UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

July 15, 2008

GRADUATE PROGRAMS IN THE BIOMEDICAL SCIENCES AND PUBLIC HEALTH

Ph.D. Degrees

Interdisciplinary
-Emerging Infectious Diseases
-Molecular & Cell Biology
-Neuroscience

Departmental
-Clinical Psychology
-Environmental Health Sciences
-Medical Psychology
-Medical Zoology
-Pathology

Doctor of Public Health (Dr.P.H.)

Physician Scientist (MD/Ph.D.)

Master of Science Degrees

-Public Health

Masters Degrees

-Military Medical History
-Public Health
-Tropical Medicine & Hygiene

Graduate Education Office

Dr. Eleanor S. Metcalf, Associate Dean
Janet Anastasi, Program Coordinator
Tanice Acevedo, Education Technician

Web Site
http://www.usuhs.mil/graded/

E-mail Address
graduateprogram@usuhs.mil

Phone Numbers
Commercial: 301-295-9474
Toll Free: 800-772-1747
DSN: 295-9474
FAX: 301-295-6772

APPROVAL SHEET

Title of Dissertation: "Functional and Immunological Analyses of Superoxide Dismutases and Other Spore-Associated Proteins of *Bacillus anthracis*"

Name of Candidate: Robert Cybulski, Jr.
Doctor of Philosophy Degree
20 August 2008

Dissertation and Abstract Approved:

Ann Jerse — 8/20/08
Ann Jerse, Ph.D. — Date
Department of Microbiology & Immunology
Committee Chairperson

D S Merrell — 8/20/08
D. Scott Merrell, Ph.D. — Date
Department of Microbiology & Immunology
Committee Member

8-20-08
Ernest Maynard, Ph.D. — Date
Department of Biochemistry
Committee Member

8-20-08
Scott Stibitz, Ph.D. — Date
FDA
Committee Member

8/22/08
Alison O'Brien, Ph.D. — Date
Department of Microbiology & Immunology
Committee Member

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

July 15, 2008

FINAL EXAMINATION FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

Name of Student: Robert Cybulski, Jr.

Date of Examination: 20 August 2008

Time: 1:00

Place: Lecture Room F

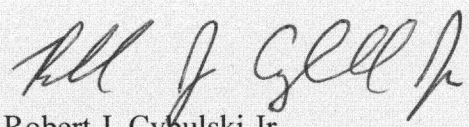
DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
Ann Jerse, Ph.D. Department of Microbiology & Immunology Chairperson	✓	
D. Scott Merrell, Ph.D. Department of Microbiology & Immunology ~~Major~~ Advisor	✓	
Ernest Maynard, Ph.D. Department of Biochemistry Member	✓	
Scott Stibitz, P.D. FDA Member	✓	
Allison O'Brien, Ph.D. Department of Microbiology & Immunology Member	✓	

The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled:

"Functional and immunological analyses of superoxide dismutases and other spore-associated proteins of *Bacillus anthracis*"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.



Robert J. Cybulski Jr.

Department of Microbiology and Immunology

Uniformed Services University of the Health Sciences

Abstract

Title of Dissertation:

"Functional and immunological analyses of superoxide dismutases and other spore-associated proteins of *Bacillus anthracis*"

Robert J. Cybulski Jr., Doctor of Philosophy, 2008

Thesis Directed by:

Alison D. O'Brien, Ph.D.

Professor, Department of Microbiology and Immunology

Bacillus anthracis pathogenesis depends on the highly-resistant spore form of the organism. The outermost structure of the spore, the exosporium, contains proteins that are visible to the host and are potential targets for a protective immunological response. We tested whether exosporium components could enhance the protection afforded by immunization with *B. anthacis* protective antigen (PA). Mice were challenged with *B. anthracis* spores after they were immunized with suboptimal doses of PA and boosted with individual exosporium proteins. Successful enhancement of PA-based protection was achieved with two proteins, BxpB (also known as ExsF) and p5303. Antibodies generated against these proteins did not recapitulate the germination inhibitory effect seen *in vitro* with whole spore antisera. However, individually, anti-BxpB and anti-

p5303 did enhance uptake and destruction of spores by macrophages. These data indicate that by facilitating greater clearance of spores and reducing the effective spore inoculum, antibodies against single exosporium proteins can enhance protection against *B. anthracis* spore challenge.

The *B. anthracis* genome encodes four superoxide dismutases (SODs), a class of enzymes that contribute to spore resistance by protecting against oxidative stress. Proteomic evidence indicates that two of these SODs are present in the spore. We hypothesized that these SODs play a role in virulence by increasing spore resistance to phagocyte-generated oxygen radicals. To test this theory, we constructed an isogenic mutant called Δ*sod15*Δ*sodA1* that lacked both spore-bound SODs and examined the double mutant's SOD activity and virulence in a mouse model. Surprisingly, spores of Δ*sod15*Δ*sodA1* proved no less virulent and possessed greater spore-bound SOD activity than did wild type spores. We next deleted the remaining two *sod* genes to construct a Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* mutant. This strain lacked spore-bound SOD activity and was attenuated *in vivo*. The attenuation correlated with *in vitro* studies in which Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* had increased sensitivity to oxidative stress and macrophage-mediated killing. Restoration of *sod15* and *sodA1* in the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* background reconstituted wild-type characteristics. We conclude that functional redundancy among chromosomally-encoded *sod* genes enhances *B. anthracis* pathogenicity by protecting the spore from oxidative stress.

Functional and immunological analyses of superoxide dismutases and other spore-associated proteins of *Bacillus anthracis*

By

Robert John Cybulski Jr.

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of the Health Sciences

F. Edward Hèbert School of Medicine

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy 2008

Acknowledgements

This work would not have been possible without the support and assistance of numerous people and organizations. In no particular order, I would like to thank:

The United States Army Long Term Health Education and Training Scholarship for providing me the time and funding with which to pursue this education.

My thesis advisor for accepting me into her lab and providing me with the support I needed to succeed.

My thesis committee for their guidance, feedback and understanding.

Dr. Patrick Sanz and Dr. Trupti Brahmbhatt for helpful discussions and debates.

My lab mates for providing me with the assistance I required to succeed on time.

Dr. Jill Czarnecki for help with polyclonal antibody generation.

Dr. Cara Olson for expert statistical analysis.

Mr. Michael Flora for primer synthesis and DNA sequencing.

My friend, Jess, for helping me to stay sane through the four-year sprint.

Michael Peterman, Colonel, U.S. Army for teaching me what it meant to be a leader and providing me with the opportunity to pursue the career I desired.

My wife, Shannon, for tolerating the working weekends and the moodiness and for learning to live with me in the midst of this whole process.

Table of Contents

Table of Tables

Table of Figures

Chapter 1

Introduction

Preface

The etiological agent of the disease anthrax is the Gram-positive, spore-forming rod *Bacillus anthracis.* Interest in this organism has grown significantly since 2001, when *B. anthracis* was used against the American population as an agent of bioterror. This event highlighted the potential dangers associated with *B. anthracis,* from the persistence and transmissibility of anthrax spores to the morbidity and mortality of the disease resulting from spore exposure. While the disease is treatable, a combination of rapid diagnosis, aggressive care and antibiotic therapy must be achieved in order to ensure a favorable outcome. Consequently, much research effort has been devoted to the area of prophylaxis. The currently licensed anthrax vaccine, adsorbed (AVA), strives to protect vaccinees from the lethal effects of the toxins elaborated by the bacteria following spore germination. However, concerns associated with the safety, efficacy and practicality of the AVA regimen have led to efforts to find an improved vaccine. Meanwhile, another focus of intense research interest is anthrax pathogenesis and the interactions that occur between host and bacterium during infection. Much disagreement exists regarding the early events of the infectious process and the host and bacterial factors that determine disease outcome. One area in which the vaccinology and pathogenesis lines of inquiry intersect is the *B. anthracis* spore. A dormant form of the organism capable of persisting in the environment for decades or more, the spore is the infectious form of the bacteria, meaning that it contains essential elements for establishing disease as well as potential targets for inclusion in a next generation anthrax vaccine.

The introduction to this dissertation is divided into several parts. The first section provides an overview of *B. anthracis* and anthrax pathogenesis. The second part includes a description of the *B. anthracis* spore and the importance of spore-host interactions in the establishment of disease. The third component presents a sumary of the current state of anthrax treatment and prevention. The fourth piece focuses on superoxide dismutases, a class of enzymes present not only in the vegetative bacilli but also in anthrax spores. The fifth and final portion of the introduction gives an outline of the hypothesis and specific aims of this dissertation.

History of *Bacillus anthracis*

Anthrax is an ancient disease, one that arguably dates back at least as far as the fifth and/or sixth plagues of Egypt during Biblical times (Exodus 9:6). The disease gets its name from the Greek work *anthrakos* (meaning "coal", a description of the characteristic black eschar associated with the cutaneous form of the disease) and was known to Greeks and Romans of the Classical Age (209). Accounts from the 17th and 18th centuries describe outbreaks of anthrax occuring in both livestock and human populations in Europe, and later in North America (209). The association between occupational hazards such as animal contact and the disease was not lost on people of the times, as the affliction came to be known by the terms "woolsorter's disease" or "ragpicker's disease". Microbiological study of anthrax dates back at least as far as 1850, when Casimir Devaine and Pierre Rayer first identified rod-like organisms in the blood of animals and humans infected with the disease (209). Later, as part of the historic investigations that led to the formulation of Koch's Postulates, Robert Koch demonstrated that the disease anthrax was specifically caused by the microorganism now known as *Bacillus anthracis* (209). During this same period, Louis Pasteur and W.S. Greenfield performed independent investigations that led to the development of attenuated vaccine strains (209,216), though it wasn't until the licensing of an attenuated live spore vaccine in 1937 that the intermittent afflictions to livestock populations and public health were quelled throughout most of the industrialized world (219). Even still, the disease remains a concern in developing countries that lack access to an effective vaccine and proper

veterinary and public health measures, as demonstrated by a 2000 outbreak in Zimbabwe that infected more than 1100 people with the cutaneous form of the illness (239).

The advent of an effective livestock vaccine and the diminution of anthrax as a general public health threat did not mark the end of *B. anthracis* as a health concern. Anthrax was used as a biological warfare agent in the first World War, when German agents operating in the United States injected horses and mules bound for Europe with anthrax in the hopes of infecting the population of horses supporting the Allied war effort (18). In World War II, both the Germans and the British developed plans to drop contaminated animals and animal waste in enemy territory in the hopes of causing human disease and mayhem within the enemy's food industry, while the Japanese tested anthrax on prisoners in Manchuria as part of a biological weapons development program (18). British scientists also performed experiments on aerial dispersal of spores in the Guinard Islands near Scotland, a study that ultimately led to observations on the capacity of spores to persist for decades in the environment (39). During the Cold War, the United States, Great Britain and the Soviet Union were among a group of nations that developed the capability to weaponize anthrax spores, a process halted in the U.S. by a 1969 Presidential Order and officially brought to an end in 1972 as part of the International Biological Weapons Convention (39). An outbreak in Sverdlovsk in 1979 that infected 96 and killed 64 resulted from the accidental release of weaponized spores being produced in secret by the Soviet Union (3,149). Most recently, an attack that infected 22 and killed 5 was conducted through the U.S. mail system in 2001 (85). This event highlighted the potential threat of anthrax as a biological agent of terror.

Organism and epidemiology

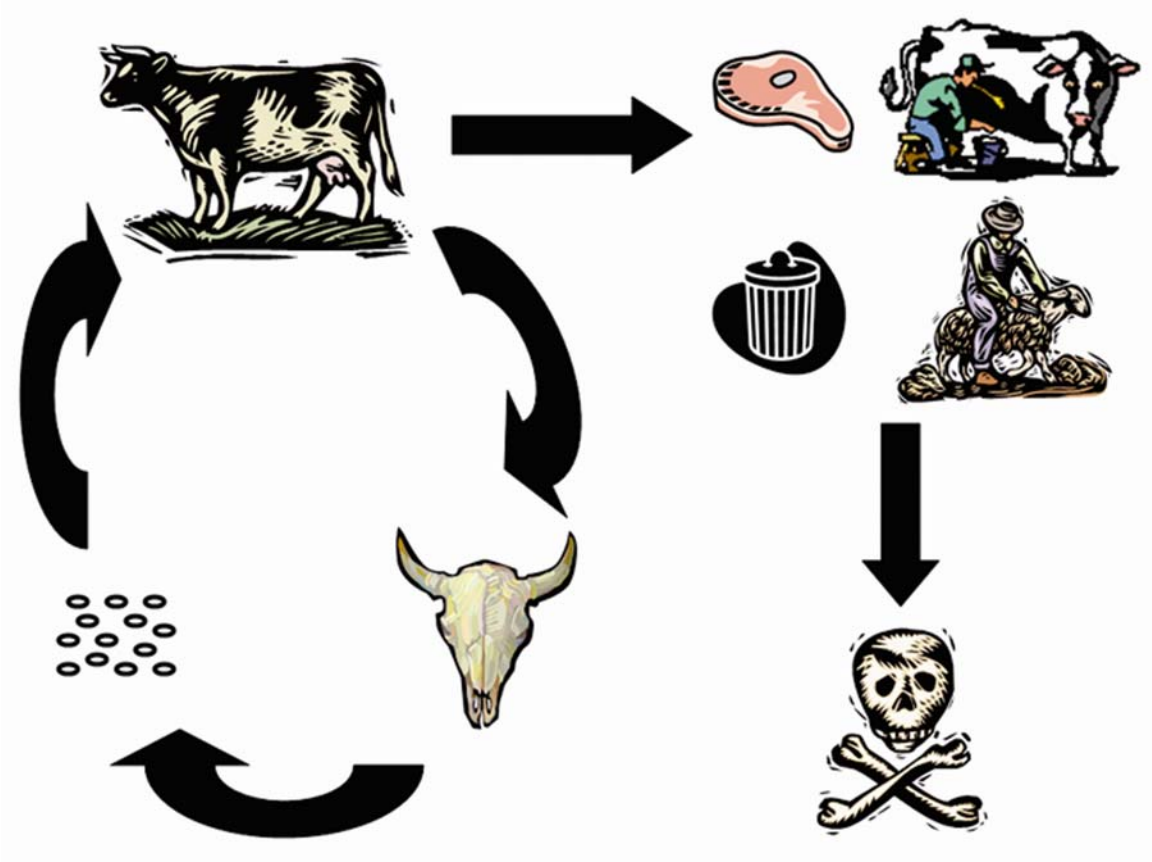
B. anthracis belongs to the *B. cereus* family of the *Bacillus* genus. In addition to *B. anthracis,* this family includes the organisms *B. cereus*, *B. thuringiensis* and *B. mycoides* (54). Differentiation of *B. anthracis* from other organisms in the *B.cereus* group can be achieved by evaluation of a number of phenotypic features. Unlike others in the *B. cereus group, B. anthracis* is non-motile, non-hemolytic on blood agar plates, generally sensitive to penicillin, and capable of expressing a capsule (54). The bacterium is large (1-2 μm by 1-10 μm), rod-shaped, non-motile, facultatively anaerobic, and Gram-positive (54). The organism grows well on non-selective media at 37ºC; in fact, its colonies can reach 4-5 mm after 18 hours of incubation. Colonies are off-white in color, have a tacky consistency and a rough, "ground glass" appearance with an irregular border (54). Vegetative growth *in vitro* results in the formation of long "boxcar" chains of bacilli, whereas growth inside the host typically results in single or paired bacillus (61). Under nutrient-deprived conditions that are not favorable to growth, *B. anthracis* is capable of undergoing sporulation, a process that results in the formation of a metabolically dormant form of the organism that is highly resistant to environmental factors such as heat, desiccation, radiation, toxic chemicals and pH extremes (157). This spore is the infectious form of the organism, and is normally ingested or inhaled by livestock while they graze in contaminated pastures. Outbreaks tend to occur sporadically and are associated with ecological changes such as heavy rainfall, flooding or drought (1). Following the death of the host, sporulation and subsequent dissemination of spores is dependent on the carcass opening its contents coming in contact with oxygen, as the vegetative cells are killed rapidly by putrefaction (115). Given that vegetative growth

and sporulation is retarded below 20ºC, warmer climates favor sporulation and dissemination into the soil with subsequent reestablishment of the infectious cycle, while colder climates tend to favor self-limited disease (115). In part for this reason, human anthrax is seen most commonly today in South and Central American, sub-Saharan Africa, southern Europe and Asia (239). Human cases are strictly zoonotic in origin (with the exception of intentional, bioterror-releated cases) as they result from contact with infected animals or contaminated animal products [Figure 2, (1)]. There is no evidence to support human-to-human (or animal-to-animal) transmission (54).

