antigen yields were low, they were consistent and capable of protecting rabbits and guinea pigs against virulent spore challenge. If alum-precipitated, this antigenic preparation could also induce a degree of immunity in mice (Hambleton et al., 1984;

Wright et al., 1954). In 1955, Boor and Tresselt cultivated *B. anthracis* CD-2 strain in a medium containing serum from the species to be vaccinated or from genetically related species (Boor and Tresselt, 1955). Other medium constituents included yeast extract, inorganic phosphate, and HCO_3^-. The antigen preparation was partially purified by ethanol or ammonium sulfate precipitation, concentrated by lyophilization and subsequently analyzed by electrophoresis and immunoprecipitation. The "PA" first described by Cromartie was now considered an α-globulin protein and the Boor–Tresselt vaccine successfully protected guinea pigs, rabbits, sheep, and nonhuman primates against challenge with the virulent *B. anthracis* strain CD-25 (also reported as "M36," a *B. anthracis* Vollum strain derivative) (Boor and Tresselt, 1955). The large amounts of serum proteins in the medium, however, precluded "PA" purification and identification, and also limited the utility of the Boor–Tresselt vaccine.

Belton and Strange (1954) in the United Kingdom succeeded in improving yields of protective antigens in a chemically defined medium supplemented with casamino acids, yeast extract, and charcoal which was found to increase the potency of the final product by "absorbing or removing" undefined toxin inhibitors (Belton and Strange, 1954). The antigen preparation was concentrated by lyophilization or precipitation with aluminum potassium sulfate ("alum"). The concentrate was capable of protecting both rabbits and nonhuman primates against intramuscular (IM) and aerosol challenge with the *B. anthracis* Vollum M36 strain. The U.K. vaccine differed from the vaccine of Wright et al. in that it was produced using protein hydrolysate growth medium rather than a chemically defined medium, and the production strain was *B. anthracis* Sterne $34F_2$ (Turnbull, 2000). The Belton and Strange vaccine formulation is the basis of the current U.K. licensed anthrax vaccine. It includes detectable levels of all three anthrax toxin components (PA, lethal factor [LF], and edema factor [EF]) and two cell-associated "S-layer" proteins, Sap and EA1. Each of these proteins is antigenic, although with the exception of PA, their contributions to the immunizing potential of the vaccine are unclear (Baillie et al., 2003). The U.K. vaccine is increasingly referred to as "anthrax vaccine precipitated" (AVP) to distinguish it from the U.S. "anthrax vaccine adsorbed" (AVA) (Grabenstein, 2008; Hepburn et al., 2007). The U.K. AVP formulation has the interesting characteristics that the alum can be dissolved using citrate salts and functional toxin components recovered and quantified (Hallis et al., 2002). These characteristics distinguish the vaccine from the current U.S. AVA formulation which contains preformed aluminum hydroxide, $Al(OH)_3$, as the adjuvant and a low level of formaldehyde as a stabilizing agent. Successful desorption of functional toxin from this adjuvant has not been reported and it is likely that the formaldehyde inactivates any residual enzymatic activity of any LF and EF, if present. The Belton and Strange vaccine (AVP) was licensed in the United Kingdom for human use in 1979 under the European Directive 75\319\EEC (license numbers 1511/0037 and 0058) (Turnbull, 2000). AVP is an aluminum potassium sulfate precipitate of sterile culture filtrate from the attenuated *B. anthracis* Sterne $34F_2$ strain (Grabenstein, 2008; Turnbull, 2000). AVP is administered by the IM route, and the primary course of four single injections is 0, 3, 6, and 30 weeks, followed by an annual booster. The vaccine is manufactured exclusively by the U.K. HPA for and on behalf of the U.K. Government. It is supplied to the U.K. Department of Health (DoH) for occupational health purposes and to the U.K. Ministry of Defence (MoD) to vaccinate service personnel against the use of anthrax as a weapon.

Prior to 1962, the U.S. vaccine was an alum precipitate similar to AVP (Grabenstein, 2008, Wright et al., 1954). It was this "Fort Detrick" formulation of Wright and coworkers—occasionally referred to as the "Merck Anthrax Vaccine"—that was used in the field study

of Brachman et al. (1962). The current AVA formulation also derives from studies in the United States by Wright and coworkers who described improved antigen production using *B. anthracis* V770-NP1-R grown in a defined medium under microaerophilic conditions (Wright et al., 1962). Antigen prepared in this way was adsorbed onto preformed aluminum hydroxide gel, $Al(OH)_3$, tested in guinea pigs, and used to immunize humans. A single dose of this vaccine preparation was capable of protecting guinea pigs against challenge with a virulent *B. anthracis* Vollum strain. In humans, the mild local reactions to vaccination were similar to those reported by Darlow and coworkers for the U.K. AVP formulation (Darlow et al., 1956; Puziss and Wright, 1963). In 1963, Puziss et al. reported the large-scale microaerophilic production of the PA from *B. anthracis*, and this has formed the basis of the manufacturing process for the current U.S. licensed anthrax vaccine (Puziss et al., 1963). This formulation of anthrax vaccine adsorbed (AVA, U.S. patent number 3,208,909, September 28, 1965) was the forerunner of the vaccine approved for use in the United States (Auerbach and Wright, 1955; Turnbull, 2000; Wright et al., 1962). The *B. anthracis* V770-NP1-R production strain secretes only low levels of anthrax toxin components EF and LF in comparison to the PA—an aspect considered important in reducing reactogenicity (Johnson and Spero, 1981). AVA prepared using *B. anthracis* V770-NP1-R was licensed in the United States in 1972 for subcutaneous (SC) administration to those in at-risk occupations and until 2008 was the only licensed aluminum-adjuvant vaccine used in this manner (Marano et al., 2008; Pittman, 2002). On December 11, 2008, the FDA approved IM administration of AVA and a reduction in the number of primary vaccinations from 6 to 5, with omission of the vaccination at 2 weeks (http://www.fda.gov/cber/approvltr/biothrax121108L.htm). AVA is not licensed for use in children, pregnant women, or those older than 65 years. The vaccine is now marketed as "BioThrax" by Emergent BioSolutions (Lansing, MI).

The U.S. Advisory Committee on Immunization Practices (ACIP) recommends routine vaccination with AVA for persons engaged in work involving production quantities or concentrations of *Bacillus anthracis* cultures or activities with a high potential for aerosol production. In practice, the vaccine is administered primarily to military service personnel and laboratory workers. In 2008, the ACIP reiterated its guidance that emergency and other responders are not recommended for routine pre-event anthrax vaccination due to lack of a calculable risk assessment. However, responder units engaged in response activities that may lead to exposure to aerosolized *B. anthracis* spores may choose to offer their workers pre-event vaccination on a voluntary basis. The ACIP recommended that any such vaccination program be carried out under the direction of a comprehensive occupational health and safety program (http://www.cdc.gov/vaccines/pubs/ACIP-list.htm#vacc).

VACCINE EFFICACY

Animal Models of Efficacy

Due to the low prevalence of anthrax in humans in the western hemisphere, evaluation of a human anthrax vaccine relies almost exclusively on animal models (Ivins and Welkos, 1988), and a range of animal genera, species, and strains have been used in this context. However, animal genera and species can differ in their susceptibility to both *in vitro* produced toxin and to *B. anthracis* infection. For example, although relatively resistant to spore challenge, the Fischer 334 rat strain is uniquely sensitive to anthrax lethal toxin and

was used routinely in the 1980s to assay lethal toxin potency (Ezzell et al., 1984). Mice are uniquely susceptible to capsulated, non-toxigenic strains of *B. anthracis* and this susceptibility varies by strain. Consequently, most mouse studies of vaccine candidate efficacy use toxigenic ($pXO1^+$), non-capsulating ($pXO2^-$) challenge strains such as *B. anthracis* Sterne or *B. anthracis* STI (Flick-Smith et al., 2002a; Ivins and Welkos, 1988; Welkos and Friedlander, 1988a,b). Guinea pigs and rabbits have been used extensively in development of skin tests for determination of the antigen content of early cell-free vaccines, pathology studies, and vaccine potency testing (Belton and Henderson, 1956; Darlow et al., 1956; Phipps et al., 2004; Ross, 1957). Vaccination using either *in vitro* produced antigen or live spores of non-capsulating, toxin antigen-producing strains can protect both of these genera against virulent spore challenge (Ivins et al., 1986, 1992, 1994).

Although PA is undoubtedly the critical antigen in any effective anthrax vaccine, the manner in which the antigen is formulated and presented to the immune system affects the level of protection afforded in different genera, with the PA-alum formulations being the least effective in mice, rats, and guinea pigs but highly effective in rabbits and nonhuman primates (Phipps et al., 2004). The apparent lower efficacy of PA-alum formulations in rodents has lead to the misconception that PA-based vaccines are not fully protective against anthrax, although there are extensive data to show that these levels of potency are most likely due to the specific effects of alum in these genera. Turnbull and coworkers (1990), for example, demonstrated that the efficacy in guinea pigs of PA-alum formulations could be enhanced using whole cell pertussis vaccine or whole cell extracts of *Corynebacterium ovis*, and there are several later reports of this being accomplished using inactivated spores of *B. anthracis* (Brossier et al., 2002; Gauthier et al., 2009; Rijpkema et al., 2005; Turnbull et al., 1990). Multiple studies over several decades have demonstrated the potency of PA in rats, guinea pigs, and rabbits using a range of non-alum adjuvants. Selection of the appropriate adjuvant for the target species is therefore a critical part of effective vaccine formulation (Ivins et al., 1992). Several reports have demonstrated that synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs enhanced the efficacy of AVA both in animal models (mice, rhesus macaques) and in humans (Gu et al., 2007; Klinman et al., 2006, 2007; Krieg et al., 1995; Tross and Klinman, 2008). Some of these CpG motif ODNs have been evaluated in human clinical trials, thus providing a level of confidence in their safety and efficacy if used in human vaccines (Klinman, 2006). The presence of all three anthrax toxin components PA, LF, and EF in a polyvalent vaccine formulation also enhances antibody responses to all three components (Ivins and Welkos, 1988; Kelly et al., 2007; Kim et al., 2008; Kolla et al., 2007; McLachlan et al., 2008; Park et al., 2008; Pezard et al., 1995; Quesnel-Hellmann et al., 2006; Sloat and Cui, 2006). These studies confirm the pivotal role of PA in protection and that efficacy can be enhanced by considered formulation, and serve as timely reminders that design of anthrax vaccines should be based firmly on the host in which the vaccine will eventually be used. At this time however, alum is the only adjuvant licensed for use in humans (Marciani, 2003; McKee et al., 2007; Ruiz et al., 2005).

The rabbit and the nonhuman primate models of anthrax vaccine efficacy have gained increasing prominence since 2001 for the evaluation of PA vaccine formulations for humans (Fritz et al., 1995; Joellenbeck et al., 2002; Leffel and Pitt, 2005; Phipps et al., 2004; Vasconcelos et al., 2003; Zaucha et al., 1998). These genera have been shown to be sensitive to *B. anthracis* infection and to have similar pathology to anthrax in humans (Fritz et al., 1995; Zaucha et al., 1998). Data on PA vaccine efficacy in rabbits and nonhuman primates have demonstrated consistently that as few as one dose of AVA and PA vaccines provides some protection against aerosolized spores of highly virulent *B. anthracis* and

that 2–3 doses separated by 7–14 days can provide complete protection for extended periods (Fellows et al., 2001; Marano et al., 2008; Pitt et al., 2001; Williamson et al., 2005).

Similar to interpretation of alum-based vaccine efficacy in rodents, the evaluation of early animal model vaccine efficacy data is complicated also by the use of a range of different *B. anthracis* strains of reportedly different levels of virulence. In the mid-twentieth century for example, the most frequently used challenge strains in laboratory studies were derived from *B. anthracis* Vollum. Of strains considered potentially "vaccine resistant" in a guinea pig model (Ivins et al., 1994; Little and Knudson, 1986), none were *B. anthracis* Vollum derivatives. Consequently, the highly virulent *B. anthracis* Ames strain has emerged as the standard for animal infection studies. Aerosol exposure and pulmonary or intratracheal liquid instillation are the preferred routes of administration for modeling inhalation anthrax (Fellows et al., 2001; Lyons et al., 2004).

Because rates of disease are low and human challenge studies are unethical, licensure of anthrax vaccines relies heavily on animal studies. In 2002, the U.S. FDA finalized an amendment of 21 CFR Parts 314 and 601; the so-called "animal rule." This amendment enables appropriate studies in animals in certain cases to provide substantial evidence toward licensure for new drug and biological products for which adequate and well-controlled clinical studies in humans cannot be ethically conducted and field efficacy studies are not feasible (Food and Drug Administration, 21 CFR Parts 314 and 601, [Docket No. 98N–0237], RIN 0910–AC05, New Drug and Biological Drug Products; Evidence Needed to Demonstrate Effectiveness of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible; Federal Register/Vol. 67, No. 105/Friday, May 31, 2002, pp. 37988–37998) (http://www.upmc-biosecurity.org/website/resources/govt_docs/countermeasures/hhs/fda_21_cfr_parts_314_and_601_docket_98n-0237_rin_0910-aco5.html). Thus, the path toward licensure for anthrax vaccines may encompass several stages in animal models of efficacy, such as a proof of concept stage in murine models using a toxigenic, non-capsulating challenge strain, followed by corroborative proof of concept in rabbit models of infection using fully virulent *B. anthracis* Ames, and finally, completing the pivotal regulatory compliant preclinical licensure studies in rabbits and nonhuman primate models of inhalation anthrax.

Efficacy in Humans

Naturally occurring anthrax is considered a rare disease in the United Kingdom and the United States. Where it does occur, it is associated with handling of contaminated animal materials such as bone meal and hides (Brachman and Friedlander, 1999; Inglesby et al., 1999; Plotkin et al., 1960; Quinn and Turnbull, 1998). Efficacy analyses of human anthrax vaccines are hampered by this low prevalence. Consequently, there is only one controlled study of a human anthrax vaccine in the United States and no similar study of the U.K. vaccine. Nonetheless, although improved industrial hygiene practices most likely account for the reduction in human anthrax, there is still indication of vaccine efficacy. For example, subsequent to the introduction in 1965 in the United Kingdom of vaccination for workers in at-risk occupations, there was a fourfold decrease in the number of reported anthrax cases in the period 1961–1980 with only one case recorded in 1980 (CDSC, 1982; Vaccine against Anthrax, 1965). Between 1981 and 2008, only 19 cases of possible anthrax were reported in the United Kingdom, including two deaths from confirmed inhalation anthrax; one in Scotland in 2006 and one in England in 2008. Both of these fatalities were associated with handling or playing drums made with contaminated

animal hides and neither individual was vaccinated (http://www.hpa.org.uk/webw/HPAweb&Page&HPAwebAutoListDate/Page/1191857565963?p=1191857565963).

In the United States prior to 1900, about 200 human anthrax cases were reported (Plotkin et al., 1960). Although vaccination for workers considered at risk became only mandatory in the 1960s, there were just 18 cases of inhalation anthrax reported in the United States between 1900 and 2000, none of which were in vaccinees (Inglesby et al., 1999). Until the bioterrorist attack in 2001, 95% of the 238 cases of anthrax reported in the United States since 1955 were cutaneous infections (Brachman and Friedlander, 1999). Because of the low prevalence of anthrax, the human efficacy data for anthrax vaccines in the United States derive primarily from one field study of an acellular, alum-precipitated PA-based anthrax vaccine—the formulation of Wright and coworkers and a forerunner to AVA—and a surveillance study completed by the U.S. Centers for Disease Control (CDC) from 1962 to 1974 (Joellenbeck et al., 2002) (http://www.cdc.gov/vaccines/pubs/ACIP-list.htm#vacc).

The Brachman study was a randomized, placebo-controlled efficacy trial conducted in four textile mills in the northeastern United States from January 1955 to March 1959. The mills processed imported goat hair that was typically contaminated with *B. anthracis* spores. Typical infection rates were 0.6–1.8% (Brachman et al., 1962). There were 379 participants who completed the vaccination course and 414 participants in the placebo group. In the course of the study, a total of 26 cases of anthrax were reported among the four mills. The cases were reported essentially throughout the entire evaluation period indicating continuous exposure throughout the study duration. Twenty-one of the cases were cutaneous anthrax and five were inhalation anthrax. Three of these cases occurred among individuals who had received the vaccine. Of the remaining 23 cases, 17 were in the placebo group and 6 were unvaccinated individuals. Inhalation cases had a fatality ratio of 80% (4/5) and occurred only among unvaccinated workers. There were too few inhalation cases to permit an assessment of the effectiveness of the vaccine against this form of the disease. Overall, combining the cutaneous and inhalation cases, however, the data indicated that vaccination provided 92.5% protection against anthrax compared with the placebo group (95% confidence interval, 65–100%).

The CDC observational study data on the occurrence of anthrax in workers in goat hair processing mills or those living near mills in the United States were collected during the period 1962–1974. During the surveillance period, individuals received either the "Fort Detrick" vaccine or AVA (Brachman et al., 1962; Grabenstein, 2008; Joellenbeck et al., 2002). There were 27 cases of cutaneous anthrax confirmed and no cases of inhalation anthrax. Of the confirmed cases, 24 occurred in mill workers and 3 occurred in persons who worked in or near mills but who were not mill employees. Of the confirmed cases, two were in mill workers who had received two doses of vaccine and one was in a mill worker that had received only one dose of vaccine. The remaining 89% of cases was in unvaccinated individuals. The study concluded that no cases of anthrax occurred in individuals who had received at least three of the recommended six doses of anthrax vaccine although the risk of infection was continuous for the surveillance period (FDA, 1985, 2005).

Although the acellular PA-based alum vaccines have undoubtedly contributed to the decline in anthrax in humans, there is considerable room for improvement in several areas of vaccine design. The preparations of both the U.K. and U.S. licensed vaccines are essentially of undefined composition; at least two doses are required to produce substantial antibody responses in humans and the origins and supporting data for the licensed vaccination schedules for both vaccines are not well documented (Darlow et al., 1956; Hepburn

et al., 2007; Marano et al., 2008; Pittman, 2002). To address this issue for AVA, Pittman et al. conducted a pilot clinical trial in which a reduced AVA vaccination schedule and a change to the IM route elicited similar antibody responses with fewer injection site adverse events (AEs) than the licensed regimen (Pittman et al., 2002). These data provided impetus for the U.S. Congress in 1999 to mandate the U.S. CDC to complete a pivotal randomized, double-blind, placebo controlled, Phase 4 clinical trial to assess the serological noninferiority of reduced dose schedules and a change in route of administration of AVA. The CDC Anthrax Vaccine Research Program (AVRP) will undoubtedly resolve some of the issues surrounding the vaccination regimen for AVA. Based on data from interim analyses of the CDC AVRP, the FDA in December 2008 approved the use of AVA by the IM route and, significantly, eliminating the dose at week 2 from the first four injections of the vaccine priming series. These were the first data-driven FDA-approved changes in the use of AVA since it was licensed in 1971. The conclusion of the CDC study in 2009 will likely provide additional data on optimal use of AVA (Marano et al., 2008).

Specific literature for the efficacy of live spore vaccines in humans is even more limiting than those for acellular vaccines. Demicheli et al. (1998) published a meta-analysis comparing the field study of Brachman et al. (1962) with an observational study of the live spore STI vaccine. The authors concluded that, despite the limitations of the analysis, both of the anthrax vaccines evaluated were efficacious and safe (Demicheli et al., 1998).

DURATION OF IMMUNITY

The duration of anthrax vaccine induced immunity in humans following the initial priming series is not known. Data from animal studies suggest that two SC inoculations with a PA vaccine may provide protection for 1–2 years and that three IM inoculations with AVA provide significant levels of protection in rhesus macaques for up to 4 years (Darlow et al., 1956; Ivins et al., 1998; Marano et al., 2008; Turnbull et al., 1986). These periods of efficacy were defined by the duration of those studies, rather than waning immunity. Anti-PA antibodies are an important serological indicator for protection against anthrax (Little et al., 2004; Pitt et al., 2001; Reuveny et al., 2001). Persisting PA-specific IgG memory B cell activity may also contribute to immunity, due to these cells' ability to proliferate and differentiate rapidly into anti-PA antibody-secreting plasma cells. For example, in rhesus macaque models of anthrax, serum anti-PA IgG levels have been reported to decrease over time to low or undetectable levels with only a partial loss of protection against aerosol challenge with virulent *B. anthracis* (Ivins et al., 1998; Marano et al., 2008). Persisting memory B cells capable of replenishing neutralizing antibody after *B. anthracis* challenge may be responsible for protection at late time points. For anthrax vaccination and clinical disease, therefore, quantitative analysis of PA-specific IgG B cell memory may be a useful predictor of the duration of immunity against anthrax, and peak anti-PA IgG levels after infection or vaccination may be a surrogate marker for the presence and magnitude of PA-specific IgG memory B cells (Quinn et al., 2004). PA-specific IgG memory B cell frequencies observed in patients with inhalation anthrax were comparable to or higher than those observed in AVA vaccinees at approximately 12 months after infection or booster vaccination, respectively. These data suggest that both survivors of inhalation anthrax and AVA vaccinees may develop long-term protective immunity to anthrax (Quinn et al., 2004). This interpretation is supported by data from mouse studies in which elevated levels of antigen-specific B cells were associated with extended duration of protection (Tross and Klinman, 2008). Although direct data for duration of immunity against human anthrax are not

available, data for other vaccines indicate that antigen-specific memory B cells may persist in immunized individuals for more than 50 years (Crotty et al., 2003). The required frequency of booster vaccinations to maintain such long-term immunity using anthrax vaccines in humans is being investigated (Marano et al., 2008).