Figure 1. The *B. anthracis* transmission cycle.

Herbivores serve as the natural host for *B. anthracis*. Infection occurs through the ingestion or inhalation of anthrax spores, typically from contaminated soil. Rapid germination and vegetative dissemination results in the death of the host, and the opening of the carcass and exposure of its contents to oxygen promotes sporulation and a return of spores to the soil. Humans are incidental hosts who can contract the disease cutaneously, gastrointestinally or inhalationally by contact with contaminated animals or animals products such as meat, hides, wool or waste.

Adapted from (26,154).



The spore

Structure and function

The anthrax spore is the cause of both great interest and great concern. Spores are metabolically dormant structures capable of persisting in the environment for decades or more (39,227) in the face of harsh stresses that include heating, drying, UV irradiation, and chemical exposure. Spore can resist as much as 10 minutes of boiling and treatment with most available disinfectants (157). Exposure to bleach, ozone, or temperatures as high as 120ºC for no less than 15 minutes are among the few methods known to be effective for spore decontamination (239). The purpose of these extreme resistance qualities are to protect the *B. anthracis* genetic material which is contained deep within the spore core (Figure 2A), complexed with protective small, acid-soluble proteins (SASPs) (196) and surrounded by a basic architecture common to all *Bacillus* species (8,101). The core structure is surrounded by two membranes, with a specialized peptidoglycan layer known as the cortex located between the inner and outer membranes (174). The outer membrane serves as the initiation site for the spore coat, the structure most responsible for the resistant qualities of the spore (56,99). The spore coat is a tough, layered protein shield that provides the spore with mechanical strength, structural elasticity and biochemical and physical resistance (56,99,157) while also encompassing the germinant receptors and associated enzymes that are capable of monitoring the spore environment and triggering spore germination under the proper conditions (56,99,131,197). While for most *Bacillus* species the spore coat is the outermost spore

structure, *B. anthracis* and other organism's of the *B. cereus* family possess an additional layer known as the exosporium (184).

Exosporium

The exosporium is a complex, paracrystalline shell separated from the exterior of the spore coat by a significant gap (80,144,184). Referred to as a "balloon-like" structure (184), the exosporium consists of a collection of lipids, carbohydrates, proteins and glycoproteins that include both structural components and enzymes and is responsible for the physical characteristics of the spore surface (21,37,139,144,182,206,207,213,217). The role of the exosporium in overall spore fitness is not well understood (13,113), particularly given observations in certain animal models that spores stripped of their exosporium remain as infectious as those in which the exosporium is intact (36,81). The most prominent and well-examined component of the exosporium is the immunodominant protein *Bacillus* Collagen-Like Antigen, or BclA (206,213). BclA is responsible for the "hair-like" nap (91) present on the exterior of the exosporium, and, like the exosporium as a whole, the importance of the spore-surface appendage remains a subject of intense study and debate (20,22,23,28,161,213).

Sporulation

The complex structure of the spore is assembled through an equally-complex process known as sporulation (Figure 2B). Most of what is known about *B. anthracis* spores and sporulation comes through work intially done in the related organism *B. subtilis* and confirmed wherever possible with organisms from the *B. cereus* family. The process,

which requires nearly 8 hours to complete, is essentially a stress response to a nutrient-starved environment (169,212). The first step of sporulation is the assymetrical division of the vegative cell cytoplasm into large and small compartments known as the mother cell and forespore, respectively (169). The division of cytoplasms mirrors a simultaneous division of gene regulation, as the mother cell and forespore undergo different transcription programs using compartment-specific sigma factors (56). In the forespore, SASPs that protect the spore DNA from damage during dormancy and serve as a source of energy during germination are synthesized under the control of spore-specific σ^F- and σ^G-regulated promoters (196). Meanwhile, the septum dividing the two compartments gradually engulfs the forespore in a double membrane, creating a situation in which the developing forespore is surrounded by the cytoplasm of the mother cell (56). The forespore takes up large deposits of dipicolinic acid and divalent cations provided by the mother cell (56). The thick, peptidoglycan layer that makes up the cortex of the spore is produced under the control of the mother cell and deposited between the two membranes that encompass the forespore (174). Assembly of the cortex helps bring about the dehydration of the forespore, which presumably shuts down spore-specific protein synthesis (148,175). However, a signal transmitted from the increasingly-quiescent forespore triggers the production of the spore coat (140). This proteinaceous shell consists of species synthesized in the mother cell cytoplasm and assembled on top of the outer membrane of the forespore (56).

The current model for spore coat construction (36,56,57,98,224), which reliably assembles as many as 60 proteins (128,131) in an arrangement that produces both resistance to outside stresses and responsiveness to signals of growth-permissive

environs, depends on a small subset of structural proteins known as morphogenetic proteins. These morphogenetic proteins serve as anchors to the underlying layers (59,185,210) and nucleation points for futher protein deposition (242). At the direction of these critical spore scaffolding constituents, the remainder of the spore coat is believed to assemble through a complex network of protein-protein interactions (96,243). Deletion of the genes encoding these critical proteins results in the failure of entire layers of the spore to properly coalesce (56). Within the remaining population of coat proteins, there appears to be some degree of functional redundancy. In fact, protein-protein interactions are substantially generic and promiscuous and certain structural proteins with closely-related homologs have been deleted from the chromosome with no apparent effect on spore morphology, resistance properties or germination kinetics (56).

The construction of the exosporium occurs concomitantly with that of the coat (15,160), and, thus, the exosporium grows around the spore and gradually envelops it while still within the mother cell cytoplasm. As with the spore coat, certain structural proteins are essential for proper exosporium construction, with elimination of these proteins resulting in a failure of the growing exosporium to associate with the spore coat (52). Eventually, the mother cell lyses, releasing the spore into the surrounding environment. Of significance to the life cycle of the organism during the course of infection, sporulation requires oxygen (154). Therefore, reintroduction of spores to the environment after infection requires the corpse to undergo rupture and exposure to the surrounding air before the toxic chemicals produced by putrefaction kill the susceptible vegetative bacilli (115).

Figure 2. Spores and sporulation.

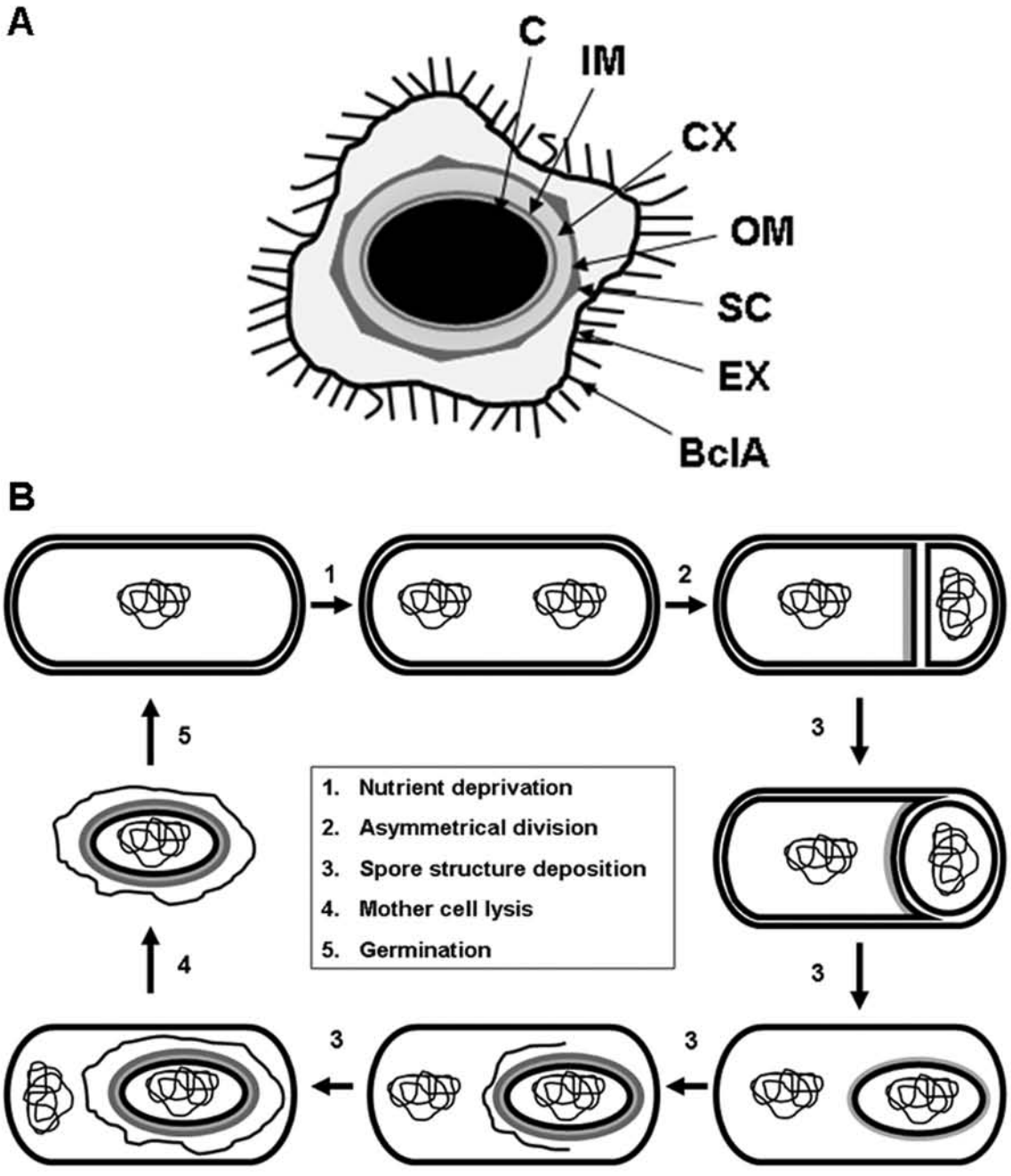
(A) *B. anthracis* spore

Spore core (C), inner spore membrance (IM), spore cortex (CX), outer spore membrane (OM), spore coat (SC), exosporium basal layer (EX), *Bacillus* Collagen-Like Antigen (BclA)

(B) *B. anthracis* sporulation and germination

In the absence of sufficient nutrients and the presence of oxygen (1), vegetative bacilli undergo sporulation. The process begins with an asymmetirical cell division (2), followed by a layering of proteins onto the developing endospore (3). Ultimately, the mother cell lyses and the spore is released (4). The metabolically inert yet highly resistant spore remains dormant until it becomes exposed to a nutrient-rich environment, such as a host. At this time, specific nutrients trigger the process of germination (5), in which the spore rehydrates and the vegetative bacillus reemerges.

Adapted from (26,56,58).


A
C
IM
CX
OM
SC
EX
BclA
B
1
2
3
3
3
3
4
5
1. Nutrient deprivation
2. Asymmetrical division
3. Spore structure deposition
4. Mother cell lysis
5. Germination

Disease pathology

Anthrax infections result from the entry of *B. anthracis* spores into the body from the outside environment through one of three routes: the skin, the gut or the respiratory mucosa. The spore is the essential infectious form, with no evidence that vegetative cells can persist in the environment, transmit between hosts or establish infection (1,54). Regardless of the route of infection, once inside the host, spores rapidly germinate into bacilli that multiply throughout the lymphatics and bloodstream (187). Bacterial levels can reach as high as 1×10^8 vegetative bacilli/ml of blood at the time of the host's death (54). However, death is not the direct result of the overwhelming bacteremia facilitated by an antiphagocytic capsule but rather of the intoxication mediated by the high levels of lethal toxin (LT) and edema toxin (ET) produced by that overwhelming population of bacteria (203).

Cutaneous anthrax

Approximately 95% of all human anthrax cases worldwide are transmitted through the cutaneous route of infection (238). Cutanous anthrax results when spores of *B. anthracis* are introduced into the skin through cuts or abrasions, usually while handling infected animals or animal products. Incubation is typically 2-5 days, although early clinical symptoms can be seen as early as 12 hours and as late as two weeks post-exposure (54). Cutaneous manifestations begin as a small, painless, erythematous macule that becomes vesicular and edematous within 24-72 hours. This edema is extensive and contains culturable bacilli (54). Rupture of the vesicles results in the characteristic black

eschar for which the disease was originally named. This lesion is usually painless and self-resolves in 1-2 weeks, though antibiotic therapy is still recommended due to mortality reaching as high as 20% in untreated cases (222). Fever, malaise and regional lympthadenopathy often accompany the cutaneous pathology (54).

Gastrointestinal anthrax

The gastrointestinal form of anthrax is the most rare overall, but occurs most frequently in the developing world as a result of consumption of contaminated and undercooked meat (112). Symptoms generally appear within 2 to 5 days of ingestion, presumably as a result of spores entering the body via a breach in the mucosa (130). Disease begins with fainting spells, fever and headache followed by abdominal pain and nausea. Symptoms progress to ascites, GI bleeding and shock, with most cases proving fatal (130). Postmordem examination indicates proliferation of bacilli in the gut mucosa and lymphatic tissue with resulting edema and necrotic ulcers (112).

Inhalational anthrax

Historically, inhalational anthrax was seen only sporadically among wool handlers and other groups in close contact with susceptible animal hosts (198). However, with the onset of an age in which mankind weaponized biological agents, inhalational anthrax has become a much more general threat. In addition to studies performed with non-human primates, much is known about the progression of inhalational anthrax in humans due to two well-documented events: the accidental release of anthrax spores from a Soviet biological warfare facility located in the city of Sverdlovsk in 1979 (3,149) and the

intentional distribution of spores through the U.S. mail system in 2001 (85,110,111). Anthrax spores are 1 to 2 μm in diameter, making them an optimal size for inhalation and deposition in the alveolar spaces of the lung (166). The 50% lethal dose (LD_{50}) for humans via the inhalational route of inoculation is estimated to be ~10,000 spores (72). The incubation period is as short as 5-6 days, though extended delays of more than 45 days between exposure and onset have been seen following aerosol inoculation of monkeys (107). Initial symptoms of fever, malaise, myalgia and non-productive cough are commonly confused with flu or the common cold and persist for 3-4 days before progressing within 24 hours to severe acute respiratory stress, hypoxemia, cyanosis and septic shock (6). These symptoms, which culminate in death in nearly 100% of untreated cases, correspond with the overwhelming septicemia, edematous hemorrhagic mediastinitis and multi-organ failure visible upon post-mortem examination (6). This damage is the result of toxin-producing bacilli that mutliply rapidly in the mediastinal lymphnodes and spread throughout the body in the blood after intial spore introduction to the lungs (6,187).

Pathogenesis

Germination

Following introduction of spores into a host system, the first step in infection is spore germination. Germination, the process by which the metabolically dormant spore undergoes conversion to an actively growing vegetative bacillus, occurs when conditions favorable to the growth of the bacteria are met. These conditions include a temperature between 8 and 45ºC, a pH between 5 and 9, and a relative humidity greater than 95% (197). Of note, these necessary conditions do not include the presence of oxygen. While necessary for vegetative outgrowth, oxygen is not required for germination itself (188). Essential to a permissive germination environment is the presence of germinants, molecules such as sugars, single amino acids including L-alanine or purine nucleosides including inosine, which serve as signals of a nutrient-rich environment (197). Germinants are recognized by germinant receptors, a family of sensor molecules recognizing an overlapping array of molecules whose activation is the triggering event of germination (32,141,194). This activation is an irreversible event, as upon activation of receptors, germination will proceed even if the germinants are rapidly removed (32). Through a series of events and interactions not well understood, the spore releases a deposit of hydrogen ions stored in the core, thereby elevating the core pH from ~6.5 to ~7.7 (71). This event triggers the release of concentrated dipicolinic acid (DPA) and associated divalent cations (such as Ca^{2+}), which are rapidly replaced by water to achieve rehydration of the spore core (71). These early events coincide with the loss of the heat-resistance and the light refractility that are commonly used as differentiating features for

spores (176). DPA release and core rehydration serves as a signal to initiate hydrolysis of the spore cortex and spore coat, which allows further hydration of the spore and restoration of metabolic activity to the reawakening cell (155). While the conditions for germination are most easily met inside a host organism, germination will occur in the environment if and when the necessary conditions are properly met (197).

Virulence factors

Essential to the ability of *B. anthracis* to acheive full virulence in mammals is the presence of several key virulence factors, particularly the poly-γ-D-glutamic acid capsule and a full complement of anthrax toxins (223). The generation of antibodies to each of these factors in patients recovering from cutaneous anthrax (201) is indication not only of their immunogenicity, but likely also of the importance of blocking their function in order to effectively combat anthrax infection. The capsule, which is composed of γ-D-glutamic acid residues held together with peptide bonds to form complexes 215 kDA in size *in vivo* (84,143), has been shown to provide the bacillus with antiphagocytic characteristics that greatly enhance virulence (64). The toxins, whose combined effects of edema and shock ultimately bring about host death (167), were first described in studies in which animals succumbed to death despite elimination of the bacteria through antibiotic treatment, and lethality was conferred between animals through the sterile, filtered serum of an infected animal (116,203,204).

The anthrax toxin components protective antigen (PA), edema factor (EF) and lethal factor (LF) act as binary pairs of the A-B toxin class (129,153,205) and are ineffective if administered alone. Protective antigen is the element common to both edema toxin

(PA+EF) and lethal toxin (PA+LF). Responsible for the assembly, internalization and delivery of anthrax toxins (9), PA derives its name from the fact that antibodies generated against the element are capable of preventing intoxication and thereby protecting against disease (205). PA is a 83 kDA protein containing four functional domains, including a cell receptor binding doman, an LF/EF binding domain, an insertion domain and a translocation doman (9). PA is capable of binding the host cell molecules tumor endothelium marker 8 (also known as anthrax toxin receptor 1, or ATXR1) and human capillary morphogenesis protein 2 (a.k.a. ATXR2), and variations in the level of expression of each of these receptors determine the differing sensitivities of cell types to intoxication (25,190,193). Upon binding, PA is cleaved by a furin-like protease to generate a 63-kDa protein known as PA63 (125). PA63 is capable of a heptamerization that opens the molecule's LF/EF binding domain (151,152,200) and mediates uptake of the PA-LF or PA-EF complex by receptor-mediated endocytosis (2). The final function of PA, the formation of pores in the walls of the endocytic vesicles that facilitates the translocation of LF and EF (LF is fully translocated to the cytoplasm, while EF remains vesicle-bound) is dependent upon acidification of the endocytic vesicle cell (90,126).

Lethal factor is a 85 kDA zinc metallo-protease that mediates toxic effects directly linked to the death of the host (14,167). The enzymatic activity of LF cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKKs), an alteration that inhibits a variety of host cell pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (60,226). While the direct links between these effects and the toxin-induced shock that ultimately leads to the death of the host are not well understood, the demonstrated ability of lethal toxin (LT) to induce apoptosis in

endothelial cells (121) and macrophages (86) has lead to a model in which MAPKK cleavage facilitates host cell lysis and the initiation of an uncontrolled pro-inflammatory cytokine cascade resulting in shock (93,94,167).

The ability of a then-unknown factor to induce edema upon intradermal injection along with PA (205) is the origin of the name edema factor. EF is an 88 kDA calcium- and calmodulin-dependent adenylate cyclase capable of catalyzing the converson of ATP to cyclic AMP, an activity that alters host cell electrolyte fluxes and water homeostasis and thereby induces localized edema (5,132,133,205). However, edema toxin (ET) has additional effects whose relation to the overproduction of cAMP are less well understood, including the induction of pro-inflammatory cytokines (102), the stimulation of neutrophil chemotaxis (228) and the inhibition of neutrophil phagocytosis and respiratory burst (7,159,240). The observation that strains lacking ET demonstrate a higher infectious dose than strains carrying a full complement of toxins (167) suggest that EF's impacts on the host immune system likely have a synergistic effect when combined with the immune dysregulatory function of LT.

The components of anthrax toxin are encoded on the 152 kb plasmid pXO1 (215,223), which also contains the virulence factor regulatory elements *pagR* and *atxA* (88,127). While *pagR* is a repressor of PA expression with an unclear role in virulence, *atxA* is a global virulence regulator responsible for gene expression beyond pXO1 (127). *atxA* deletion strains demonstrate not only a loss of toxin production but a reduction in capsule expression (100). The genes reponsible for capsule synthesis and degradation are located on the 96 kb plasmid pXO2 (215). Plasmid pXO2 is capable of being lost spontaneously in nature to produce $pXO2^-$ strains such as the Sterne strain that is

commonly used in laboratory research (or cattle vaccination) due to its severe attenuation (220). Virulence gene expression is in part tied to the bacteria's growth state, as increased toxin and capsule expression is seen as bacteria transition from exponential to steady-state growth (127).

Current models of spore:host interaction

B. anthracis causes an acute and fatal disease that overwhelms the host immune system through a combination of rapid and extensive bacterial spread, the antiphagocytic effect of the capsule and the immunomodulatory effects of the toxins (54). Many of the critical elements of infection and spore:host interaction have yet to be fully elucidated. A key point of interest and debate regarding anthrax infections involves the role of the macrophages and other phagocytic cells in both the establishment of infection and the initiating of the host's early immune response. Early reports dealing with this subject (187) indicated that macrophages play a role in the lymphatic migration and germination of *B. anthracis* spores, events which are followed by vegetative outgrowth and dissemination of extracellular bacilli within the host. Later studies indicated that upon phagocytosis of the dormant spore, macrophages provide a critical environment in which germination may occur (53,87,89,191,231). Whereas numerous reports substantiated the notion that germination is followed by intracellular bacterial replication, toxin elaboration and subsequent killing of the phagocyte (53,163,189,231), other reports failed to observe replication within macrophages (89). More recently, reports of extracellular germination in both the lung and at a subcutaneous site of infection suggest that macrophages may not be an essential germination environment (19,191,230). Additionally, several reports

suggest that dendritic cells, whose critical role in inducing a proinflammatory response and activating the adaptive arm of the host immune response is impaired by exposure to LT (4,40), may serve as an important vehicle for carrying spores from the site of infection to the regional lymphnodes (29,41).

In addition to questions regarding the essentiality of phagocytes in early spore dissemination and germination, the exact role of macrophages, neutrophils, and the adaptive immune response in controlling anthrax infection remains an open question. Mayer-Scholl *et al.* (145) demonstrated that human neutrophils were capable of engulfing spores, which subsequently germinated intracellularly and were rapidly and efficiently killed. Combined with evidence of neutrophil infiltration at the site of infection (149), these data suggest a role for neutrophils in controlling anthrax infection. However, studies by Cote *et al.* (44,46) suggest that whereas systemic depletion of neutrophils has no deleterious effect on the host response to *B. anthracis*, a similar depletion of macrophages was lethal. These data were generated using an otherwise-resistant strain of mice and introduction of spores via an inhalational, parenteral or intraperitoneal route. Cote *et al.*'s results support the notion that, far from simply serving as a permissive environment for germination and propagation, macrophages are capable of destroying anthrax, and serve as an important line of defense in the host's immune response (44,46,103,113). Additionally, recent evidence (82) implicates the cellular immune response as playing an important role in controlling anthrax infection through the induction of a spore-specific Th1 response. These data suggest that there is much yet to be understood about the dynamics of spore:host interactions and the factors which determine the balance of overwhelming infection versus protective immune response.

Treatment

Current post-exposure treatment for anthrax is dependent upon one of two approved antibiotics: ciprofloxacin or tetracyclines. The presence of inducible β-lactamases in the Ames strain used in the 2001 attack has caused penicillin, previously among the antibiotics included among available options, to be removed from the list of recommended therapeutics (35). Ciprofloxacin is especially attractive as a therapeutic given its demonstrated benefit as a prophylactic treatment for *Yersinia pestis and Franciscella tularensis*, two organisms also considered to be threats for intentional use as bioterror agents (34). *B. anthracis* has been shown *in vitro* to also be susceptible to chloramphenicol, aminoglycosides, macrolides, imipenem, rifampicin, and vancomycin (35). Exposed patients without symptoms are placed on a 60-day antibiotic regimen to allow for killing of bacteria that persist in the lung as spores for extended periods (77). Alternatively, exposed personnel can receive four weekly doses of ciprofloxacin combined with three doses of the currently-approved anthrax vaccine, adsorbed (AVA) (35). Prognosis for patients treated for cutaneous anthrax is excellent if treatment is begun promptly, although the black eschar that is the disease's namesake may still occur. However, treatment of pulmonary and gastrointestinal anthrax is usually ineffective, owing largely to the fact that the early stages of the disease are often mistaken for influenza or the common cold, and proper treatment is begun too late (54).

Vaccines

Current vaccine and established correlates of protection

Although post-exposure antibiotic therapy offers hope for a patient who is rapidly diagnosed (77), prophylactic immunization is the more feasible form of protection for large populations. The ability to generate an effective immune response is demonstrated by individuals and animals who suffer from cutaneous infection and show a reduced susceptibility to subsequent infection (76). Serum taken from animals vaccinated with PA is capable of protecting against disease when administered to non-vaccinated animals, a finding that suggests that antibodies that protect against intoxication are a key to an effective response (135). The currently licensed AVA vaccine, a preparation comprised of a formalin-treated, aluminum salt-adsorbed, cell-free culture filtrate from a non-encapsulated attenuated strain of *B. anthracis* (24), is predicated on the importance of a response to PA. It is generally accepted that while anti-PA antibodies do not guarantee protection, protection is not possible without them. Established correlates of protection for anthrax immunizations are based on anti-PA antibody titers (136,170-172,183,221). However, AVA is not a perfect vaccine and is unlikely to be the final answer to the question of anthrax prophylaxis. Immunization studies in various animals models suggest that the humoral response generated by PA and the level of protection conferred by anti-PA antibodies are more variable than was first thought (69,106-108,234). Additionally, while the vaccine is considered safe and effective despite its less than favorable public reputation (75), recurrent problems exist with reactogenicity,

availability, lot-to-lot PA dosage variability, a cumbersome multi-dose vaccine regimen and outstanding concerns regarding long-term efficacy (24,219).

New approaches

Much of the effort devoted to next generation anthrax vaccines involves making improvements on the current, PA-exclusive formulation. On-going efforts include the use of defined recombinant PA (rPA) sources (67), more effective adjuvants (202), novel delivery platforms (31,150), and prime-boost strategies with PA-encoding DNA vaccines in combination with rPA (237). These strategies may improve the degree and reliability of the immune response to PA but do not provide additional targets for generating a more diverse response. Therefore, these schemes do not deal with concerns regarding the potential for an engineered strain in which the anthrax toxins are replaced by foreign virulence factors. Alternative approaches involve targeting components of the bacteria itself, either in the spore or vegetative forms.

Early success with Louis Pasteur's vaccine strain, as well as with the attenuated Sterne strain isolated in the 1930's and still in use as a cattle vaccine today, was based on administration of whole spores (219). Studies involving attenuated live spore vaccines have repeatedly demonstrated a capacity to confer protection against anthrax spore challenge (42,124,137,199,234). Certain studies indicate that live-spore vaccines are more protective than PA-based vaccines (137,234), perhaps due to a broader immune response compared to immunization with PA alone. The facility of formaldehyde-inactivated spores added to a PA-based vaccine to confer greater protection against spore challenge than PA alone, despite eliciting similar levels of anti-PA antibodies (30),

suggest protection is afforded by an immune response to the spore. An attenuated live spore anthrax vaccine, developed by scientists in the former Soviet Union (199), is still in use in Russia and China. Despite the data indicating that an immune response generated against *B. anthracis* spores can protect against anthrax infection, whole spore-based vaccines are impractical for human use in the United States due to safety concerns. This limitation necessitates the identification of individual spore antigens that might be added to the vaccine to obtain a meaningful anti-spore response and achieve a more protective vaccine.

Superoxide dismutase

Sources and mechanisms of oxidative stress

Oxidative stress is a normal byproduct of aerobic metabolism. Superoxide anion ($O_2^{\cdot-}$) is generated when molecular oxygen (O_2) interacts with and spontaneously oxidizes reduced flavoprotein complexes such as NADH dehydrogenase II that are involved in the respiratory chain (104,105). Superoxide anions can be further converted to hydrogen peroxide (H_2O_2), and the more reactive oxygen species hydroxyl radical ($HO^{\cdot}$), through a series of reactions depicted in Figure 3 [adapted from (65)]. These oxygen radicals are capable of inducing diverse forms of damage within the typical cell. Hydrogen peroxide is a powerful oxidizer of cysteine residues and is therefore capable of inactivating a variety of enzymes dependent on cysteine in a reduced form (179). Superoxide anions are capable of the peroxidation of lipids (142), as well as the destruction of iron sulfur (Fe-S) clusters that are essential components of enzymes such as dehydratases (70). These enzymes play important roles in both amino acid biosynthesis and the catabolism of non-fermentable sugar sources, and so the inactivation of these enzymes can have a critical impact on metabolism and cell growth. Worse still, $O_2^{\cdot-}$ and H_2O_2 work synergistically to damage DNA. Iron released from Fe-S clusters by superoxide anion associates tightly with DNA and reacts with hydrogen peroxide to produce hydroxyl radical via the Fenton reaction. The consequence of this interaction is to place a powerful oxidizer of nucleic acid ($HO^{\cdot}$) in direct proximity of its target (117,134). Through these direct effects, and the effects of secondary radicals such as hypochlorous acid (HOCl)

that are generated in the presence of $O_2^{\cdot-}$ (83), endogenously-generated oxidative stress poses a critical threat to the health of the cell.

Oxygen radicals are such an effective means of inflicting damage on a cell that immune systems have evolved to harness the power of reactive oxygen species (ROSs) in a targeted, compartmentalized fashion to eliminate pathogens. Professional phagocytes such as macrophages and neutrophils employ a variety of defenses to destroy phagocytosed pathogens. These defense mechanisms include the generation of ROSs by NADPH oxidase and the generation of reactive nitrogen species (RNSs) by nitric oxide synthetase (iNOS), in addition to other antimicrobial weapons such as proteases and antimicrobial peptides, all of which can be introduced into the phagolysosome (10,49,65,173). Ongoing debates regarding such issues as the actual levels of individual radical species within the phagolysosomal compartment and the capacity of superoxide anion to diffuse across the bacterial lipid membranes at lower pH (65,83,142) leave the relevant mechanisms of pathogen destruction unclear, but a general model of contributing pathways is outlined in Figure 3B. In sum, the phagolysosome mediates the death of internalized pathogens through the direct toxic effects of oxygen radical and the synergistic interaction of ROSs with RNSs that lead to the generation of still more reactive species [(Figure 3A), (33,65,78,95,118,179)].

Figure 3. Phagocyte-generated oxidative stress.