VACCINE SAFETY

Anthrax Vaccine Adsorbed and Anthrax Vaccine Precipitated

AVA and AVP are currently the only licensed anthrax vaccines in the United States and the United Kingdom, respectively. The history, development, and context of the U.K. anthrax vaccine have been described in detail elsewhere (Turnbull, 1991, 2000). There are few published data on the safety in humans of AVP. In the early studies of this vaccine, Darlow and coworkers reported data from the administration of 1057 SC doses to 373 recipients. They reported a localized inflammatory reaction to repeated vaccination with the AVP preparation. This was associated with mild erythema and an increased reaction frequency following annual booster inoculations. There were no other local AEs reported in humans in that study and systemic AEs ("febrile reactions") were low at 0.19% (Darlow et al., 1956). More recent reports indicate a low association between self-reported anthrax vaccination and multiple AEs in the U.K. military service personnel (Murphy et al., 2006, Unwin et al., 1999). A retrospective safety study in Royal Air Force (RAF) personnel receiving 1–4 doses of a single batch of AVP reported mild side effects in 11% of recipients and no serious side effects (Enstone et al., 2003). A toxicity study in mice raised concern regarding coadministration of AVP with whole-cell pertussis vaccine. This combination resulted not only in an adjuvant effect for AVP but also caused a mouse strain-dependent toxic response including splenomegaly, weight loss, and a lethal ascites. These effects were attributed to potentiation of the pertussis toxin activity in the formulation rather than an effect of AVP, and only a cautious extrapolation was made regarding safety of this vaccine combination in humans (Rijpkema et al., 2005). The literature on AVP administered alone is consistent with information available from the U.K. HPA which reports that "No serious side effects have been reported. Reactions are uncommon but occasionally a mild rash or swelling at the site of injection or even at the site of an earlier injection may occur and last for a couple of days. More rarely, swollen glands, mild fever, flu-like symptoms, rash, itching or other allergic reactions may occur. The occurrence of a reaction after a first injection of the vaccine does not necessarily indicate a pre-disposition to subsequent reactions on further injections" (http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1216883803565).

Extensive recent studies of AVA and a comprehensive Institute of Medicine (IOM) review have asserted that AVA is reasonably safe (Brachman et al., 1962; Grabenstein, 2003; Joellenbeck et al., 2002; Marano et al., 2008; Pittman, 2002; Pittman et al., 2001, 2002, Sato et al., 2000). Martin and coworkers reported that over a 2-year period, 1998–2001, more than 750,000 U.S. service personnel received AVA without persistent systemic symptoms (Martin et al., 2002). In its 2002 report, the IOM concluded that local injection site AE reactions such as redness, swelling, or nodules were similar to those experienced following receipt of other aluminum containing vaccines in use by adults. Systemic AEs such as fever, malaise, and myalgia associated with receipt of AVA were similar to those observed with other vaccines, but of much lower frequency than local AEs. Immediate

local AEs such as arm motion limitation occurred in some individuals, but these effects were self-limited and had no known long-term effects (Joellenbeck et al., 2002). This interpretation is in agreement with data from the Vaccine Adverse Event Reporting System (VAERS)—a passive surveillance program for vaccine safety sponsored by the Centers for Disease Control and Prevention (CDC) and the FDA. Although not a definitive representation of AVA safety—the data represent unverified reports of health events, both minor and serious, that occur after vaccination—these data may provide a reasonable source of trends in AVA-associated events. From 1990 through March 31, 2005, VAERS evaluated 4279 reports of AEs following administration of 5.3 million doses of AVA to approximately 1.3 million DOD personnel and were unable to conclude that there was a causal relationship between serious AEs or deaths and the vaccine.

Influence of Route of Administration and Gender on Local AEs to Vaccination

AVP is routinely administered by the IM route (Hepburn et al., 2007). AVA, however, was originally licensed for SC administration and only recently has been approved for IM administration (http://www.fda.gov/cber/approvltr/biothrax121108L.htm). The benefits of IM compared to SC administration have been demonstrated in human clinical trials of AVA (Marano et al., 2008; Pittman et al., 2002). In the most recent of these reports, 1005 volunteers were evaluated in a randomized, double blind, noninferiority Phase 4 clinical trial. In that study, solicited injection site AEs assessed during examinations occurred at lower proportions in the IM group compared to SC, and there was a significant ($p < 0.01$) odds ratio (OR) reduction of occurrence for warmth, tenderness, itching, erythema, induration, edema, and nodules. Administration of the vaccine IM compared to SC was associated with fewer moderate and severe injection site AEs (10.2% vs. 7.0%; $p = 0.04$) and a reduction of 50% in the odds of experiencing greater pain upon injection ($p < 0.01$). IM administration did not significantly influence the occurrence of systemic AEs (Marano et al., 2008).

Gender-related immunogenicity and AE differences have also been reported for AVA and a relationship to the route of administration demonstrated. In the study by Marano et al., females were almost twice as likely as males to experience any injection site AE (OR = 1.93, $p < 0.01$), and the absolute differences between females and males for warmth, itching, erythema, induration, and nodules were largest in those receiving the vaccine SC. Females had a significant increase in the odds of experiencing greater pain upon injection compared to males (OR = 1.61, $p < 0.01$). By changing to the IM route of administration, however, the proportions of females and males with an injection site AE were 69% and 53%, respectively, similar to those from safety evaluations of other aluminum-containing vaccines given IM and comparable to the preceding pilot study of AVA by Pittman et al. (Gorse et al., 2006; Pittman, 2002; Pittman et al., 2002).

Marano et al. (2008) concluded that changing from SC to IM reduced the frequency of most injection site AEs in men and women and resulted in substantially diminished absolute differences in AEs between men and women. In addition to route of administration, the frequency and severity of AVA-associated AEs in women have also, to some extent, been attributed to body mass index and progesterone levels although this and other sex-related factors are still under investigation (Brachman et al., 1962; Grabenstein, 2003; Joellenbeck et al., 2002; Marano et al., 2008; Pittman, 2002; Pittman et al., 2001, 2002; Sato et al., 2000; Zhang et al., 2008).

RECOMBINANT PA VACCINES

The current licensed anthrax vaccines AVA and AVP have long been recognized as difficult to standardize and incompletely characterized (Charlton et al., 2007; Hallis et al., 2002; Joellenbeck et al., 2002) and an extensive body of research has been generated on developing and evaluating defined, efficacious anthrax vaccines containing PA (Chawla et al., 2009; Fasanella et al., 2008; Ivins et al., 1998; McBride et al., 1998). The native PA protein from *B. anthracis* and its recombinant counterpart (rPA) from a variety of expression systems have been the central components to all of these approaches.

Native PA is secreted from *B. anthracis* as an 83-kDa (PA83) monomeric polypeptide (Welkos et al., 1988). The 3-dimensional crystal structure of PA has been resolved to 2.1Å (Petosa et al., 1997). The PA83 monomer is organized into four domains. Domain 1 comprises the amino terminal residues 1-258 including the protease sensitive motif $S_{163}RKKRS_{168}$ required for activation of PA to the toxin complex-forming 63-kDa (PA63) conformer (Klimpel et al., 1992). Domain 1′ (residues 168-258) constitutes the new amino-terminus of the PA63 polypeptide after proteolytic activation and contains a solvent-exposed hydrophobic region implicated in the high-affinity interactions with the catalytic polypeptides EF and LF. Domain 2 extends from residues 259-487 and includes a chymotrypsin-sensitive flexible loop (residues 302-325) implicated in membrane insertion and toxin translocation (Novak et al., 1992; Sellman et al., 2001; Singh et al., 1994). Domain 3 is encompassed by residues 488-595, and Domain 4 comprises the carboxyl-terminal region of residues 596-735. During the intoxication process PA63 conformer forms a heptameric complex to which LF and EF bind with high affinity (Petosa et al., 1997). Domains 1′ and 2 are predominantly exposed on the interior of the heptameric conformer with Domains 3 and 4 predominantly on the exterior surfaces of the conformer. While domains 1′, 2, and 3 are in close proximity and coordinate together for heptamerization and membrane insertion of PA63 during translocation of anthrax toxin complexes into host cells, Domain 4 is spatially distinct from the other domains and is implicated in target cell receptor binding (Petosa et al., 1997). In the context of anthrax vaccine development, multiple regions on PA can potentially serve as epitopes, and a variety of linear B cell, T cell, and conformational epitopes have been defined, or proposed, that contribute to the immunizing effect of PA. For example, Abboud and Casadevall (2008) used a combination of predictive surface exposure and empirical analyses to identify linear peptide epitopes in PA83. These regions included Q_{121}-N_{150}, K_{143}-Q_{158}, S_{339}-R_{359}, and L_{421}-L_{440} (Abboud and Casadevall, 2008). Specific linear regions of PA83 recognized by monoclonal antibodies that neutralize anthrax toxin *in vitro* and *in vivo* include D_{648}-Y_{660}, E_{712}-G_{720}, and K_{679}-N_{693} recognized by, or involved in the binding of, murine monoclonal 14B7 and derivatives (Sivasubramanian et al., 2008; Varughese et al., 1999) and the linear sequence S_{301}-S_{325} recognized by the murine monoclonal F20G75 (Gubbins et al., 2006). Monoclonal 14B7 is a forerunner for some of the many anti-PA-based immunotherapeutics currently under development (Mohamed et al., 2005). The epitope recognized by murine monoclonal F20G75 was also a target for antibodies in serum from human recipients of the AVA (Gubbins et al., 2007).

Kwok et al. (2008) identified multiple CD4+ T cell epitopes within PA83 using tetramer-guided epitope mapping and T cells from human AVA recipients. The regions identified included T_{169}-E_{188}, T_{369}-N_{388}, L_{385}-I_{404}, T_{393}-P_{412}, I_{505}-K_{524}, D_{593}-V_{612}, and N_{713}-Y_{732}. Of particular note was that the AVA vaccination promoted a predominantly Th2 type lineage of memory T cells. PA-specific Th2 development is associated with a predominantly IgG_1 response which concurs with animal model data indicating that

facets of the anti-PA antibody response are reasonable correlates of protection against anthrax.

The PA63 comformer has received some attention as a vaccine component, whether as the recombinant protein, as a truncated gene transcript expressed in *Salmonella* spp. or as a component of purified DNA vaccines (Coulson et al., 1994; Garmory et al., 2003; Ivins et al., 1989). The rationale for this approach resides in the concept that the PA63 conformer may present different, more relevant, epitopes to the immunized host than does PA83 and that direct presentation of PA63 obviates the need for proteolytic processing of the full-length molecule. Despite some success in animal models, the development of PA63 as a vaccine component in its own right does not seem to have gained momentum, and the focus of recombinant PA vaccine research remains on full-length PA83.

The most prominent candidates for rPA-based anthrax vaccines in humans have included "rPA102" and "SparVax™" manufactured by VaxGen and PharmAthene, respectively. Although rPA102 successfully completed Phase I clinical trials, its development has been discontinued (Gorse et al., 2006; Russell, 2007). SparVax, initially developed by Avecia prior to their acquisition by PharmAthene, has demonstrated efficacy in nonhuman primate infection studies (Williamson et al., 2005), and the manufacturer reports that "Phase I and Phase II clinical trials involving more than 700 healthy human subjects have been completed and showed that SparVax™ appears to be well tolerated and induces an immune response in humans. These studies suggest that three doses of SparVax™, administered several weeks apart, should be sufficient to induce protective immunity. In preclinical studies, SparVax™ has also demonstrated the capability to protect rabbits and non-human primates against a lethal aerosol spore challenge of the anthrax Ames strain" (Duchars, 2007; Duchars et al., 2009) (http://media.corporate-ir.net/media_files/irol/19/191999/FactSheet-SparVax_Nov2008.pdf). Both rPA102 and SparVax were formulated for IM injection.

ANTHRAX VACCINE RESEARCH

Notwithstanding the extensive data on PA vaccine efficacy, a significant component of recent literature has focused on augmenting PA vaccines by employing novel delivery technologies, adjuvants, and including additional antigens such as capsule poly-D-glutamic acid (PGA) oligomers, formalin inactivated *B. anthracis* spores, and other toxin components (Wang and Roehrl, 2005). This research has included analyses of PA polypeptide domains (Flick-Smith et al., 2002b; Yan et al., 2008), DNA vaccines (Galloway and Baillie, 2004), alternative delivery technologies (Luxembourg et al., 2008; Mikszta et al., 2005), evaluation of novel adjuvants (Kelly et al., 2007; Kim et al., 2008; Park et al., 2008), oral and mucosal vaccines (Aloni-Grinstein et al., 2005; Baillie et al., 2008; Bielinska et al., 2007; Jiang et al., 2006; Stokes et al., 2007), and PA-producing live-attenuated vaccines (Ivins et al., 1990; Skoble et al., 2009).

Additional antigens have been explored both in their own right as vaccines and as supplements to PA. These additional antigens have included other toxin components such as LF (Mendelson et al., 2005), spore and vegetative cell antigens (Brahmbhatt et al., 2007; Brossier et al., 2002; Cohen et al., 2000; Gauthier et al., 2009; Skoble et al., 2009), capsule amino acid oligomers (Chabot et al., 2004; Joyce et al., 2006; Kubler-Kielb et al., 2006; Makino et al., 2002; Rhie et al., 2003; Schneerson et al., 2003; Wang et al., 2004), and toxin-encoding DNA sequences (Galloway and Baillie, 2004; Galloway et al., 2004; Gu et al., 1999; Price et al., 2001). The rationale for these approaches is to enhance the vaccine

induced anti-toxin immune response by additional targeting of the spore and vegetative cell. Such approaches have the potential to broaden the defensive repertoire of the vaccinated host by recruiting opsonophagocytotic activity and enhanced bacterial clearance. It is noteworthy that the existing undefined vaccines may already have achieved this breadth of targeting, though perhaps through necessity and serendipity rather than design (Baillie et al., 2003; Grunow et al., 2007; Turnbull, 2000).

Vaccine Delivery Technologies

The vaccination regimens for the current licensed anthrax vaccines and second-generation vaccines in development involve multiple vaccinations by needle injection over weeks and months. Single-dose or infrequent-dosing, noninvasive, and self-administered vaccinations would therefore be attractive propositions for the development of third-generation vaccines. New vaccine delivery technologies have been investigated both to simplify the vaccination procedure and to potentially enhance vaccine efficacy. These technologies have included transdermal skin patches, transcutaneous electroporation, oral vaccination, and intranasal delivery.

Transcutaneous rPA vaccination using skin patches was reported protective against *B. anthracis* spore challenge in mice (Kenney et al., 2004; Matyas et al., 2004), and Mikszta et al. compared microneedle-based intradermal injection, epidermal delivery by microabrasion, and intranasal delivery of liquid and powder formulations of rPA with IM delivery in mice and rabbits. Intradermal and epidermal delivery of rPA with CpG or aluminum hydroxide and intranasal delivery of rPA with CpG or a CpG/chitosan combination protected rabbits against *B. anthracis* aerosol spore challenge. However, the authors noted the dependence on specialized delivery technologies and that, with the exception of jet injection, such technologies are in very early stages of development and have yet to be proven clinically (Mikszta et al., 2005).

Oral and nasal administration of vaccines may be less invasive than injection or intradermal microneedle delivery, and these strategies have been effective in a variety of animal models to elicit antitoxin antibody responses and to protect against lethal toxin injection and *B. anthracis* infection (Flick-Smith et al., 2002a; Gaur et al., 2002; Klas et al., 2008; Sloat and Cui, 2006, Wimer-Mackin et al., 2006). Oral administration of *Salmonella* expressing different rPA constructs (Stokes et al., 2007), or *B. anthracis* spores from attenuated strains expressing rPA (Aloni-Grinstein et al., 2005), have been successful in the guinea pig model. Edible anthrax vaccines for humans and livestock have received considerable attention because theoretically, production costs could be lowered, costs for stockpiling, storage, and transport could be reduced, and large numbers of individuals could be vaccinated without the need for medical personnel. Kim et al. (2004) showed expression of a cholera toxin B subunit-LF fusion protein in potato; Aziz et al. (2005) and Watson et al. (2004) were able to demonstrate expression of PA in tobacco chloroplasts or nuclear transgenic tomato plants. However, while these plant-expressed proteins were functionally active in cytotoxicity assays and immunogenic in mice, these studies have not progressed beyond the proof of concept stage, and it has not been demonstrated whether vaccines produced in crops used as animal feed or for human consumption would confer protection against anthrax in any animal model.

Nasal and oral vaccine delivery may induce both mucosal and systemic immune responses (Boyaka et al., 2003). Baillie et al. used *Salmonella enterica* serovar Typhi Ty21a to express and export a ClyA-PA fusion protein and demonstrated protective immu-

nity after mucosal priming followed by a parenteral PA or AVA boost (Baillie et al., 2008). The fact that in mice a protein boost is still necessary illustrates the difficulty in achieving consistent protection by nasal or oral vaccination alone. The prime-boost approach also introduces additional complexities into any potential vaccination regimen based on this system. Recently, several strategies specifically for mucosal immunization against anthrax have been proposed. Intranasal mucosal immunization with AVA protected mice against *B. anthracis* Sterne spore challenge (Zeng et al., 2007), and rPA administered nasally in combination with a soybean oil-and-water nanoemulsion protected guinea pigs against intranasal challenge with *B. anthracis* Ames (Bielinska et al., 2007). Mikszta et al. reported that intranasal delivery of an rPA powder formulation adjuvanted with CpG or CpG plus chitosan protected rabbits against *B. anthracis* Ames strain aerosol challenge (Mikszta et al., 2005). Intranasal delivery of rPA formulations may therefore be a promising alternative delivery technology for effective PA vaccines (Jiang et al., 2006). The protective value of eliciting specific anti-PA responses has not yet been clarified. Similarly, the use of commensal bacteria in the human gastrointestinal tract, specifically *Lactobacillus* species, has been proposed as a delivery vehicle and as an adjuvant, targeting antigens like PA to dendritic cells (Mohamadzadeh et al., 2008; Zegers et al., 1999). Additional studies are needed to demonstrate whether this line of investigation can deliver enhanced protection in the animal models available.

Novel Antigens

One of the strategies to broaden the vaccine-induced immune response is to include protein conjugates of synthetic oligomers of the poly-D-glutamic acid (PGA) capsule in PA-based vaccine preparations. The perceived dogma for the PGA has been that it is weakly antigenic except for low molecular weight species released *in vivo*, it is a poor immunogen, and perhaps an immune decoy. This dogma has been challenged in studies reporting that, when conjugated to rPA as a carrier protein, PGA oligomers were capable of eliciting a specific IgG response with opsonophagocytic properties and thus PGA has potential as a vaccine component (Chabot et al., 2004; Joyce et al., 2006; Kubler-Kielb et al., 2006; Makino et al., 2002; Rhie et al., 2003; Schneerson et al., 2003; Wang et al., 2004).

The evaluation of novel antigens of *B. anthracis* has also extended into the relatively unexplored area of carbohydrates. Recently, the immunogenicity of the carbohydrate moiety of the exosporium glycoprotein BclA has been demonstrated (Mehta et al., 2006; Wang et al., 2007). The addition of carbohydrate antigens from *B. anthracis* spores or structurally similar sugars from the capsular polysaccharide (CPS) of *Shewanella* spp. (Kubler-Kielb et al., 2008) or oligosaccharide antigens from the *B. anthracis* cell wall (Vasan et al., 2008) could potentially confer protection against the germinating spore and proliferating vegetative bacterium as well as the anthrax toxemia (Leoff et al., 2009; Wang and Roehrl, 2005). Hahn et al. demonstrated that spore protein antigens delivered as DNA vaccines improved protective efficacy in mice of a PA- and BclA-encoding plasmid DNA combination as compared to vaccination with either of the individual plasmids (Hahn et al., 2006b).