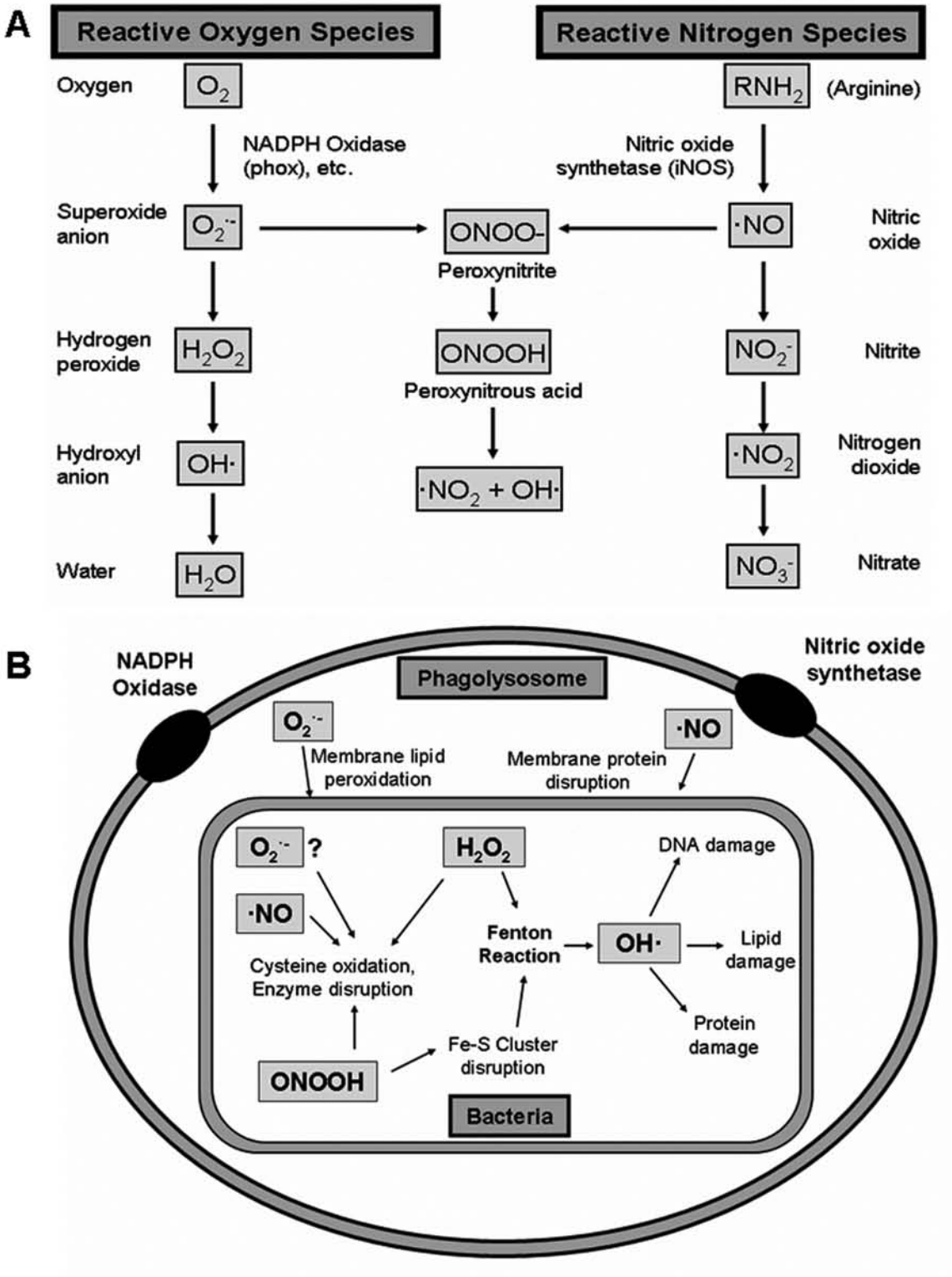
(A) Production of reactive oxygen and reactive nitrogen species

Superoxide anion ($O_2^{\cdot-}$) is generated both as an accidental by-product of aerobic respiration and by the enzyme NADPH oxidase, which is expressed in professional phagocytes. These cells also express nitric oxide synthetase, an enzyme that generates nitric oxide (NO·). These radicals are capable of inducing damage directly, or through the establishment of separate and synergistic cascades that lead to the production of more reactive intermediates.

(B) Mechanisms of oxidative damage within the phagolysosome

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by enzyme complexes bound to the phagolysosomal membrane serve as mediators of pathogen destruction. $O_2^{\cdot-}$ is believed to have limited facility to diffuse across lipid bilayers and may be restricted to the extracellular or periplasmic regions, where these radicals generate damage through the peroxidation of lipids and the disruption of membrane-bound proteins. Other ROS either diffuse across the membrane (H_2O_2, NO·, ONOOH) or are generated intracellularly (OH·) and cause damage to metabolic enzymes and/or DNA.

Adapted from (65).


A
Reactive Oxygen Species
Reactive Nitrogen Species
Oxygen
O_2
RNH_2
(Arginine)
NADPH Oxidase (phox), etc.
Nitric oxide synthetase (iNOS)
Superoxide anion
$O_2^{\cdot-}$
ONOO-
Peroxynitrite
·NO
Nitric oxide
Hydrogen peroxide
H_2O_2
ONOOH
Peroxynitrous acid
NO_2^-
Nitrite
Hydroxyl anion
OH·
$\cdot NO_2 + OH\cdot$
$\cdot NO_2$
Nitrogen dioxide
Water
H_2O
NO_3^-
Nitrate
B
NADPH Oxidase
Nitric oxide synthetase
Phagolysosome
$O_2^{\cdot-}$
·NO
Membrane lipid peroxidation
Membrane protein disruption
$O_2^{\cdot-}$?
H_2O_2
DNA damage
·NO
Fenton Reaction
OH·
Lipid damage
Cysteine oxidation, Enzyme disruption
Protein damage
Fe-S Cluster disruption
ONOOH
Bacteria

Defenses against oxidative stress

Studies with *Escherichia coli* have shown that while intracellular concentrations of $O_2^{\cdot-}$ and H_2O_2 as low as 10^{-5} M are toxic, bacteria in steady-state or mild oxidative stress conditions successfully maintain [$O_2^{\cdot-}$] and [H_2O_2] around 10^{-10} M and 10^{-7} M, respectively (83). Successful detoxification of otherwise-harmful oxygen radicals is achieved through the utilization of a number of antioxidant molecules and mechanisms. These defenses include alkaline phosphatases that inhibit ROS production, DNA repair enzymes to mitigate DNA damage, and superoxide-resistant isozymes of critical metabolic enzymes (211). Also important are enzymes capable of neutralizing ROSs. This line of defense includes catalases and peroxidases that detoxify hydrogen peroxide, and superoxide dismutases that detoxify superoxide anion.

Superoxide dismutases (SODs) are enzymes that catalyze the dismutation of superoxide anion to hydrogen peroxide and molecular oxygen (146). SODs serve to scavenge $O_2^{\cdot-}$ anions before they can cause cellular damage (122,123), and, in combination with catalases or peroxidases that breakdown the H_2O_2 byproduct of superoxide dismutation, they achieve a detoxification of imminent oxidative stressors (211). Since first being discovered in bovine erythrocytes in the 1960s (146), SODs have been found in almost all aerobic organisms studied, including aerobic (as well as some anaerobic) bacteria. SODs are divided primarily into two main structural classes, based on the capacity of the enzyme to complex either with the combination of copper and zinc (Cu-Zn SODs) or with either manganese or iron (Mn/Fe SODs). Early work with erythrocytes (142), later extended to prokaryotes, demonstrated that Mn/Fe SODs are primarily localized to the cell cytoplasm (74). Conversely, Cu-Zn SODs are typically

found in the periplasm of Gram negative bacteria or extracellularly (74,208). The knowledge that many ROSs have limited facility to diffuse across lipid membranes between subcellular compartments (65,83,142), and the fact that many bacterial species possess SODs of both classes, suggests that each class of SOD possesses a specific role regarding exogenously- and endogenously-produced oxygen radicals.

SODs in pathogenic bacteria

While the ubiquity of SODs among aerobic bacteria is probably best explained by the need to protect against endogenously-generated oxygen radicals, the utilization of ROSs by professional phagocytes strongly suggests that intracellular bacteria require SODs for more than just protection against self-induced oxidative stress. Indeed, the list of organisms for which the presence of functional SODs are an important or even essential contributor to pathogenicity is continually growing. Organisms such as *Brucella abortus* (79) rely on the presence of Cu-Zn SODs to maintain fitness. Meanwhile, *Francisella tularensis* (12), *Staphylococcus aureus (114)*, *Streptococcus agalactiae* (177), *Bordatella pertussis* (119), *Shigella flexneri* (73), *Campylobacter jejuni* (178), and *Enterococcus faecalis* (225) only display maximum virulence in the presence of Mn- or Fe-SODs. Certain pathogens, such as *Salmonella enterica* serovar Typhimurium (66,68,218), *Mycobacterium tuberculosis* (62,168), and *E.coli* (17,158) employ enzymes of both classes in important roles. Common among many of these pathogens is the requirement that they survive interaction with ROS-generating phagocytic cells such as macrophages while establishing an infection.

Hypothesis and specific aims of this dissertation

The goal of this work is to investigate individual components of the *B. anthracis* exosporium to better understand the contribution of these substances to the pathogenic mechanisms of the organism and to define which of these factors might serve as protective immunogens for inclusion in a future anthrax vaccine. The work is guided by two distinct hypotheses, each with associated specific aims.

Hypothesis #1: Antibodies to individual components of the *B. anthracis* exosporium may contribute to a protective immune response.

Specific Aims:

1. Identify and localize proteins within the exosporium.
2. Determine which proteins are immunogenic and might contribute to protection against a *B. anthracis* spore challenge.

Hypothesis #2: Superoxide dismutases present in the spore contribute to the virulence of *B. anthracis.*

Specific Aims:

1. Demonstrate the presence of functional SODs within the spore
2. Identify the contribution that spore-bound SODs make to *B. anthracis* pathogenicity.

CHAPTER 2: RECOMBINANT *BACILLUS ANTHRACIS* SPORE PROTEINS ENHANCE PROTECTION OF MICE PRIMED WITH SUBOPTIMAL AMOUNTS OF PROTECTIVE ANTIGEN

Published as: Robert J. Cybulski Jr., Patrick Sanz, Dennis McDaniel, Steve Darnell, Robert L. Bull and Alison D. O'Brien. 2008. Recombinant *Bacillus anthracis* spore proteins enhance protection of mice primed with suboptimal amounts of protective antigen. Vaccine. XX(X):XX-XX

Note: The figures and tables shown reflect the work of Robert Cybulski, with several exceptions. Dr. Patrick Sanz performed the cloning and construction of the plasmid constructs listed in Tables 1-3. Dr. Robert Bull generated the anti-spore polyclonal antisera 311001-01 as well as the polyclonal antisera against the individual spore proteins. Mr. Stephen Darnell performed the preliminary mouse immunization experiment, described in Table 4, as well as the screening of recombinant spore proteins by Western Blot as depicted in Figure 4A. Dr. Dennis McDaniel performed the immuno-electron microscopy depicted in Figure 6. Additionally, Drs. Sanz and O'Brien contributed to the experimental design and interpretation of the data as well as preparation of the manuscript.

Abstract

Inactivated *Bacillus anthracis* spores given with protective antigen (PA) contribute to immunity against anthrax in several animal models. Antiserum raised against whole irradiated *B. anthracis* spores has been shown to have anti-germination and opsonic activities *in vitro*. Based on these observations, we hypothesized that surface-exposed spore proteins might serve as supplemental components of a PA-based anthrax vaccine. The protective anti-spore serum was tested for reactivity with recombinant forms of 30 proteins known, or believed to be, present within the *B. anthracis* exosporium. Eleven of those proteins were reactive with this antiserum, and, subsequently a subset of this group was used to generate rabbit polyclonal antibodies. These sera were evaluated for recognition of the immunogens on intact spores generated from Sterne strain, as well as from an isogenic mutant lacking the spore surface protein *Bacillus* collagen-like antigen (BclA). The data were consistent with the notion that the antigens in question were located beneath BclA on the basal surface of the exosporium. A/J mice immunized with either the here-to-fore hypothetical protein p5303 or the structural protein BxpB, each in combination with subprotective levels of PA, showed enhanced protection against subcutaneous spore challenge. While neither anti-BxpB or anti-p5303 antibodies reduced the rate of spore germination *in vitro*, both caused increased uptake and lead to a higher rate of destruction by phagocytic cells. We conclude that by facilitating more efficient phagocytic clearance of spores, antibodies against individual exosporium components can contribute to protection against *B. anthracis* infection.

Introduction

Bacillus anthracis, the etiological agent of anthrax, is a spore-forming, Gram-positive bacterium. *B. anthracis* spores, the highly resistant infectious stage of the organism capable of remaining dormant yet viable for decades, form in response to stressful environmental conditions such as desiccation and inadequate supplies of nutrients (154). Spores can establish infection in a host through the cutaneous, oral, or inhalational routes, each of which provide permissive environments for spores to germinate, grow into bacilli, and elaborate the toxins that ultimately kill the host (54). Although herbivores are the primary target of anthrax spores within the environment and natural human infection is incidental, the potential for the infection of large populations by intentional distribution of spores was highlighted by the biological attack conducted through the U.S. mail system in 2001 (111).

While post-exposure antibiotic therapy is an effective treatment for rapidly-diagnosed anthrax infection (77), prophylactic immunization offers the possibility of protection to potentially vulnerable populations prior to exposure. The currently licensed vaccine is the anthrax vaccine adsorbed (AVA), a preparation consisting of a formalin-treated, aluminum salt-adsorbed, cell-free culture filtrate from a non-encapsulated attenuated strain of *B. anthracis* (24). The protection afforded by AVA is primarily attributable to antibodies raised against protective antigen (PA), the cell-binding component of both edema toxin and lethal toxin and an essential element of the deadly toxemia mediated by anthrax infection (156). Correlates of protection for anthrax immunizations are based on anti-PA antibody titers (136,170-172,183,221), and as a

result, PA is an essential component in any potential future anthrax vaccine candidate. However, though considered safe and effective (75), AVA is plagued by issues related to reactogenicity, availability, lot-to-lot PA dosage variability, a multi-dose vaccine regimen, and adverse public perception toward the anthrax vaccine (24,219). Furthermore, while defined recombinant PA (rPA) sources (67), novel delivery platforms (31,150,165), and more effective adjuvants (202) offer the possibility of improved immune responses to PA, immunization studies in a variety of animals models cite the variability of the protection conferred by vaccines based solely on PA (69,106-108,234).

An alternative approach to anthrax vaccinology might be to target not only the toxin produced by the vegetative bacillus but also the spore that is the essential component for establishing infection. Multiple studies demonstrate the capacity of attenuated live spore vaccines to confer protection against anthrax spore challenge (42,124,137,199,234). Live-spore vaccines appear to be more protective than PA-based vaccines against challenge with virulent strains of *B. anthracis* (137,234), perhaps because of the broader immune response these spore vaccines likely generate. Brossier *et al.*. (30) demonstrated that the addition of formaldehyde-inactivated spores to a PA-based vaccine conferred greater protection against spore challenge than PA alone, despite both formulations eliciting similar levels of toxin-neutralizing activity. While these studies in aggregate strongly indicate that responses to spore-associated antigens contribute to protective immunity, the fact that whole spore-based vaccines are unacceptable for human use in the United States due to safety concerns requires the identification of individual spore antigens that might be added to the current PA-based vaccine to

recapitulate the benefits of whole spores and create a more efficacious vaccine formulation.