DNA Vaccines, Viral Vectors, and Combination Vaccines

Vaccination against anthrax using DNA theoretically offers advantages in terms of eliciting an immune response against a defined antigen delivered via a nucleic acid coding

region, most often in a plasmid or viral construct. The approach is currently limited to protein antigens and for anthrax at least, precludes the use of novel PGA and carbohydrate antigens. In addition, the efficacy of this approach in nonhuman primates and human subjects still poses challenges. In order to provide strong immunogenicity in the nonhuman primate model and in humans, the efficiency of DNA uptake, gene expression, and antigen presentation must be optimized so that one of the major advantages of DNA vaccination (i.e., defined antigen translational products) is not compromised by the need to add bacterial cellular components or protein antigens to the formulations (Galloway and Baillie, 2004). Gu et al. were the first to demonstrate protection of mice against intravenous lethal toxin challenge after IM administration of a plasmid expressing the coding sequence for PA63 (Gu et al., 1999). While immunization of mice with DNA vectors encoding the terminal fragment of LF or PA63 resulted in protection against intravenous lethal toxin, the immune responses were enhanced in mice which received both DNA vectors when compared to the mice which received only one plasmid (Price et al., 2001). DNA vaccines encoding the full-length, 83-kDa PA protein introduced by electroporation to mice, rats, and rabbits enhanced immune responses in comparison to IM delivery, and studies in the rabbit model with PA and LF plasmid DNA alone or in combination demonstrated protection against *B. anthracis* Ames spore aerosols (Galloway et al., 2004; Hermanson et al., 2004; Luxembourg et al., 2008). A number of investigations have addressed the issue of vaccine efficacy and enhancement of protection by addition of spore protein coding sequences (Hahn et al., 2004, 2006a,b) but the safety and efficacy of DNA vaccines for anthrax in nonhuman primates and humans have not yet been addressed (Galloway and Baillie, 2004).

With regard to viral vectors, Tan et al. demonstrated that a single IM dose of a recombinant serotype 5 adenovirus vector (Ad5) which expressed the *pag*A coding sequence for PA elicited an anti-PA antibody response and *in vitro* lethal toxin neutralization activity, and provided protection against intravenous anthrax lethal toxin challenge in BALB/c mice (Tan et al., 2003). A follow-up study investigated the use of a nonhuman primate adenovirus serotype (AdC7) as a strategy to circumvent the widespread preexisting immunity to adenovirus in the human population. When mice immune to Ad5 were vaccinated with AdC7 expressing *pag*A (AdC7PA), they developed high anti-PA antibody titers and were protected against lethal toxin challenge, whereas Ad5 immune mice vaccinated with a similar Ad5 construct expressing *pag*A elicited only low anti-PA neutralizing titers and were not protected against lethal toxin challenge. Thus, the AdC7PA vaccine proved effective in the presence of anti-Ad5 immunity, and this suggests that AdC7PA may be effective in human populations regardless of prior adenovirus exposure (Hashimoto et al., 2005).

A conceptually appealing strategy for protection against several potential bioweapons or emerging diseases is to immunize with a combination of antigens from different pathogens, either as protein vaccines (DuBois et al., 2007; Morefield et al., 2008), plasmid DNA (Williamson et al., 2002), or borne on virus vectors (Lee et al., 2006; Li et al., 2005). An additional new approach consists in the combination of vaccination and therapy. Manayani and coworkers generated viral nanoparticles that polyvalently displayed the PA-binding anthrax toxin receptor ANTXR2 on the surface. The recombinant virus-like particles neutralized lethal toxin activity in a cellular assay with RAW264.7 cells and a single dose protected male Fischer 344 rats against intravenous injection of lethal toxin (Manayani et al., 2007). The combination of vaccine and antitoxin in one reagent offers clear benefits and adds a new perspective to anthrax vaccine research. The Flock House Virus platform theoretically may be used to display additional *B. anthracis* antigens with or without the

VWA domain of ANTXR2 being present (Manayani et al., 2007), possibly improving its efficacy when used as a vaccine. However, in addition to demonstrating efficient protection against *B. anthracis* aerosol exposure in the nonhuman primate model, it will be necessary to explore the issue of potential autoimmune responses to the receptor protein. While Fischer rats did not mount an immune response to the human ANTXR2 receptor, autoimmune reactions in human patients may be a concern, particularly when the chimeric virus-like particles are administered as a therapeutic.

CONCLUSIONS AND FUTURE PROSPECTS

Motivated by the apparent need to protect military personnel and civilians from biological weapons and bioterrorism, the first decade of the twenty-first century has witnessed a resurgence of interest in anthrax vaccine research and development (Russell, 2007). A body of elegant and detailed investigative science has ensued using innovative technologies and new approaches to a long-standing objective: to create a safe, defined, rapid onset, and effective vaccine that protects against inhalation anthrax. Ideally, such a vaccine will also be cold-chain independent and require only a single dose to establish protective long-term immunological memory. Approaches such as the inclusion of capsule peptides or supplementary protein antigens and changing the PA vaccine adjuvant formulations have received considerable attention. These modifications may enhance the utility of PA-based vaccines by improving antigen presentation and broadening the immune response to target *B. anthracis* vegetative cells in addition to neutralizing the effects of the secreted toxin. The resulting data have confirmed much that was already known about the pathology and dissemination of fulminant systemic *B. anthracis* infection, but they have also changed perceptions on the antigenic properties of the poly-D-glutamate capsule and brought new levels of standardization to animal model design and to quantification of the immune response following both the disease and vaccination. Vaccine research data consistently demonstrate that PA provides the foundation for successful vaccine-induced protection against inhalation anthrax although one non-PA vaccine based on LF DNA showed potential in a rabbit model of infection (Hermanson et al., 2004). An anthrax vaccine that requires only a single dose to establish long-term protective immunological memory remains elusive. Though not exclusive to anthrax, a single-dose vaccine for human use is perhaps where the future of anthrax vaccine research needs to focus.

The highest public health impacts of anthrax vaccine research in the first decade of the twenty-first century have been the completion of Phase I and Phase II human clinical trials of two new, commercially produced, defined rPA protein vaccines and the first data-driven regimen change for the U.S. licensed AVA (BioThrax) vaccine in almost four decades. Of the two new rPA vaccines, at least one is progressing through clinical trials toward licensure in the United States. Independently, through a significant reversal of fortune, AVA has become perhaps the most studied anthrax vaccine in terms of safety and immunogenicity in humans. A reduced dose schedule and IM route of administration has brought AVA in line with conventional vaccine use, has improved the local side effects profile, and has the potential to increase both the acceptability and availability of the vaccine. Crude, but to all intents safe and effective, AVA and AVP remain the first-line anthrax vaccines for general and post-event human prophylaxis in the United States and the United Kingdom until such time as a next-generation vaccine meets the regulatory requirements for safety, efficacy, and licensure.

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Chapter 15

Anthrax as a Weapon of War and Terrorism

Leonard A. Cole

Biological weapons—microorganisms used deliberately to cause illness and death—are unlike any other category of weapons. Because they reproduce and multiply, some biological agents can make an environment more dangerous over time. If an organism is contagious, infected humans themselves become biological bombs that can spread disease to others. Moreover, bioweapons can be cheap, easy to produce, and, in worst-case scenarios, utterly devastating (Cole, 1997). *Bacillus anthracis*, the bacterium that causes anthrax, is neither transmissible from person to person nor has it been the source of pandemics to the extent that some other microorganisms have. Yet today, it is regarded as among the most likely of possible bioweapons. This chapter reviews the reasons for this perception, the past use of *B. anthracis* for hostile purposes, and its emergence as a favored weapon of war and terrorism.

THE POWER OF MICROBES

The lethal power of microbes has been demonstrated through the ages in the form of natural outbreaks of disease. Smallpox, caused by the variola virus, was mentioned in Egyptian writings as far back as 3700 BCE. In time, smallpox epidemics in the ancient world reached across Greece, India, and China. In more recent times, outbreaks were known to decimate local populations. In 1616–1619, smallpox killed 90% of the Native Americans in the Massachusetts Bay area. An epidemic in Iceland in 1707–1709 killed one-fourth of that island's population. In 1763, during the French and Indian War, British General Sir Jeffrey Amherst contrived to infect the native population with the disease. After blankets from the smallpox hospital were given to a delegation of Delaware Indians, smallpox raged among the tribes. The disease wiped out whole communities of Native Americans, though whether the blankets prompted the massive epidemic is uncertain (Tucker, 2001). Before smallpox was eradicated from the planet in 1980, it had killed more than 300 million people in the twentieth century alone.

The bubonic plague, caused by the bacterium *Yersinia pestis*, is responsible for equally miserable periods in human history. Three massive plague epidemics engulfed

Bacillus anthracis *and Anthrax*, Edited by Nicholas H. Bergman

Europe and Asia during the past 1500 years. The first, known as the Justinian Plague (named for the Byzantine Emperor Justinian), began in 542. At the height of the outbreak, 5000 people died every day in Constantinople. As the disease spread throughout the Roman Empire and beyond, it eventually claimed 25 million lives (Rosen, 2008).

The second plague pandemic, the Black Death, 1345–1350, is believed to have killed 75 million people worldwide, including 45 million in Europe, which was one-third of the European population. The spread of disease was partially deliberate: During a battle in 1346, the Tartars catapulted plague-infected corpses into Caffa, a walled city on the Crimean coast of Russia. According to a contemporary account by Gabriel de Mussis, Christians, Chinese, Indians, and Persians were among the traders and inhabitants of the city who died in droves (Derbes, 1996). The third pandemic surfaced in China and India beginning in 1855 and lasted for three decades during which 12 million people died of the disease (Orent, 2004).

More recently, the most notable infectious disease outbreak occurred in 1918–1919, when a highly virulent strain of the influenza virus spread throughout the world. Labeled the Spanish Flu Pandemic, the outbreak may have been the most deadly in human history. The number of fatalities was estimated to be as high as 100 million (Barry, 2005).

While *B. anthracis* is not known to have claimed as many victims in natural outbreaks, the bacterium holds a unique place in the lore of deliberately caused disease. The earliest mention of an intentional infliction on a human population is found in the Bible, when God purportedly punished the ancient Egyptians for refusing to release the Jews from slavery. Among the 10 "plagues" that were visited upon the Egyptians, numbers 5 and 6 are thought by many scholars to have been anthrax. The Bible describes the fifth plague as "a very severe pestilence" that struck "livestock in the fields—the horses, the asses, the camels, the cattle, and the sheep." Presumably the infected carcasses were burned to ash because God speaks of the sixth plague as "soot from the kiln" that was spread over Egypt, which resulted in "inflammation breaking out in boils on man and beast throughout the land" (*Tanakh: A New Translation of the Holy Scripture*, 1985, 96–97).

The nature and sequence of the biblical descriptions are highly suggestive. *B. anthracis* normally lies in spore form near the surface of the soil in many parts of the world. There the bacterium may remain inactive and in place for centuries. But while grazing for food, an animal might come into contact with anthrax spores, whose infectiousness and virulence can quickly lead to illness and death. Once introduced into an animal or human through inhalation, ingestion, or abraded skin, the spore may transform into an active germinating state. It is in this vegetative form that the bacterium reproduces and releases toxin. If a skin area has been infected, a dark lesion will appear. If inhaled or ingested, the resulting destruction of the body's organs can lead to death in days.

The biblical recounting of the infectious effects of the soot is also relevant. A spore can survive extremely stressful environmental conditions including the high temperature of fire. This was poignantly demonstrated during World War II on Gruinard Island off the northwest coast of Scotland. British and American scientists had conducted outdoor tests there with anthrax, as part of a secret biological warfare program. Sheep were tethered at various distances from explosive devices that released anthrax spores. After inhaling the spores, most sheep died within 24 h.

In 1944, when testing on Gruinard was ended, scientists found that the area was contaminated with anthrax spores. They then set fire to the island to destroy them. But when the scientists returned to evaluate the effects, they discovered that the anthrax spores were as widespread as before (Cole, 1990, 25). (The island remained off limits for nearly 50 years. After several more decontamination efforts failed, eventually, in the 1980s, the

application of massive amounts of formaldehyde and seawater proved effective. In 1990, the British government declared the island safe for people; Cole, 2003, 212–214 (see also Cole, 2009).

Thus, the durability of some microbes and their ability to infect humans even after going through fire is echoed in the biblical tale. Of course, actual bacterial spores were unknown to the people of the ancient world or to any others until the dawn of modern history. But for thousands of years, though the microscopic source was not yet recognized, the distinctive manifestations of anthrax and other diseases had been apparent.

Also clear was the fact that anthrax outbreaks largely affected field animals. Human infection has usually been limited to wool sorters and others who may be subjected to occupational exposure. Outbreaks involving multiple victims have been rare. A presumed exception occurred in 1770 when an explosive epidemic occurred in Saint Domingue—modern Haiti. Fifteen thousand people apparently died of intestinal anthrax after eating infected meat (Morens, 2002). But even this impressive number of victims was minuscule compared to those in the historic smallpox, plague, and influenza pandemics.

A major turning point in understanding the nature of disease occurred in the late nineteenth century, when microorganisms were found to be the causative agents of anthrax and other infectious illnesses. In fact, it was work with *B. anthracis* by the German physician Robert Koch that led him in1876 to propose the germ theory of disease. Five years later, the French scientist Louis Pasteur advanced this understanding with his development of a vaccine against anthrax (Ullmann, 2007). For all the good that came from these discoveries, it was recognized as well that disease-causing microorganisms could be employed for hostile purposes.

THE POISON TABOO

For most of human history, poison and disease were largely viewed as the same. Pre-twentieth-century observers could not have categorically differentiated biological weapons (living microorganisms) from chemical weapons (nonliving materials) or toxins (chemical products of organisms). Previously, unleashing arrows whose tips had been dipped in toxin or catapulting plague-infected cadavers would have been virtual equivalents. The cadavers were more likely to cause contagion because, as was thought, the air nearby somehow became infected. But whether biological or chemical, no matter the manner of delivery, poison weaponry was often disparaged as insidious, unchivalrous, sneaky.

Ancient Greek and Roman writings characterized the use of poison weapons as "abominable," "contrary to the laws of the gods," and "a violation of nature" (Gentili, 1933). This theme was later reiterated by western scholars who continued to castigate the use of poison weapons as contrary to the laws of war and nature—among them the seventeenth-century Dutch jurist Hugo Grotius, the eighteenth-century British historian Robert Ward, and the nineteenth-century American jurist and philosopher Francis Lieber (Cole, 1998).

This long-standing aversion to poison weapons was formalized in late-nineteenth and early-twentieth-century international agreements. The use of poisons in war was barred in the 1874 Brussels Declaration and at the Hague Conferences of 1899 and 1907. Still, as shown throughout history, laws and edicts might lessen the frequency of forbidden behavior, but not eliminate it. The taboo was disregarded in World War I when the major combatants used poison gas extensively on the battlefield. Anthrax was also reportedly

used by the Germans to infect livestock and contaminate animal feed to be exported to the Allies (Christopher et al., 1977).

The agony of the 1.3 million wartime victims of poison gas, nearly 100,000 of whom died, was sufficiently horrifying to prompt a call for a ban (Stockholm International Peace Research Institute [SIPRI], 1979, 129). The resulting 1925 Geneva Protocol, which prohibited the use of chemical *and* bacteriological agents in war, was the first international agreement to refer to biological ("bacteriological") agents as possible warfare weapons. Appealing to "the conscience of mankind," the protocol declared that their use "has been justly condemned by the general opinion of the civilized world." These words gave contemporary expression to the age-old taboo. Still, several signatory nations qualified their adherence to the protocol to mean only no first use. Thus, some continued to stockpile chemical and, in the case of Japan, biological agents, ostensibly to retaliate in kind if they were attacked with such weapons.

CHALLENGES TO THE NORM

Japan had signed though had not ratified the Geneva Protocol, but in the biological sphere soon became its most egregious violator. From 1932 to 1945, the Japanese engaged in a highly secret biological program that involved both testing agents on human subjects and disseminating them over populated areas. The program was established at a large military complex near Harbin, Manchuria, after Japanese troops invaded and occupied that part of China in 1932. During the following years, some 3000 mostly Chinese victims were deliberately infected with plague, typhoid, cholera, anthrax, and other agents in a facility known as Unit 731. All were killed during these experiments, many of them after agonizing vivisections by Japanese doctors. Without anesthesia, the doctors cut them open to observe the effects of the disease on the organs of a living person. In the late 1930s and early 1940s, the Japanese released *Yersinia pestis* over several Chinese villages. Infected fleas had been packed into porcelain bombs that were dropped from low flying planes. The number of deaths from the ensuing plague epidemics has been estimated to be as high as 100,000 (Harris, 1991).

Although the plague bacterium was the principal microorganism that the Japanese used in their warfare scenarios, they also had a keen interest in anthrax. In fact, Japanese open-air tests with anthrax spores were eerily similar to those underway at the same time halfway around the world on Gruinard Island. The major difference was that on Gruinard, the British and American scientists were using sheep as experimental animals, and in Manchuria, the Japanese were using humans. The Japanese variously referred to the human subjects of their medical experiments as *murata* or "logs." A description by the American historian Sheldon Harris also suggests why the Japanese did not use anthrax as an attack weapon to the extent that they used plague:

> *Anthrax experiments were conducted periodically at Anda [north of Harbin] throughout 1943 and 1944. In general, scientists worked with ten murata in each test. The head of [an] anthrax production team visited Anda on several occasions in 1943 and 1944 to supervise experiments, and observed that the murata tested "looked like Chinese." They, too, as with the plague 'logs,' were tied to stakes in the ground. Then anthrax-filled bombs were exploded nearby. The anthrax expert did note with some professional pride that 'some of the experimentees were infected with anthrax and … died.' Nevertheless, experiments at Anda were disappointing. Unit 731 experts failed to develop a viable anthrax delivery system by the end of the war. (Harris, 1994, 68–69)*

Soon after entering World War II, the United States received information about the Japanese biological program, and in 1942, President Franklin Roosevelt approved a secret plan to develop an American capability. By the end of the war, the United States and the United Kingdom had amassed stockpiles of germ weapons that could unleash a host of diseases including anthrax. None was used in battle, however, nor did the Japanese launch biological attacks against any population except the Chinese.

Postwar demobilization of America's military forces included cutbacks in its biological warfare program. But increased tensions with the Soviet Union began to spur interest in rebuilding the country's military capabilities. A Committee on Biological Warfare that was established in 1948 by the Secretary of Defense issued a report recommending development of both defensive and offensive biological warfare capabilities (Baldwin, 1948). During the next 20 years, while building huge arsenals of biological agents, the U.S. Army also extensively tested methods of dissemination, detection, and effectiveness of various pathogens including anthrax.

Between 1949 and 1969, the army conducted more than 240 outdoor experiments in which bacteria and chemical particles were released over populated areas. The test agents, described as simulants, were disseminated from boats, planes, and automobiles targeting dozens of cities including San Francisco, St. Louis, Minneapolis, Key West, and Panama City, Florida. In 1966, bacteria were released into the New York subway during peak travel hours. The purpose was to assess the dispersion patterns and viability of the organism. The results indicated that if the test bacteria had been actual warfare agents like plague or anthrax, many of the exposed humans would have been killed (Cole, 1990, 57–91).

Spraying bacteria in populated areas ended in 1969, when President Richard Nixon declared that the United States was unilaterally ending its offensive biological warfare program. By then, millions of Americans had been unwittingly exposed to the army's simulant agents. In the late 1970s when the public learned about the tests from news reports, the army said it had assumed the safety of the program, though it never monitored the health effects on the exposed population.

The most common test bacteria were *Serratia marcescens* and *Bacillus subtilis*. Employment of the serratia was suspended in the mid-1960s after recognition by some in the army that the bacteria posed a risk to the exposed population. But *B. subtilis* was a mainstay of the open-air testing until the end of the decade. Since then, the bacterium has remained a workhorse in the army's more limited defensive testing program. *B. subtilis* is attractive as a mock warfare agent because, like *B. anthracis*, it exists in spore form. Its broad use in tests echoes a widely accepted view that anthrax is among the most likely of biological weapons.