To identify the best spore antigen candidates, we chose to focus on proteins located on the outermost surface of the spore. Unlike most *Bacillus* species, *B. anthracis* spores are covered with an exosporium, a "balloon-like" structure that loosely envelops the outer surface of the spore coat and consists of a latticework basal layer and a covering of hair-like projections (80). The hair-like appendages are constructed from the immunodominant spore glycoprotein BclA (206,213) and represent a tantalizing target for vaccine efforts. Recent studies demonstrated that mice challenged with *B. anthracis* spores were better protected following immunization with PA plus BclA, administered either in recombinant form (27) or on a BclA-encoding plasmid (92), as compared to immunization with PA alone. Given this proof of concept for targeting spore antigens as effective immunogens, in this investigation we sought to screen known components of the exosporium and spore coat for additional potential vaccine candidates. From a subset of spore antigens that we verified to be present in the outer structures of the spore, and to be targets of the immune response generated against whole spores, we identified a pair of proteins capable of enhancing protection against lethal anthrax spore challenge in A/J mice following immunization with suboptimal amounts of rPA. Unlike antiserum generated against whole spores, antibodies directed against individual spore components were not capable of significantly reducing spore germination. However, a possible explanation for the protective efficacy of these spore antigens might be found in the capacity of the antibodies to enhance spore uptake and killing by professional phagocytes.

Materials and Methods

Bacterial strains. *B. anthracis* Sterne strain (toxigenic, unencapsulated, Naval Medical Research Center, Silver Spring, MD) and an isogenic deletion mutant lacking the *Bacillus* collagen-like antigen (BclA) gene (Δ*bclA*) (28) were used for *in vitro* and *in vivo* experiments.

Preparation of *B. anthracis* spores**.** Spores were produced as previously described (191). Briefly, single colonies of *B. anthracis* Sterne strain or Δ*bclA* were taken from brain heart infusion (BHI) agar plates and inoculated into BHI broth for culture overnight at 37°C. Each culture was spread onto modified germination (G) medium (120) agar plates (0.2% yeast extract, 0.2% $(NH_4)2SO_4$, 1.5% Bacto agar, 0.0025% $CaCl_2$ dihydrate, 0.05% K_2HPO_4, 0.02% $MgSO_4$ heptahydrate, 0.005% $MnSO_4$ quatrahydrate, 0.0005% $ZnSO_4$ dihydrate, 0.0005% $CuSO_4$ pentahydrate, 0.00005% $FeSO_4$ heptahydrate). The plates were incubated at 30°C for 10 to 14 days in the dark. Colonies scraped from the surface of the agar were re-suspended in distilled water, washed twice in distilled water, and heat-treated at 65°C for 1 hour to kill any viable vegetative cells. Purification of spores was done with 58% (vol/vol) Renografin (Renocal-76, Bracco Diagnostics, Princeton, N.J., USA) diluted in dH_2O. Spores were layered onto the 58% Renografin solution and centrifuged at 6,000 g for 60 min in a swinging bucket rotor. The sedimented spores were washed twice with distilled water. After the final sedimentation, the spores were re-suspended in distilled water to yield a final concentration of 10^9 - 10^{10} colony-forming units (cfu)/ml, as determined by vegetative outgrowth on BHI plates.

Preparation of spore surface protein extract (SSPE). Analysis of the proteins contained within the exosporium was achieved by removal of the exosporium from whole spores via chemical extraction according to a method previously described (8). Briefly, 0.5 ml of heat-activated spores (10^9-10^{10} cfu/ml) were pelleted at 7000 g for 15 minutes at 4°C. Spores were washed twice with water and resuspended in 0.5 ml of extraction buffer that contained 0.1M DTT, 0.1M NaCl, 0.5% SDS, pH 10. Samples were incubated in a 37°C shaking water bath for 2.5 hours and pelleted, and the resulting supernatant was collected. Supernatants were then filtered through a 0.2 μm Corning filter and dialyzed against PBS.

Preparation of recombinant proteins. Our procedures for the construction of *E.coli* strains that expressed recombinant proteins with an N-terminal six-histidine tag (Table 1), and for purification of those proteins by nickel affinity chromatography are described in detail elsewhere (28). Briefly, genomic DNA was extracted from *B. anthracis* strains with the Easy-DNA kit (Invitrogen, Carlsbad, CA, USA). NdeI-BamHI, XhoI-BamHI or XhoI-BglII fragments that contained each gene of interest were amplified from the Sterne genome by polymerase chain reactions (PCR) using the Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, IN, USA) in a PTC200 Peltier Thermal Cycler (MJ Research, Bio-Rad, Hercules, CA, USA). Primer sets designed from the flanking sequences of the targeted gene [National Center for Biotechnology Information (NCBI) at website http://www.ncbi.nlm.nih.gov.lrc1.usuhs.edu/entrez/viewer.fcgi?db=nucleotide&val=AE0

17225] are listed in Table 2. PCR products were purified with the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA 91355, USA), and the DNA fragments were ligated into the expression vector pET15b (Novagen, San Diego, CA). The resulting expression plasmids are listed in Table 3. DNA sequences were verified with the ABI Prism Big Dye method (Applied Biosystems, Forest City, CA, USA) by the Biomedical Instrumentation Center at the Uniformed Services University, then recombinant plasmids were transformed into *E. coli* BL21(DE3) pLysS according to the pET system manual (Novagen, San Diego, CA). His-tagged proteins were expressed from transformants and subjected to His-Trap nickel affinity column chromatography with the AKTA fast protein liquid chromatography system (GE Healthcare, Piscataway, NJ).

Immune sera and antibody preparation. Polyclonal antibodies were generated against each recombinant protein by methods previously described (28). Briefly, rabbits were vaccinated monthly with 50 μg of purified recombinant protein in Freund's complete adjuvant for the first inoculation and Freund's incomplete adjuvant for all subsequent immunizations. Production bleeds were taken and analyzed for specific antibodies beginning at month 5, with final bleeds taken at month 12. Immunoglobulins G were purified from each final serum sample by passage over a protein G column (Pierce Biotechnology, Rockford, IL). IgG preparations were then affinity-purified on an UltraLink® Biosupport column (Pierce Biotechnology, Rockford, IL) conjugated to 5 mg of recombinant target protein. Resulting antibody concentrations were determined by a microtiter BCA assay (Pierce Biotechnology, Rockford, IL).

Western blot analysis. *Screening of recombinant protein candidates.* Purified recombinant forms of spore proteins were screened as follows. Purified His-tagged proteins (100 ng/lane) were separated by electrophoresis on a 4-20% Tris-Glycine sodium dodecyl sulfate (SDS) polyacrylamide gel (Invitrogen, Carlsbad, CA), transferred onto Optitran BA-S 83 reinforced nitrocellulose (Whatman GmbH, Dassel, GE), and blocked overnight at 4ºC in PBST (0.5% Tween-20) with 5% nonfat dry milk (PBSTM). To verify the presence of the recombinant protein, blots were then incubated for one hour at room temperature with a His-Tag® monoclonal antibody (Qiagen, Madison, WI) diluted 1:4000 in PBSTM, washed 3 times for 5 minutes in PBST, incubated for 1 hour at room temperature with a secondary antibody [goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA)] diluted 1:16,000 in PBST, washed 3 times for 5 minutes each in PBST, and developed with Lumigen PS-3 Acridan (GE Healthcare UK, Buchinghamshire, UK) and Kodak BioMax XAR film (Kodak, Rochester, NY). To verify recognition of the recombinant protein by antiserum directed against whole spores, blots were incubated for one hour at room temperature with affinity-purified anti-spore rabbit polyclonal serum (designated 311001-01 and kindly provided by Naval Medical Research Center, protein G purified, original concentration 4.2 mg/ml) diluted to 0.1µg/ml in PBSTM. The blot was subsequently washed 3 times for 5 minutes each in PBST, incubated for 1 hour at room temperature with a secondary antibody [goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA)] diluted 1:16,000 in PBST, washed 3 times for 5 minutes in PBST, and developed as described above. *Screening of SSPE.* Antigens within SSPE were separated by electrophoresis on 4-20% Tris-glycine SDS-PAGE gels loaded with 5 µl of SSPE diluted

to a final volume of 20 µl in distilled water and transferred to nitrocellulose. The nitrocellulose was blocked with PBSTM and incubated with affinity-purified polyclonal anti-spore protein IgG standardized at 1 mg/ml and diluted 1:5000 in PBSTM for 1 hour. Following three, 5 minute washes at room temperature with PBST, the nitrocellulose was probed with goat anti-rabbit IgG conjugated to horseradish peroxidase and developed as above.

Immunoelectron microscopy. Bacterial spores were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde crystals) in PBS (pH 7.0) for a minimum of 8 hours. Following three 10-minute washes in PBS, spores were labeled by an immunogold technique with either pre- or post-embedding methods. For pre-embedding immunolabeling, spores were suspended in blocking buffer [PBS supplemented with 1mg/ml BSA Fraction V (EMD Chemicals. Inc, Gibbstown, NJ), 2% normal rabbit serum and 0.5% cold water fish skin gelatin (Electron Microscopy Sciences, Hatfield, PA)] for 45 minutes. Spores were then incubated with primary antibody (affinity-purified anti-spore protein polyclonal IgG) for 1 hour followed by secondary for 1 hour. The secondary antibody consisted of goat-anti-rabbit IgG conjugated to 10 nm gold particles (Electron Microscopy Sciences, Hatfield, PA). The spores were then incubated for 10 minutes in 4% aqueous electron microscopy grade glutaraldehyde (Tousimis, Rockville, MD) followed by 10 minutes in 50mM glycine to quench unreacted aldehyde groups. Three 10 minute washes in PBS were done between each step in the labeling procedure. Spores were dehydrated in a graduated series of ethanol and infiltrated with Spurr's epoxy resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections of samples were

then prepared and collected on 200 mesh copper grids. For post-embedding labeling, spores were dehydrated in a graduated series of ethanol and then infiltrated in LR White acrylic resin (London Resin Company Limited, Berkshire, England) at room temperature. Following overnight polymerization of the resin at -20°C in a UV Cryo Chamber (Electron Microscopy Sciences, Hatfield, PA), thin sections were collected on 200 mesh nickel grids. Grids that contained sections were then floated on a drop of blocking buffer for 30 minutes, and the grids were then incubated in primary followed by secondary antibodies (see above) for 1 hour each. Three 10-minute washes in PBS were done between each step in the labeling procedure. All grids that contained samples were then stained for 10 minutes in 2% aqueous uranyl acetate and for 5 minutes in Reynold's lead citrate. Samples were examined on a Philips CM100 electron microscope (FEI Company, Hillsboro, OR), and images were collected with a Spot Insight 4MP digital camera (Diagnostic Instruments, Sterling Heights, MI).

Mouse immunization and challenge protocol. Immunization experiments were done as previously described (27), with modifications. Briefly, six- to eight-week-old female A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and quarantined for 1 week before use. Preimmune serum samples were collected from mice by tail nicks. Two immunization protocols were used. In all cases, proteins were suspended in PBS (pH 7.4) and mixed 1:1 by volume with TiterMax Gold (TiterMax USA Inc., Norcross, GA) for a total volume of 100 µl. In protocol #1, the mice were immunized intraperitoneally (i.p.) on Day 1 with 10 µg of recombinant spore protein (BxpB, YwdL, SOD15, SODA1 or p5303). Control mice received PBS plus TiterMax Gold mixed 1:1

by volume. On Day 15, mice received a boost containing 10 μg of recombinant spore protein plus 50 ng of rPA. Control mice received either 50 ng of rPA ("PA Only" Control) or simply PBS with TiterMax Gold ("PBS Only" Control). In protocol #2, the mice were immunized i.p. on Day 1 with 50 ng of purified rPA, followed on Day 15 with 10 μg of recombinant spore protein. Following each protocol, mice were bled by tail nick on Day 28 and challenged on Day 29 with 10 times the 50% lethal dose (LD_{50}) of Sterne spores (about 10^4 spores) injected subcutaneously (s.c.) behind the right foreleg. The animals were then monitored for survival twice daily for 14 days after challenge.

ELISA analysis. *Anti-spore ELISA.* Analysis of antigen target accessibility on the spore surface was done by enzyme-linked immunosorbent assays (ELISA) with affinity-purified anti-spore protein polyclonal IgG directed against whole spores as a probe. Briefly, 96-well plates were coated with 0.1 ml of a 1×10^8 cfu/ml solution of Sterne or Δ*bclA* spores and incubated overnight at 4°C. Spores were removed and wells blocked overnight at 4°C in PBST with 3% BSA and 0.05% Tween-20 (PBSTB). After removal of the PBSTB from the wells, antibodies standardized at 1 mg/ml were added to wells at an initial dilution of 1:50 [in PBST (0.05% Tween-20)] and serially diluted 1:2 in PBST to a final dilution of 1:51,200. After a 1-hour incubation at room temperature, wells were washed four times with PBST, and a secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) was added at a dilution of 1:5,000 in PBST. After a 1-hour incubation at room temperature, wells were washed four times with PBST. The secondary antibody was detected with 3,3,5,5-tetramethylbenzidine peroxidase (Bio-Rad, Hercules, CA), and the microtiter plates were incubated at room temperature for 15 min.

An aliquot (100 µl) of 1M H_2SO_4 was then added to each well to quench the reaction, and the color intensity in each well was assessed by measuring the optical density at 450 nm (OD_{450}) with an ELISA microtiter reader (Microtek; Molecular Devices, Sunnyvale, CA). *ELISAs to measure mouse anti-rPA and anti-spore protein antibodies.* ELISAs were done to assess anti-rPA and anti-recombinant protein mouse sera IgG responses by methods described above, with modifications. Briefly, purified protein (100 ng of each in 100 µl PBS) was used to coat the wells of a "U"-bottom 96-well microtiter plate (Thermo Electron Corp., Milford, MA), and the microtiter plates were incubated at 4°C overnight. Wells were blocked and washed as described above before 100 µl of a 1:100 dilution of either pre- or post-immunization serum sample was added to the first well of the plate and serially diluted 1:2 out to 1:12,800. The microtiter plates were incubated for 2 hour at 37°C, after which the wells of the plates were washed three times with PBST. Next, 100-µl aliquots of the secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) diluted 1:10,000 in PBS were added to wells, and plates were incubated at room temperature for 1 hour. Each mouse serum was assessed in triplicate. Samples were developed and measured by techniques described above. The average intensity of the ELISA reading from the postimmune serum samples was determined after subtracting the average OD_{450} readings of the preimmune serum from the same animal. The positive controls for the anti-rPA, anti-BxpB and anti-p5303 protein ELISAs were a monoclonal anti-PA antibody (14B7, originally prepared by S.F. Little and provided by the National Naval Medical Center (138)) and affinity-purified anti-BxpB and anti-p5303 polyclonal IgGs standardized to 1 mg/ml in PBS. The negative control was protein-G

purified normal rabbit sera (NRS) that was kindly provided by Dr. Jill Czarnecki, National Naval Medical Center.

Germination assay. To examine the possibility of germination inhibition by polyclonal antibodies directed against spore proteins, 1×10^8 heat-activated spores were incubated at 37ºC for 30 minutes with 500 μg of anti-spore protein IgG prior to the measurement of germination rate. Anti-spore serum 311001-01 was used as a positive control, while protein-G purified NRS was used as a negative control. Germination efficiencies of the strains were compared by culturing spores in 5% BHI broth at 37ºC and 225 rpm, taking samples from the broth cultures at various times, and heat-inactivating the vegetative cells at 68ºC for 1 hour, as previously described (28). The heat-treated samples were then sub-cultured onto trypticase soy agar (TSA) plates to enumerate the residual heat-resistant spores. Within each experiment three samples of each strain were heat-treated and counted, and the geometric mean CFU for like samples was determined. The heat-resistant CFU over time was determined for each strain in three independent experiments.