THE BIOLOGICAL WEAPONS CONVENTION (BWC) AND SOVIET PERFIDY

Three years after the United States destroyed its own biological arsenal, it joined with the United Kingdom, the Soviet Union, and scores of other nations to establish the 1972 BWC, which went into force in 1975. In the words of the convention, state parties are "determined for the sake of all mankind, to exclude completely the possibility of bacteriological (biological) agents and toxins being used as weapons, [and are] convinced that such use would be repugnant to the conscience of mankind and that no effort should be spared to minimize this risk." Accordingly, adherents undertake "never in any circumstances to develop, produce, stockpile or otherwise acquire or retain [biological or toxin agents] in quantities

that have no justification for prophylactic, protective, or other peaceful purpose" (*Convention on the Prohibition*, 1972).

The document sought to fulfill its goals by encouraging consultation and information exchanges among states. Allegations of violations could be brought to the United Nations Security Council, but the council was empowered only to investigate and report its findings. Absent from the treaty are provisions to verify compliance. Nor are penalties provided for countries that cheat. Assessments of the BWC have variously stressed its promise and disappointment (Sims, 1988, 225).

In fact by 1980, the treaty's weakness had become manifest. Allegations that the Soviet Union had been acting in violation of the convention could not be independently assessed. The initial charges were based on a 1979 outbreak of anthrax in Sverdlovsk, a city of 1.2 million. U.S. intelligence sources believed that the epidemic had been caused by an accidental release of the bacteria from a military facility. Scores of residents reportedly died of the disease in a period of weeks.

The Soviets said the outbreak was caused by anthrax-contaminated meat and was unrelated to a biological warfare program. Insisting that the incident was a matter of public health and therefore not subject to the requirements of the BWC, Soviet officials refused requests for an on-site investigation by outsiders. While regretting their inability to visit the area, some western observers concluded that the Soviet contentions could have been accurate. Subsequent briefings to U.S. scientists by Soviet health officials convinced many that the Soviet version of the epidemic seemed "credible" and "plausible" (Cushman, 1988; Marshall, 1988).

In 1992, the year after the collapse of the Soviet Union, Russian President Boris Yeltsin acknowledged that the Soviet claims about Sverdlovsk had been fabricated. The outbreak had indeed resulted from an accidental release of anthrax bacilli from a military research facility. In that same year, 1992, Ken Alibek had resigned from his position as deputy chief of Biopreparat and fled with his family to the United States. Biopreparat, the Soviet state pharmaceutical agency, had been the hub of a secret program to develop weapons from dangerous bacteria, viruses, and other microbes. Largely unknown to the United States, the agency oversaw the stockpiling of "hundreds of tons of anthrax and dozens of tons of plague and smallpox near Moscow and other Russian cities for use against the United States and its Western allies," according to Alibek. "What went on in Biopeparat's labs was one of the most closely guarded secrets of the Cold War" (Alibek, 1999, x).

From Alibek and other Soviet scientists, the United States learned that the leakage of anthrax in Sverdlovsk had infected about 100 people and killed at least 66. But Sverdlovsk offered only a tiny glimpse into a massive clandestine biological warfare program that employed more than 30,000 people (60,000 according to Alibek) in two dozen military and civilian laboratories and research institutions (Federation of American Scientists, Biological weapons). The program's accomplishments included not only the production and stockpiling of biological weapons, but also the development of ever more lethal forms. Alibek recounts a 1989 conference of Biopreparat scientists and military officials at which investigators proudly announced advances in this regard with a variety of agents. As usual, anthrax was prominent among them. Soviet investigators maintained that they had developed a genetically altered strain of anthrax that was resistant to antibiotics. Additional reports indicated that resistance to anthrax vaccines had also been achieved (Alibek, 1999, 167, 261).

Thus, the United States and others in the West learned belatedly that the Soviet Union had been operating a huge biological warfare program in flagrant violation of the BWC.

The long-standing effort, which came to light only after it had ended in the final years of the Soviet Union, became a cause for alarm to many in the West. What other countries and subnational groups might be surreptitiously developing and acquiring these weapons as well?

MORE ANTHRAX WORRIES: AUM SHINRIKYO AND IRAQ

This concern was intensified by two other events that surfaced soon after. The first was related to the 1995 release of sarin nerve agent in the Tokyo subway by the Japanese cult Aum Shinrikyo. The attack killed 12 people, made some 1000 ill, and frightened thousands of others who believed they might have suffered from exposure to the poison. As was learned from apprehended cult members, Aum also had tried unsuccessfully to infect people with anthrax. In 1993, the cult released spores from the roof of an eight-story building in downtown Tokyo. A later investigation found that this attempted attack with anthrax spores was among several that had failed, largely because the bacillus used was of the Sterne strain, an attenuated bacterium that was ordinarily used as a vaccine for animals (Takahashi et al., 2004).

The second disquieting discovery was the finding in 1995 that Iraq had developed a biological weapons program far more advanced than previously supposed. The agreement by the United States and other coalition forces to end the first Gulf War in 1991 included the requirement that President Saddam Hussein's regime account for, and end, all its programs involving weapons of mass destruction. To verify compliance, Iraq was subject to inspection by the United Nations Special Commission (UNSCOM). But Iraqi authorities often thwarted efforts by UNSCOM inspectors to gain access to locations suspected of past or current illicit activity.

In July 1995, after previous denials by Iraqi officials, an Iraqi scientist admitted to UNSCOM biological inspectors that the regime had produced thousands of gallons of anthrax and botulinum toxin. The scientist insisted, however, that the agents were never weaponized and that they had been destroyed in 1990. Then in August 1995, two of Saddam Hussein's sons-in-law defected to Jordan. One of them was Hussein Kamel, who had run Iraq's biological warfare program. Hours after the defection, Iraqi authorities told UNSCOM that they had just discovered documents about their country's biological program on a chicken farm owned by Kamel. The "newly-found" material confirmed that Iraq had produced a variety of biological weapons, including *B. anthracis*, which could be delivered by missiles, bombs, and aerosols from tanks (Miller et al., 2001, 183–188). Kamel reaffirmed the accuracy of the documents to interrogators in Jordan. (The following January, the two defectors returned to Iraq after assurances from Saddam that they would be welcomed back. Three days after their return, they were killed; Jehl, 1996.)

Between information in the documents and subsequent acknowledgments by Iraqi officials, UN inspectors learned that the Iraqis had a biological arsenal in place during the 1991 Gulf War. Their reluctance to use these weapons seems attributable to uncertainty that the agents could be disseminated effectively and perhaps fear that retribution by United States and other coalition forces would be unsparing. But the information about Iraq's program, as about the programs of Aum Shinrikyo and the Soviet Union, underscored several disquieting facts.

First, in all three cases, the United States and other outside powers either underestimated or knew nothing about the biological programs. Second, the cases demonstrated the relative ease with which a government or subnational group could act in violation of the

BWC under the existing means of oversight. (Many individuals and countries support adding verification and enforcement procedures to the BWC as well as provisions that would deter acquisition of biological agents by terrorists; Harris, 2008.) Third, *B. anthracis* was deemed an important, if not preeminent agent in all three programs. Fourth, these discoveries elevated concerns in the United States about the threat of biological weapons, and anthrax in particular.

By the end of the 1990s, U.S. health and military officials had devised a hierarchy of three categories of bioagents according to their perceived threat. They are listed by the Centers for Disease and Prevention (CDC) under the title "Bioterrorism Agents/Diseases" (U.S. Centers for Disease Control and Prevention, Bioterrorism Agents/Diseases). Category C agents and diseases comprise the lowest priority of the three. These are described as emerging pathogens that could be relatively easy to produce and engineer for dissemination and have a potential to create a major health impact. The CDC offers two examples: Nipah virus and hantavirus.

Category B agents and diseases are moderately easy to disseminate, result in moderate morbidity and low mortality rates, and require enhancements of CDC's diagnostic capability and disease surveillance. This category comprises nine listings. They range from specific agents/diseases such as brucellosis and staphylococcal enterotoxin B to broader descriptions like Food Safety Threats (e.g., species of *Salmonella* and *Escherichia coli*) and Water Safety Threats (e.g., *Vibrio cholerae* and *Cryptosporidium parvum*).

Category A, the highest priority, constitutes organisms that pose the greatest risk to U.S. national security. They can be easily disseminated or transmitted from person to person, result in high mortality rates, and have the potential for major health impact. The CDC also describes the six agents/diseases that comprise this category as having the potential to cause public panic and social disruption, and to require special action for public health preparedness. At the top of the list is anthrax, followed by botulism, plague, smallpox, tularemia, and viral hemorrhagic fevers (U.S. Centers for Disease Control and Prevention, Bioterrorism Agents/Diseases).

Thus, as the twentieth century drew to a close, the cumulative experience of the previous 50 years, and especially the last 10, led policymakers to view anthrax as the preeminent biothreat to the nation. Worries about anthrax and other threats prompted the CDC in 1999 to establish a Bioterrorism and Response Program. The program's priorities included equipping federal and state public health laboratories with the capability to test specimens for the presence of anthrax and other threat agents. By late 2001, 80 laboratories throughout the country had become part of the CDC's new Laboratory Response Network (LRN). As such, they had achieved a level of competency to test for some select agents.

Having the LRN in place in September–October 2001 proved very fortunate. Philip Lee, a microbiology technician in one of the network labs—the state facility in Jacksonville, Florida—had recently undergone CDC training to identify anthrax and a few other agents. On October 3, Lee's tests on blood specimens from a critically ill patient confirmed the suspicions of Dr. Larry Bush, an infectious disease specialist at the JFK Medical Center in Lake Worth, Florida. His patient Robert Stevens, as Bush suspected, had anthrax (Cole, 2003, 9–17).

The next day, Lee's tests were validated by the CDC in Atlanta. Stephen A. Morse had become associate director of the CDC's Bioterrorism and Response Program when it was established in 1999. For months after his appointment he worried that "something was going to happen before we got the Laboratory Response Network up and running (Cole, 2003, 130–131)." Although distressed about the finding of anthrax in October 2001, he felt a measure of relief that the LRN had been in place in time. But any satisfaction

about the new LRN was quickly overshadowed by the gravity of the larger reality: the United States was confronting the largest bioterrorism event in the country's history.

THE U.S. ANTHRAX ATTACKS

Robert Stevens died of inhalation anthrax on October 5, almost certainly from spores that had arrived by mail to his newspaper's office. The 63-year-old photo-editor worked for the *Sun*, a supermarket tabloid published by the American Media, Inc. (AMI) in Boca Raton, Florida. Two days after his death, anthrax spores were found on his office computer keyboard and in the mailroom of the AMI building (Cole, 2003, 2008, 2009).