Macrophage infection model. The interaction of spores with macrophages of the RAW264.7 cell line (American Type Culture Collection, ATCC, Rockville, MD, USA) was studied by methods detailed earlier (63,231), with modifications. Briefly, samples of 1×10^8 heat-activated spores were pre-incubated for one hour at 37ºC with 50μg of anti-spore protein IgG. Anti-spore serum 311001-01 was used as a positive control, while protein-G purified NRS was used as a negative control. After one hour, spores were diluted in serum-free DMEM to a final concentration of 5×10^6 spores/ml. The cell line

was grown in Dulbecco's Minimal Essential Medium (DMEM) with 10% fetal bovine serum (FBS, Lonza, Walkersville, MD, USA). Seed cultures were subcultured onto 6-well plates and incubated at 37ºC in air with 5% CO_2 for 3-4 days, until cells reached (0.8-1.2) × 10^6 cells/well. Cells were washed three times with PBS and infected with ~5 CFU per cell. Cells were incubated at 37ºC in air with 5% CO_2 for 45 minutes. Unphagocytosed spores were then removed by washing the wells three times with PBS. Serum-free DMEM that contained 50 μg/ml gentamicin was added, and cells were incubated at 37ºC in air with 5% CO_2 for 45 minutes to kill extracellular bacteria. The cells were washed three times with PBS and fresh serum-free DMEM was added. Cells were then incubated at 37ºC in air with 5% CO_2 for 0, 1, 4 and 18 hours, after which bacterial viability was assessed as follows. At each harvest time, cells were washed three times with PBS, and incubated for 5 minutes in 1 ml of 0.01% BSA in water to lyse macrophages. Wells were scraped with a sterile rubber syringe stopper and pipetted into tubes. Lysates were heated at 65ºC for 1 hour to kill germinated spores before 10-fold serial dilutions were prepared for colony counts on TSA plates. Data are presented as the number of spores per macrophage (cfu/macrophage) at the initial timepoint (t_0) and the percent survival of spores within macrophages at a given time point (tx) compared to a sample taken at t_0. Within each experiment three samples of each strain were heat-treated and counted, and the geometric mean CFU for like samples was determined.

Statistical evaluations. Differences in percent survival and mean-time-to-death (MTD) for mice challenged subcutaneously with Sterne spores following different immunization schemes were determined by Fisher's Exact Test and Kaplan-Meier Analysis,

respectively. Differences in antibody titers generated by immunization were determined by one-way analysis of variance (ANOVA) followed by Tukey's pairwise post-hoc comparisons. Fisher's Exact Test was used to analyze differences in outcomes for mice immunized with PA alone versus PA plus additional antigen(s) among low PA responders. To compare differences in germination rates among strains, changes in $\log_{10}$ counts of bacteria from t_0 to t_x were analyzed by a repeated measures ANOVA followed by one-way ANOVA and Tukey's pairwise post-hoc comparisons. Differences in spore uptake by macrophages and differences in percent survival of phagocytosed spores following incubation with different antibodies were compared by one-way ANOVA followed by Tukey's pairwise post-hoc comparisons.

Table 1. *B. anthracis* genes cloned and expressed in Chapter 2

Sterne locus	Ames locus	Function	ORF (bp)	Protein size (kDa)	Anti-spore reactivity	Reference
BAS0108	BA0108	Translation elongation factor Tu	1188	42.9	-	(139,182)
BAS0238	BA0252	Alanine racemase	1170	43.6	-	(139,181,182,206)
BAS0253	BA0267	GroEL	1635	57.4	-	(37,182)
BAS0340	BA0355	CotB homolog	522	19.4	-	(182)
BAS0766	BA0803	CotJC	570	21.6	-	(131,139)
BAS0767	BA0804	CotJB	276	10.9	-	(139)
BAS0768	BA0805	CotJA	216	8.4	-	(139)
BAS1130	BA1222	BclA	1203	36.8	+	(206,213)
BAS1141	BA1234	CotZ1	459	16.1	+	(139,182)
BAS1144	BA1237	BxpB	504	17.3	+	(139,182,206)
BAS1145	BA1238	CotZ2	471	16.8	-	(139,182)
BAS1378	BA1489	Fe-Mn superoxide dismutase (SOD15)	915	36.1	+	(139,181,206)
Δ1-96 BAS1378	Δ1-96 BA1489	Truncated Fe-Mn superoxide dismutase (SOD15)	627	24.6	+	(181,206)
BAS1655	BA1786	ExsE	957	35.8	-	(217)
BAS2008	BA2162	BxpA	705	27.7	-	(139,206)
BAS2138	BA2292	Hypothetical protein (p2138)	756	28.5	-	(139)
BA2150 homolog	BA2150	ExsG	153	5.4	-	(217)
BAS2174	BA2332	BxpC	399	14.4	-	(182)
BAS2377	BA2554	Hypothetical protein (p2377)	330	12	+	(139,182)
BAS2439	BA2617	ExsD	465	17.4	+	(217)
BAS2693	BA2888	Inosine-uridine preferring nucleoside	969	36.3	-	(131)
BAS2986	BA3211	Hypothetical protein (p2986)	474	17.8	-	(131)

BAS3402	BA3668	Glycosyl hydrolase, family 18	1293	48.1	-	(139)
BAS3619	BA3906	CotE	543	20.4	-	(217)
BAS3957	BA4266	Hypothetical protein (p3957)	363	13.4	+	(139)
BAS4177	BA4499	Mn superoxide dismutase (SODA1)	612	22.6	+	(139)
BAS4383	BA4722	ThiJ/PfpI family	663	24	+	(139)
BAS4544	BA4898	Small, acid-soluble spore protein B	198	6.8	-	(139)
BAS5241	BA5640	Cell wall hydrolase	423	16.1	-	(139)
BAS5242	BA5641	YwdL	438	16.2	+	(139)
BAS5303	BA5699	Hypothetical protein (p5303)	402	15	+	(139)

Table 2. PCR primers and reactions to synthesize constructs used in Chapter 2 (restriction site underlined)

Reaction: product/purpose	**Template**	**Primer**	**Primer sequence**
PCR1: 1188 bp / BAS0108 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0015	CACACACATATGGCTAAAGCTAAATTCG AACGTTCTAAACCCC
		PS0016	CACACAGGATCCTATTACTCAACGATAG TAGCAACTACACCG
PCR2: 1170 bp / BAS0238 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0108	CACACACTCGAGATGGAAGAAGCACCAT TTTATCG
		PS0109	CACACACAGATCTACTATATATCGTTCAA ATAATTAATTACTTCC
PCR3: 1635 bp / BAS0253 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0114	CACACACATATGGCAAAAGATATTAAAT TTAGTGAAGAAGCACGTCG
		PS0115	CACACAGGATCCTATTACATCATTCCGCC CATACCGCCCATGCCC
PCR4: 522 bp / BAS0340 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0049	CACACACATATGAGTTTATTTCATTGTGA TTTTTTGAAAGAC
		PS0050	CACACAGGATCCTATTATCTGCCTCTACT TGATTGTCTTTGTGC
PCR5: 570 bp / BAS0766 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0011	CACACACATATGTGGATTTATGAAAAAA AATTACAATACCC
		PS0012	CACACAGGATCCTATTAAAAATAAATCT TTCGATCGCG
PCR6: 276 bp / BAS0767 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0016	CACACACATATGACGACTGACGTGAACC AGCC
		PS0017	CACACAGGATCCTATTATATTTGCCATGG CCAAGGACC
PCR7: 216 bp / BAS0768 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0013	CACACACTCGAGATGGATAAATATATGA AATCATATGTGCC
		PS0014	CACACAGGATCCTATCACCGCCCTTTTTT ATAAGGATTTTCG
PCR8: 1203 bp / BAS1130 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0051	CACACACATATGTCAAATAATAATTATTC AAATGG
		PS0009	CACACAGGATCCTATTAAGCAACTTTTTC AATAATAATGGATGC

PCR9: 459 bp / BAS1141 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0102 PS0103	CACACACATATGAGTTGTAACGAAAATA AACACCATGGC CACACAGGATCCTATTAGATAGTAACGT CGCGTAAGC
PCR10: 504 bp / BAS1144 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	BA1237F BA1237R	CACACACATATGTTCTCTTCTGATTGCGA ATTTAC CACACACGGATCCTACTAGCTAATTTGTG CAACTGTTAATGC
PCR11: 471 bp / BAS1145 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0104 PS0105	CACACACATATGAGCTGCAATTGTAACG AAGACC CACACAGGATCCTATTAAATCGTAACAT CACGCAAGC
PCR12: 915 bp / BAS1378 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0110 PS0111	CACACACTCGAGATGAGTCAGCAAGGTG TATTTACAG CACACAGGATCCTACTAATACGGTGTCC ATTCAATTTGTTTTGCAC
PCR13: 627 bp / Δ1-96 a.a. BAS1378 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0125 PS0111	CACACACATATGGCTGTTCCAATTGGAG GACATACACTCCC CACACAGGATCCTACTAATACGGTGTCC ATTCAATTTGTTTTGCAC
PCR14: 957 bp / BAS1655 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0041 PS0042	CACACACATATGAGAACATGGCGCGTTG GAACATTTTCAATGGGGC CACACAGGATCCTATTATTTTTCGATAAC ACTCATATTTAAATTG
PCR15: 705 bp / BAS2008 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	BA2162F BA2162R	CACACACTCGAGATGTTCTATAATAATC AACCGCCTTACCC CACACAGGATCCTACTAAAACTGCTCAT TCTTCTCCCAAG
PCR16: 756 bp / BAS2138 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0017 PS0018	CACACACTCGAGATGTTCATGTCTCAATC TGAAATCGAAAAATATGG CACACAGGATCCTATTAATTTGTAAGTAT AACTGCTTCTAATTTAGGG
PCR17: 153 bp / BA2150 homolog loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0120 PS0121	CACACACATATGGAATTCCAATTGTTGGT GACTTGTATATTAC CACACAGGATCCTATTAAGAACATCTTG GTACACCTAAAGCTAAG

PCR18: 399 bp / BAS2174 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0112 PS0113	CACACACATATGTTTTTTGCAAAATTACG TGGTAGAAATGAAGTTCC CACACAGGATCCTATTAGCAATAATTCA GTTGTCCACGAATTTCACC
PCR19: 330 bp / BAS2377 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0021 PS0022	CACACACATATGGGATCTCGTTATAGTA ATTCTAGAAAAAAGTG3 CACACAGGATCCTATTATGTTAACAATG CTTCAATCGCTTC
PCR20: 465 bp / BAS2439 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0118 PS0119	CACACACATATGGCTGATTACTTTTATAA AGATGG CACACAGGATCCTATTAATTGCAGTCGT GATAATATTCATCACAGG
PCR21: 969 bp / BAS2693 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0047 PS0048	CACACACATATGAGTATAGTTAATAAGA AAATCATCTTTTTTGG CACACAGGATCCTATTATGGACAATCAG GTTTCAATATTGTCCGC
PCR22: 474 bp / BAS2986 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0039 PS0040	CACACACTCGAGATGAGGAATAACTTTC AAATCGTTGCGTTGC CACACAGGATCCTATCACACTCTATTAAC AAGGCAGTTATAGC
PCR23: 1293 bp / BAS3402 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0023 PS0024	CACACACATATGATTCAAATTGTAACGG TTCGTAGCGG CACACAGGATCCTATCAGCCCTTTTTCGT AATCGTAAAG
PCR24: 543 bp / BAS3619 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0124 PS0125	CACACACATATGTCCGAATTTAGAGAGA TTATTACAAAAGCAGTGG CACACAGGATCCTATTACTCTTCTTCTGC ATCAACGATAAAGTTTGG
PCR25: 363 bp / BAS3957 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0025 PS0026	CACACACATATGTTTGGATCATTTGGATG CTGTGATAAC CACACAGGATCCTATTAGATAGCGATCG CAACAATATCAGTTGGGC
PCR26: 612 bp / BAS4177 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0037 PS0038	CACACACATATGGCAAAACACGAATTAC CAAATTTACC CACACAGGATCCTATTATTTTGCTTCTTG GTAACGTTTTTCAGC

PCR27: 663 bp / BAS4383 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0027 PS0028	CACACACTCGAGATGTTGAAAAGGATATTGTTAGTTTCAACAAGCGC CACACAGGATCCTACTATACTTTATTTACAGCTCTTCTTATAGC
PCR28: 198 bp / BAS4544 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0029 PS0030	CACACACATATGGCACGTAGCACAAATAAATTAGCGG CACACAGGATCCTATTATTTTTGGAAACCGCCTAATTGTTGCTC
PCR29: 423 bp / BAS5241 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0031 PS0032	CACACACATATGGGCGTTATCGCGTATAACGAAGCAG CACACAGGATCCTATTAATATACGCTAGGACAATCCTCGCCAG
PCR30: 438 bp / BAS5242 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0033 PS0034	CACACACATATGGCACAGCAACAAAACCCGTATTATGG CACACAGGATCCTATTATGGTCTTGGAGCATAAGTTGC
PCR31: 402 bp / BAS5303 loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0035 PS0036	CACACACATATGCCTCTTCATTATCCACATCCCGC CACACAGGATCCTATTAATGAATGCGAACAAGTTGTTTATG

Table 3. Plasmids used or constructed in Chapter 2

Plasmid	Description	Source
pET15b	N-terminal His Tag expression vector.	Novagen
pET15b-BAS0108	BAS0108 expression vector. 1188 bp PCR fragment of BAS0108 loci in pET15b.	This study
pET15b-BAS0238	BAS0238 expression vector. 1170 bp PCR fragment of BAS0238 loci in pET15b.	This study
pET15b-BAS0253	BAS0253 expression vector. 1635 bp PCR fragment of BAS0253 loci in pET15b.	This study
pET15b-BAS0340	BAS0340 expression vector. 522 bp PCR fragment of BAS0340 loci in pET15b.	This study
pET15b-BAS0766	BAS0766 expression vector. 570 bp PCR fragment of BAS0766 loci in pET15b.	This study
pET15b-BAS0767	BAS0767 expression vector. 276 bp PCR fragment of BAS0767 loci in pET15b.	This study
pET15b-BAS0768	BAS0768 expression vector. 216 bp PCR fragment of BAS0768 loci in pET15b.	This study
pET15b-BAS1130	BAS1130 expression vector. 1203 bp PCR fragment of BAS1130 loci in pET15b.	This study
pET15b-BAS1141	BAS1141 expression vector. 459 bp PCR fragment of BAS1141 loci in pET15b.	This study
pET15b-BAS1144	BAS1144 expression vector. 504 bp PCR fragment of BAS1144 loci in pET15b.	This study
pET15b-BAS1145	BAS1145 expression vector. 471 bp PCR fragment of BAS1145 loci in pET15b.	This study
pET15b-BAS1378	BAS1378 expression vector. 915 bp PCR fragment of BAS1378 loci in pET15b.	This study
pET15b- Δ1-96 a.a. BAS1378	Δ1-96 a.a. BAS1378 expression vector. 627 bp PCR fragment of BAS1378 loci in pET15b.	This study

pET15b-BAS1655	BAS1655 expression vector. 957 bp PCR fragment of BAS1655 loci in pET15b.	This study
pET15b-BAS2008	BAS2008 expression vector. 705 bp PCR fragment of BAS2008 loci in pET15b.	This study
pET15b-BAS2138	BAS2138 expression vector. 756 bp PCR fragment of BAS2138 loci in pET15b.	This study
pET15b-BA2150 homolog	BA2150 homolog expression vector. 153 bp PCR fragment of *B. anthracis* Sterne strain's BA2150 homolog loci in pET15b.	This study
pET15b-BAS2174	BAS2174 expression vector. 399 bp PCR fragment of BAS2174 loci in pET15b.	This study
pET15b-BAS2377	BAS2377 expression vector. 330 bp PCR fragment of BAS2377 loci in pET15b.	This study
pET15b-BAS2439	BAS2439 expression vector. 465 bp PCR fragment of BAS2439 loci in pET15b.	This study
pET15b-BAS2697	BAS2697 expression vector. 444 bp PCR fragment of BAS2697loci in pET15b.	This study
pET15b-BAS2986	BAS2986 expression vector. 474 bp PCR fragment of BAS2986 loci in pET15b.	This study
pET15b-BAS3402	BAS3402 expression vector. 1293 bp PCR fragment of BAS3402 loci in pET15b.	This study
pET15b-BAS3619	BAS3619 expression vector. 543 bp PCR fragment of BAS3619 loci in pET15b.	This study
pET15b-BAS3957	BAS3957 expression vector. 363 bp PCR fragment of BAS3957 loci in pET15b.	This study
pET15b-BAS4177	BAS4177 expression vector. 612 bp PCR fragment of BAS4177 loci in pET15b.	This study

pET15b-BAS4383	BAS4383 expression vector. 663 bp PCR fragment of BAS4383 loci in pET15b.	This study
pET15b-BAS4544	BAS4544 expression vector. 198 bp PCR fragment of BAS4544 loci in pET15b.	This study
pET15b-BAS5241	BAS5241 expression vector. 423 bp PCR fragment of BAS5241 loci in pET15b.	This study
pET15b-BAS5242	BAS5242 expression vector. 438 bp PCR fragment of BAS5242 loci in pET15b.	This study
pET15b-BAS5303	BAS5303 expression vector. 402 bp PCR fragment of BAS5303 loci in pET15b.	This study

Results

Screening spore antigen candidates for recognition by anti-spore polyclonal antibody. An examination of the existing literature revealed a list of more than 30 proteins encoded by the *B. anthracis* genome for which there was proteomic evidence of localization to the exosporium (Table 1, (37,131,139,181,182,206,213,217)). Therefore, we screened this group of proteins for recognition by the anti-spore polyclonal serum 311001-01. We reasoned that such recognition by antiserum raised against whole, intact spore would suggest the presence of that antigen at or near the spore surface and warrant further investigation of that protein as a possible vaccine target. For that purpose, individual *B. anthracis* genes were cloned into the inducible expression vector pET15b and expressed in *E. coli* BL21(DE3) pLysS. Following purification of each recombinant protein via the N-terminal His-tag encoded on pET15b, proper expression was confirmed by Western blot analysis using His-Tag monoclonal antibody (data not shown). Proteins were then screened by Western blot for recognition by anti-spore serum 311001-01 (Figure 4A). Of the 31 candidates so screened, a total of 11 proteins demonstrated recognition by the anti-spore antiserum; these 11 included the exosporium component BclA (BAS1130). Data concerning BclA as a vaccine candidate were previously published (27), so BclA was not further examined as a part of this study.

Localization of spore proteins. After identifying the spore proteins that were reactive to anti spore polyclonal serum, polyclonal antiserum was prepared in rabbits against each of the recombinant proteins. Six of the proteins [CotZ1 (BAS1141), SOD15 (BAS1378),

ExsD (BAS2439), a ThiJ/PfpI family protein (BAS4383) and here-to-for hypothetical proteins p2377 (BAS2377) and p3957 (BAS3957)] demonstrated poor solubility resulting in low levels of purified protein that initially precluded efficient immunization. In the case of the superoxide dismutase SOD15 (BAS1378), solubility was improved by cloning and expressing a truncated form of the protein, minus the first 96 amino acids at the N-terminus of the protein. This Δ1-96 form of SOD15 was detected in prior proteomic analysis of the exosporium (206) and is likely the more relevant form for further study. Along with SOD15, recognition-positive candidates BxpB (BAS1144), SODA1 (BAS4177), YwdL (BAS5242), and the hypothetical protein p5303 (BAS5303) were used for rabbit immunizations. The resulting antisera, which were purified against a Protein G column to isolate the IgG fractions, were further purified by affinity to the immunization target. Affinity-purified anti-spore protein polyclonal IgGs were then used to demonstrate the presence of the target proteins in SSPE prepared from wild-type Sterne and isogenic Δ*bclA* strain spores (Fig. 4B). These Western blots demonstrated that not only were the five proteins of interest part of the whole-spore immunome, but detectable levels of each protein could be found associated with the outer structures of the spore. Furthermore, while there appeared to be a shift in the form(s) of BxpB and p5303 present on the spore in the absence of BclA, none of the proteins appeared dependent on BclA for localization to the spore exterior.

Antigens near the spore surface are partially obscured by the presence of BclA. To further examine the localization of the five spore antigens identified during the screening process, spores were incubated with antigen-specific polyclonal IgG both by ELISA

(Figure 5) and by immunoelectron microscopy (Figure 6). Recognition of the antibody targets in the context of intact Sterne spores was low when the antibodies were incubated with spore-coated ELISA plates, an observation likely attributable to the presence of BclA. The densely-packed BclA proteins that are responsible for the "hair-like nap" found on the surface of the spore (213) may cause a stearic hindrance that interferes with the recognition of targets located on the spore. However, the use of spores made from an isogenic Δ*bclA* strain that lacks the obstructive BclA demonstrated that all five proteins could in fact be recognized by the appropriate antibodies in the context of whole spores (Figure 5). Immunoelectron microscopy samples in which spores were labeled with antibody before embedding confirmed these observations, as recognition of antiserum targets was limited to Δ*bclA* spores. When antiserum staining was done after spores were first embedded and sectioned, a method that should expose epitopes buried within the structure of the spore, antigens could be identified throughout the spore. Together these data obtained by different embedding methods suggest that while the five proteins are present within the basal structures of the exosporium, they are not localized specifically to that region of the spore. One notable exception to this conclusion was the protein BxpB, a structural protein known to help anchor BclA to the exosporium (207). Whereas other proteins appeared to be present throughout the spore with no discrete localization to the exosporium, the nearly exclusive presence of BxpB at or near the surface of the spore indicated that this protein was specifically localized to the exosporium basal surface.

Select spore antigens can provide enhanced protection against spore challenge. Previous studies with BclA (27,92) demonstrated that the protective effect conferred by

immunization with formaldehyde-inactivated (FIS) spores (30) can be replicated with the use of individual spore components as immunogens in the A/J mouse model. However, these studies demonstrated that immunization with such spore antigens was not sufficient to provide protection but rather had to be used in combination with rPA. By immunizing with a single 50 ng dose of rPA, which we previously showed allows approximately 50% survival of mice after an otherwise uniformly lethal spore challenge (27), the protective effect of the spore antigen additive could be assessed by the extent of enhancement of protection beyond that afforded by rPA alone. Our first immunization scheme, which involved simultaneous immunization with 50 ng of rPA and 10 μg of recombinant spore antigen following an initial immunization with 10 μg of recombinant spore antigen alone, yielded no benefit compared to immunization with rPA alone (Table 4). This failure to protect despite the generation of a response to the spore antigens was likely due to the reduction in the anti-PA antibody response that was seen upon simultaneous immunization with a second antigen. Next, we immunized mice with a single 50 ng dose of rPA, followed two weeks later by a second immunization with 10 μg of recombinant spore protein. A preliminary experiment indicated that SOD15, SODA1 and YwdL failed to provide additional protection beyond that afforded by rPA alone (data not shown). BxpB and p5303 demonstrated positive preliminary results and were thus examined further (Table 5, Fig. 7A). The addition of single spore antigens as a second immunogen appeared to increase survival, though the improved outcomes were only statistically significant in the case of BxpB ($P = 0.022$). The improved survival for the response of Group 5 compared to the response of Group 3 corresponded to a robust humoral response generated against each spore antigen (Table 5, Fig. 7B, 7C) that was

achieved without a significant diminution in the overall response of the immunization group(s) to rPA. These anti-spore protein antibody responses actually appeared to have altered the outcomes of mice that generated poor responses to rPA (Fig. 7D). Low PA responders ($OD_{450} \leq 1.5$) that received BxpB or p5303 showed a better survival-to-death ratio (5:1, 10:3, respectively) compared to low PA responders that received no second antigen (1:9). Of note, when mice received BxpB and p5303 simultaneously as part of a second immunization the antibody response to BxpB (Fig. 7B) was diminished and added protection among low PA responders was not seen (Fig. 7A, 7D).

Anti-spore protein polyclonal IgGs do not inhibit germination. A previous report (233) indicated that polyclonal antiserum generated against whole spores is capable of reducing spore germination *in vitro*. These observations suggested that the protective benefit of FIS spores (30) may be due in part to a reduction of germination within the mouse mediated by antibodies against the spore. Given that the enhanced protection afforded by immunization with BxpB or p5303 was likely attributable to the generation of anti-BxpB or anti-p5303 antibodies, such antibody-mediated enhanced protection might be the result of an anti-germination effect. Consequently, we sought to determine whether these antibodies were capable of reducing germination *in vitro*. Spores were pre-incubated with NRS, anti-spore serum 311001-01 or antigen-specific IgG directed against individual spore-bound targets (BxpB, p5303, BclA) before incubation in 5% BHI. Only in the case of preincubation with 311001-01 was germination seen to be reduced (Fig. 8). These results indicate that any protective effect conferred by immunization with BxpB or

p5303 (or BclA, as has previously been shown (27)) was not the result of a reduction in spore germination mediated by a humoral response to the respective immunogens.

Anti-spore protein polyclonal IgG increases spore uptake and killing by macrophages. In the course of anthrax infection, macrophages are believed to serve as a site for both spore germination (53,89) and spore destruction (44,46). Therefore, aside from reducing germination, an alternative mechanism by which antibodies directed at spores might assist in the immune response to infection is by acting as opsonins that increase the uptake and perhaps killing of spores in macrophages. To test this theory, we studied the effects of anti-BxpB and anti-p5303 antibodies on the opsonization and survival of spores in RAW264.7 macrophages (Fig. 9). Spores were preincubated with NRS, anti-spore serum 311001-01, anti-BxpB, or anti-p5303 prior to incubation with macrophages. Anti-BclA was included as a control, given its previously-published (27) capacity to increase phagocytic uptake and facilitate enhanced protection in the mouse model. Not surprisingly, anti-spore serum 311001-01 showed the greatest increase in uptake compared to NRS, a rise of nearly 5-fold, followed by anti-BclA and anti-BxpB (Fig. 9A). The opsonization effect of anti-p5303 was not significant ($P = 0.272$). When killing of phagocytosed spore was examined, spores opsonized with anti-spore serum 311001-01 or anti-BxpB were consistently killed more efficiently than spores incubated with NRS alone, whereas spores incubated with anti-BclA or anti-p5303 were more readily killed only at 4 hours post-infection (Fig. 9B).

Figure 4. Detection of *B. anthracis* spore proteins by Western blot analysis.

(a) Incubation of purified His-tagged proteins (100 ng/lane) with anti-spore serum 311001-01. (b) Incubation of SSPE (5 µl/lane) prepared from Sterne or Δ*bclA* spores with affinity-purified anti-spore protein polyclonal IgG. Molecular weight standards (kDa) are shown on the left of each figure.

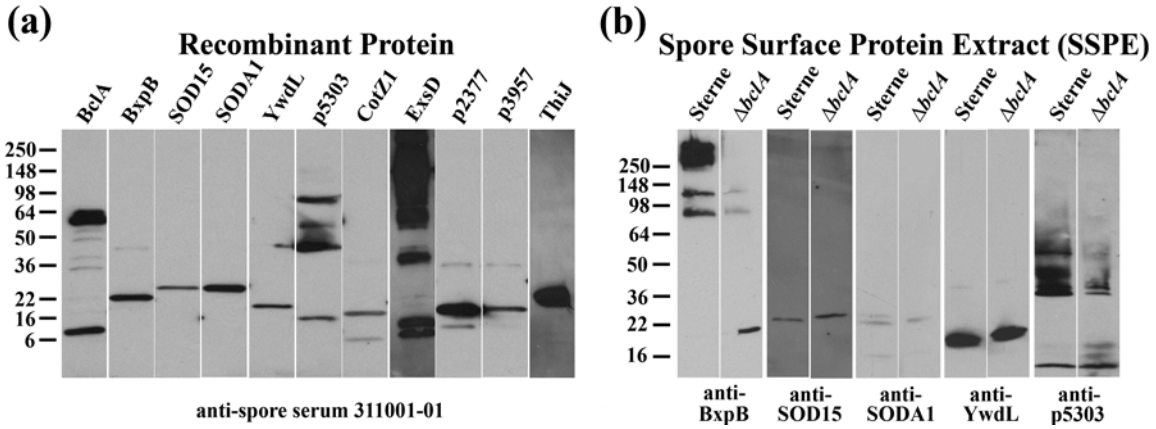

(a)
Recombinant Protein
BclA
BxpB
SOD15
SODA1
YwdL
p5303
CotZ1
ExsD
p2377
p3957
ThiJ
250
148
98
64
50
36
22
16
6
anti-spore serum 311001-01
(b)
Spore Surface Protein Extract (SSPE)
Sterne
ΔbclA
250
148
98
64
50
36
22
16
anti-BxpB
anti-SOD15
anti-SODA1
anti-YwdL
anti-p5303

Figure 5. Accessibility of *B. anthracis* surface-exposed spore proteins by ELISA. Binding of affinity-purified anti-spore protein polyclonal IgG to wells of microtiter plates coated with 1 x 10^7 cfu/well of either intact Sterne or Δ*bclA* spores was assessed by ELISA.

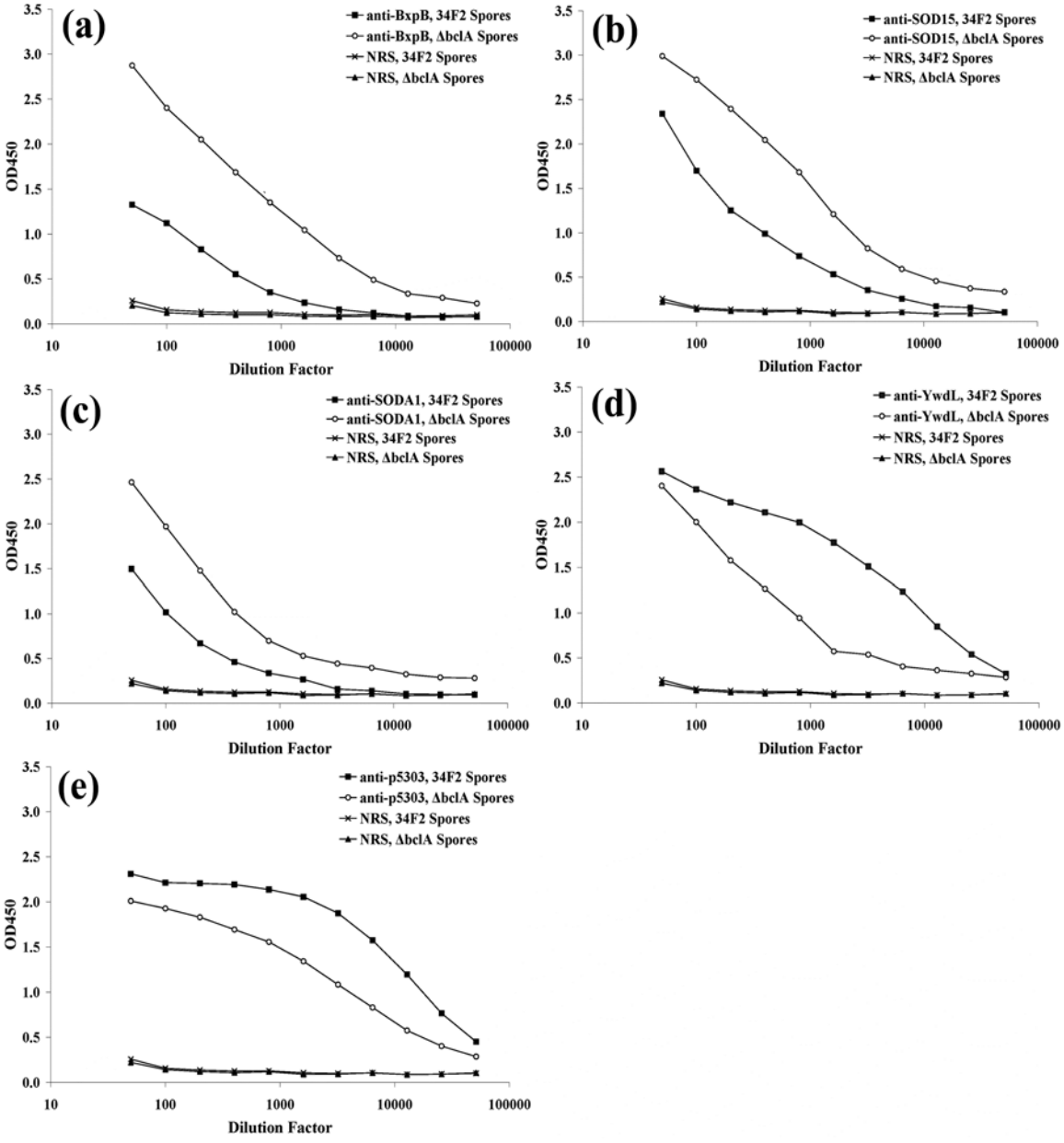

(a)
anti-BxpB, 34F2 Spores
anti-BxpB, ΔbclA Spores
NRS, 34F2 Spores
NRS, ΔbclA Spores
OD450
Dilution Factor
(b)
anti-SOD15, 34F2 Spores
anti-SOD15, ΔbclA Spores
NRS, 34F2 Spores
NRS, ΔbclA Spores
OD450
Dilution Factor
(c)
anti-SODA1, 34F2 Spores
anti-SODA1, ΔbclA Spores
NRS, 34F2 Spores
NRS, ΔbclA Spores
OD450
Dilution Factor
(d)
anti-YwdL, 34F2 Spores
anti-YwdL, ΔbclA Spores
NRS, 34F2 Spores
NRS, ΔbclA Spores
OD450
Dilution Factor
(e)
anti-p5303, 34F2 Spores
anti-p5303, ΔbclA Spores
NRS, 34F2 Spores
NRS, ΔbclA Spores
OD450
Dilution Factor

Figure 6. Localization of *B. anthracis* spore proteins within the spore by immunoelectron microscopy.