Although Stevens was the first to be identified as an anthrax victim, subsequent analyses indicated that others had been infected before as well as after. Perhaps a half-dozen letters containing powdered anthrax spores had been sent to journalists and politicians starting a week after the jetliner attacks on September 11, 2001. As a result, at least 22 had become infected, half with cutaneous (skin) anthrax and half with inhalation anthrax; five of the latter died (Jernigan et al., 2002). As was recognized retrospectively, between September 21 and October 1, nine individuals had displayed symptoms of anthrax; seven had contracted the cutaneous form and two the inhalational. None was initially diagnosed correctly. The last case to be reported was Ottilie Lundgren, a 94-year-old Connecticut woman, who died of inhalation anthrax on November 21 (Cole, 2008, 28).

During the two-month roll out of infections, it became clear that the bacterium had been disseminated through the mail. Not only were threat letters containing anthrax recovered, several postal sorting centers had become contaminated with spores that had leaked from envelopes being processed. Most victims were postal workers or otherwise known to have contacted contaminated mail. Four letters, each containing 1–2 g of dry anthrax spores, were found during this period. All were postmarked "Trenton, NJ" which meant they had been processed at the Postal Sorting and Distribution Center in nearby Hamilton, New Jersey. Two envelopes were postmarked September 18, 2001, one addressed to the editor of the *New York Post* and the other to Tom Brokaw at NBC-TV. The message on a sheet inside was a copy of an original that evidently was kept by the mailer. Under "09-11-01," in handwritten block letters, the text read:

THIS IS NEXT
TAKE PENACILIN [sic] NOW
DEATH TO AMERICA
DEATH TO ISRAEL
ALLAH IS GREAT (Compilation of Mailed Anthrax Evidence, 2001–2003)

The other two recovered envelopes, postmarked October 9, 2001, were addressed to Senator Tom Daschle and Senator Patrick Leahy. The text in both letters was identical, though somewhat different from the wording in the first pair. Dated "09-11-01" they read:

YOU CANNOT STOP US.
WE HAVE THIS ANTHRAX.
YOU DIE NOW.
ARE YOU AFRAID?
DEATH TO AMERICA.
DEATH TO ISRAEL.
ALLAH IS GREAT (Compilation of Mailed Anthrax Evidence, 2001–2003).

Anthrax letters were presumably also sent to the ABC and CBS television studios in New York as well as the offices of the *Sun* and another AMI tabloid, the *National Inquirer*. This assumption is based on the trails of spores found in these places and the identification of infected individuals who had been in their proximity. Since the envelope flaps were sealed with tape, the anthrax spores—each around 1 μ in diameter—apparently had been leaking through the envelope paper whose pores exceeded 20 μ in size. Although bacteria could have been transported to these locations by cross-contaminated mail, their large concentrations suggested the likely source was an original threat letter.

In several instances, initial diagnoses of the victims failed to include anthrax as a possible cause of their illness. Nor was the extent of contamination in scores of buildings, offices, and postal facilities quickly recognized. Retrospective assessments and belated testing eventually identified several locations as well as the individuals that had been exposed to anthrax.

As succeeding days and weeks brought information about more victims and contaminated locations, the public became increasingly anxious. The actual presence of spores, along with several false alarms, prompted much of official Washington to shut down. At various times during this period, numerous buildings and offices were closed, including the Capitol, offices of members of the Senate and House of Representatives, portions of the Pentagon, the State Department, the Federal Reserve Building, and the Supreme Court. Spores were found in postal facilities and other offices along the eastern seaboard and as far west as Kansas City, Missouri (BBC News Online, 2001). People everywhere were afraid to open mail.

Despite generating massive disruption, the anthrax attacks resulted in relatively few casualties. But they demonstrated the potential for more devastating consequences. More than 30,000 people who were considered at risk of exposure to the bacterium were treated with prophylactic antibiotics (Lane and Fauci, 2001). Without this intervention, many of them would likely have become infected and died. Moreover, if the strain of bacterium had been drug-resistant, or if hundreds of anthrax letters had been mailed, the number of casualties could have been far greater.

The outbreak was recognized as intentional soon after Stevens's death, as spores were found at several improbable locations. But the extent of illness and contamination was not grasped for several more weeks. Moreover, identification of the perpetrator proved elusive.

SEEKING THE PERPETRATOR

Not until 7 years had passed did the Federal Bureau of Investigation (FBI) name the person it believed to be the sole perpetrator. On August 1, 2008, a news story indicated that the FBI considered Bruce Ivins, a scientist who had worked at the U.S. Army Medical Institute of Infectious Diseases (USAMRIID), to be behind the anthrax attacks. The story also revealed that Ivins had committed suicide 3 days earlier, and therefore would never be tried in a court of law (Willman, 2008).

Before Ivins was deemed the culprit, the lives of others who had come under FBI scrutiny had been disrupted. Several lost their jobs and suffered emotional distress. One of them, Steven Hatfill, who had been publicly designated a "person of interest" by Attorney General John Ashcroft in 2002, sued the government. After a protracted legal fight, in 2008, the government acknowledged error and agreed to a settlement that awarded Hatfill $5.8 million (Shane and Lichtblau, 2008).

Despite several missteps, the investigation produced an important scientific advance. Because anthrax bacteria commonly exist as static spores, they rarely undergo mutation. Thus, the bacteria's DNA structure remains largely unchanged during long periods, and genetic differences are hardly perceptible among organisms of a common strain.

But during the years of investigation, the FBI's scientists along with 60 consulting experts identified four genetic mutations in the bacteria found in the letters. They then developed specific molecular assays that could detect the four mutations. This venture into microbial forensics enabled the bureau to match the anthrax spores in the letters with those in Bruce Ivins's laboratory (Enserink, 2008). Since others at Fort Detrick also had access to Ivins's laboratory, this linkage alone did not prove his guilt. But it was a central element of the FBI's case.

Moreover, the FBI requested the National Academy of Sciences to make an independent assessment of the scientific procedures. The National Academy agreed to assemble a panel of experts who began their deliberations in 2009. Based on past experience with such reviews, the process could take more than a year to complete. Meanwhile, the government made publicly available the evidence it had developed against Ivins.

It did so, according to Jeffrey Taylor, the U.S. Attorney for the District of Columbia, as a step toward concluding the investigation. Once that process was completed, "we will formally close the case" (Federal Bureau of Investigation, 2008).

In reviewing the government's contentions, Taylor highlighted the genetic match of the anthrax spores in the letters with those produced in Ivins's laboratory. Ivins's access records also showed that he had returned to his laboratory to work alone late into the night on dates just before the letters were mailed. The government's case was comprised of these and other presumptive indicators. Critics, including Paul Kemp, who had been Ivins's attorney, maintained that other individuals had access to Ivins's laboratory and that the government's case was speculative.

The FBI formally closed the case in February 2010, before the National Academy experts had completed their assessment. The bureau's action was deemed premature by many observers and fueled further criticism. Still, unless some relevant information surfaces beyond that already provided by the FBI, Ivins's guilt or innocence will be debated far into the future (Cole, 2009). Moreover, the anthrax attacks in 2001 showed how disruptive and potentially destructive a bioattack could be. The incident reinforced the understanding that anthrax spores can be very effective agents of terror.

WORLD AT RISK

Coincidentally, the revelations about Bruce Ivins appeared in the midst of deliberations by the Commission on the Prevention of Weapons of Mass Destruction Proliferation and Terrorism. This bipartisan body had been appointed in May 2008 by the leadership of the U.S. Senate and House of Representatives. Its creation was an outgrowth of the 9/11 Commission's warning in 2004 that "the greatest danger of another catastrophic attack in the United States will materialize if the world's most dangerous terrorists acquire the world's most dangerous weapons." The new congressional commission was tasked with providing a report in 6 months. After meetings and interviews with some 250 government officials and nongovernmental experts, in December 2008, the commission issued its report, titled *World at Risk*.

The report begins with a declaration that unless the world community acts urgently, a weapon of mass destruction (WMD) will probably be used somewhere in the world in

the next 5 years. The succeeding passage is more specific: "The Commission further believes that terrorists are more likely to be able to obtain and use a biological weapon than a nuclear weapon. The Commission believes that the U.S. government needs to move more aggressively to limit the proliferation of biological weapons and reduce the prospect of a bioterror attack" (*World at Risk*, 2008).

In elaborating on these assertions, *World at Risk* reviews the efforts of al-Qaeda and other terrorist groups to obtain biological and chemical weapons. To highlight the effects of a bioattack, the report recounts a scenario developed by the Homeland Security Council of how "terrorists could launch an anthrax attack in the United States—and the horrific chain of events that would follow" (*World at Risk*, 2008, 5). The attack begins with the spraying of an aerosol of anthrax spores from a truck driving through a densely populated urban area. An assumed exposure of some 300,000 people would, in the following days, result in 13,000-odd fatalities. The massive disruption to services and other normal activities would be incalculable.

When interviewed about the report's findings, the commission's chairman, former Senator Bob Graham, noted that the nine commission members were unanimous in their conclusions. (Graham is a Democrat and the commission's vice-chairman, former Senator Jim Talent, a Republican.) Graham emphasized his concern about the ability to create novel pathogens through genetic engineering. He cited as well the significance of naturally occurring agents, and specifically anthrax, which, he noted, could be obtained from dead cattle that had contracted the disease (Isikoff and Hosenball, 2008).

CONCLUSION

As underscored by the report of the congressional Commission on the Prevention of WMD Proliferation and Terrorism, the threat of biological weapons, and *B. anthracis* in particular, remains a high-priority concern. In the course of history, the effects of diseases other than anthrax have been far more formidable. Only in the biblical parable of the 10 plagues, where (presumably) anthrax was inflicted as God's punishment of the ancient Egyptians, did that disease much affect the human condition. Not until the twentieth century, as the durability and lethality of anthrax spores became better understood, was the bacterium viewed as a choice weapon. By mid-century, anthrax weapons had been incorporated into British, American, and some other countries' arsenals. Under terms of the 1972 BWC, however, states agreed to destroy all their biological and toxin weapons, and most did.

But subsequent events have reignited concerns about bioweapons: the discovery in the 1990s that Iraq and the former Soviet Union had maintained illegal biological programs; the finding that al-Qaeda and other terrorist groups were seeking to obtain anthrax bioweapons; the 2001 anthrax attacks via the U.S. mail. Recognition of anthrax as a disease agent of war or terrorism is largely a matter of recent history, but based on the evidence, the perception is justified.

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