Spores were labeled with immunogold by incubation with affinity-purified anti-spore protein polyclonal IgG followed by a gold-labeled secondary antibody. Labeling occurred either before or following embedding in 4% formaldehyde in PBS (pH 7.0) for a minimum of 8 hours as described in Materials and Methods.

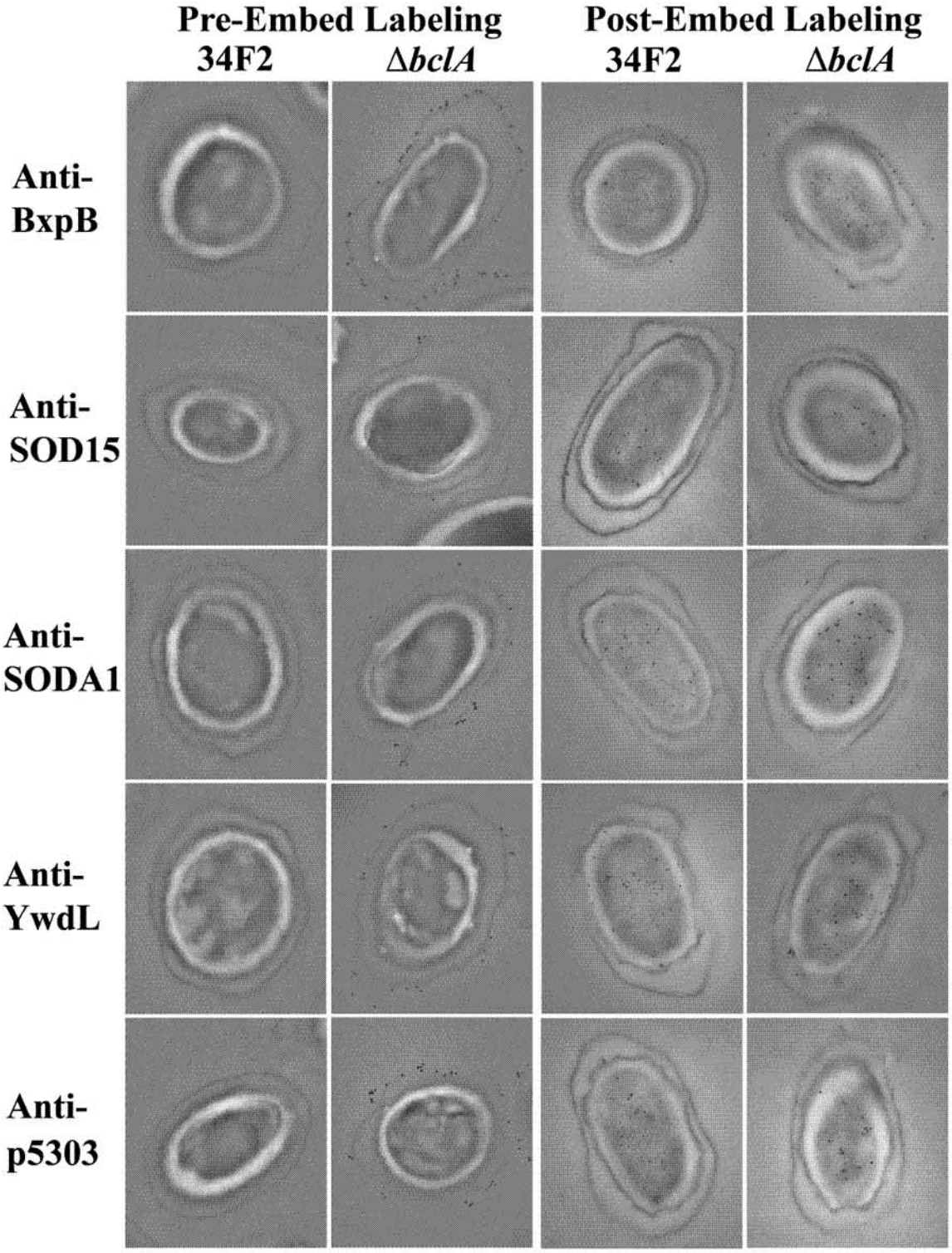

Pre-Embed Labeling
Post-Embed Labeling
34F2
ΔbclA
34F2
ΔbclA
Anti-BxpB
Anti-SOD15
Anti-SODA1
Anti-YwdL
Anti-p5303

Table 4. Survival, mean time-to-death, and serological response of mice vaccinated simultaneously with rPA plus recombinant spore protein and challenged s.c.

Group	Immunization		Survival [a]	MTD	Mean anti-PA response (OD_{450})[b]	Mean anti-spore protein response (OD_{450})[c]
	Day 1	Day 15				
1	PBS	PBS	0/5	4.2	0.07	N/A
2	PBS	PA (50 ng)	2/5	8.0	1.29*	N/A
3	CotZ1 (10 µg)	PA (50 ng) + CotZ1 (10 µg)	0/5	4.5	0.31	1.12
4	BxpB (10 µg)	PA (50 ng) + BxpB (10 µg)	0/5	5.3	0.11	2.65
5	SOD15 (10 µg)	PA (50 ng) + SOD15 (10 µg)	0/5	5.2	0.15	2.88
6	p2366 (10 µg)	PA (50 ng) + p2366 (10 µg)	0/5	4.6	0.24	0.18
7	p3957 (10 µg)	PA (50 ng) + p3957 (10 µg)	0/5	4.0	0.13	0.58
8	SODA1 (10 µg)	PA (50 ng) + SODA1 (10 µg)	0/5	4.8	0.30	3.10
9	YwdL (10 µg)	PA (50 ng) + YwdL (10 µg)	0/5	4.6	0.20	3.05
10	p5303 (10 µg)	PA (50 ng) + p5303 (10 µg)	0/5	5.8	0.19	2.94

[a] Mice were challenged on Day 28 with 10 LD_{50}s Sterne spores. The 50% lethal dose of Sterne strain is approximately 1.13×10^3 spores.

[b]Optical density measurement at 450 nm for a 1:300 antiserum dilution. OD_{450} for positive control (monoclonal anti-PA antibody) was 1.68. OD_{450} for negative control (NRS) was 0.04.

[c]Optical density measurement at 450 nm for a 1:200 antiserum dilution. OD_{450} for positive controls (polyclonal anti-spore protein IgGs) ranged from 3.23 to 3.43. OD_{450} for negative control (NRS) was 0.04.

* $P \leq 0.002$ compared to all other groups

Table 5. Serological responses for mice immunized sequentially with PA then BxpB and/or p5303 prior to s.c. spore challenge.

Group	Immunization		Mean anti-PA response (OD_{450})[a]	Mean anti-BxpB response (OD_{450})[b]	Mean anti-p5303 response (OD_{450})[c]
	Day 1	Day 15			
1	PBS	PBS	NR	NR	NR
2	PA (50ng)	PBS	1.64	NR	NR
3	PA (50ng)	BxpB (10µg)	1.79	2.23	NR
4	PA (50ng)	p5303 (10µg)	1.41	NR	3.16
5	PA (50ng)	BxpB (10µg) + p5303 (10µg)	1.54	0.99*	3.07

[a] Optical density measurement at 450 nm for a 1:400 antiserum dilution. OD_{450} for positive control (monoclonal anti-PA antibody) was 1.65. OD_{450} for negative control (NRS) was 0.03.

[b] Optical density measurement at 450 nm for a 1:800 antiserum dilution. OD_{450} for positive control (polyclonal anti-BxpB) was 3.45. OD_{450} for negative control (NRS) was 0.03.

[c] Optical density measurement at 450 nm for a 1:3200 antiserum dilution. OD_{450} for positive control (polyclonal anti-p5303) was 3.24. OD_{450} for negative control (NRS) was 0.02.

NR = no response ($OD_{450} \leq 0.15$)

* $P < 0.001$

Figure 7. BxpB provides enhanced protection against subcutaneous spore challenge.

(a) A/J mice were sequentially injected i.p. on Day 1 with 50 ng of rPA and on Day 15 with either PBS (□) or 10 μg of recombinant spore proteins BxpB (○), p5303 (▲), or BxpB+p5303 (×). Control mice (♦) received PBS on both Day 1 and Day 15. The mice were challenged subcutaneously on Day 29 with 10 LD_{50}'s of Sterne spores. The animals were then monitored for survival for 14 days after challenge. * Mice that received 10 ug of BxpB demonstrated enhanced survival (14 of 15 mice) compared to mice receiving 50 ng of rPA only (11 of 20 mice)($P = 0.022$). # Mice that received 10 ug of p5303 alone (17 of 20 mice) or 10 ug of both BxpB and p5303 (14 of 20) did not demonstrate statistically-significant enhanced survival ($P \geq 0.082$). (b-d) Serological responses were tested by incubating Day 28 serum samples (b) at a 1:800 dilution on microtiter plates coated with BxpB, (c) at a 1:3200 dilution on microtiter plates coated with p5303, or (d) at a 1:400 dilution on microtiter plates coated with rPA. ELISA titers are from mice that survived (●) or died (□) following s.c. challenge. Lines indicate one standard deviation from the mean serological response for the group. * Survival among mice with a low ($OD450 \leq 1.5$) anti-PA response was significantly greater when mice were boosted with BxpB or p5303 as compared to mice receiving only rPA ($P \leq 0.0022$).

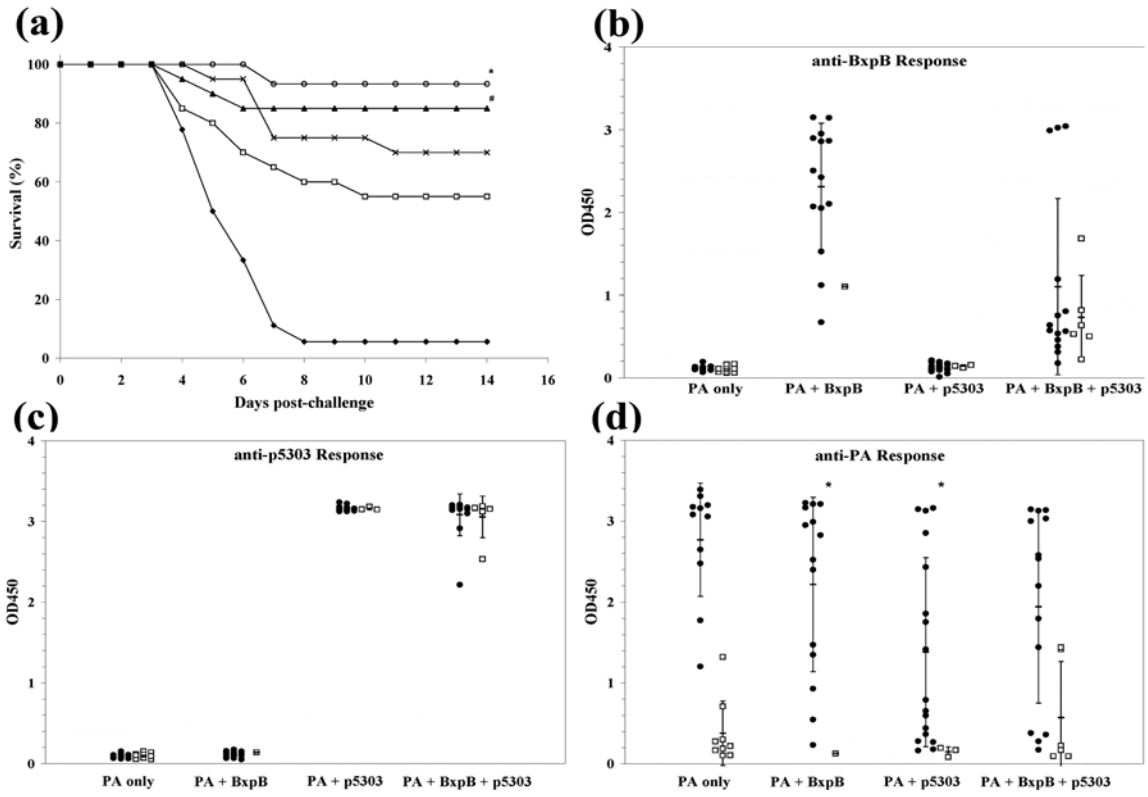

(a)
Survival (%)
Days post-challenge
(b)
anti-BxpB Response
OD450
PA only
PA + BxpB
PA + p5303
PA + BxpB + p5303
(c)
anti-p5303 Response
OD450
PA only
PA + BxpB
PA + p5303
PA + BxpB + p5303
(d)
anti-PA Response
OD450
PA only
PA + BxpB
PA + p5303
PA + BxpB + p5303

Figure 8. Antibodies against individual spore proteins do not block germination. Heat-inactivated Sterne spores (1×10^8) were pre-incubated with anti-spore serum 311001-01 (□), anti-BclA (○), anti-BxpB (▲), anti-p5303 (×), or NRS (♦) before inoculation of 5% BHI broth. Colony counts of heat-treated samples that were taken from the cultures at various time points (t_x) were plotted as $\log_{10}$ cfu against each time point. Error bars represent ± 1 standard deviation from three separate experiments. Colony counts following heat-treatment represent ungerminated spores. * The level of germination of spores pre-incubated with 311001-01 was statistically lower than wild type at 15 minutes ($P = 0.048$) and 30 minutes ($P = 0.019$).

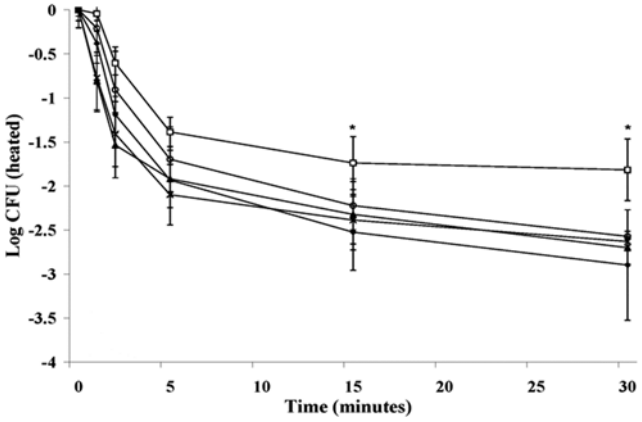
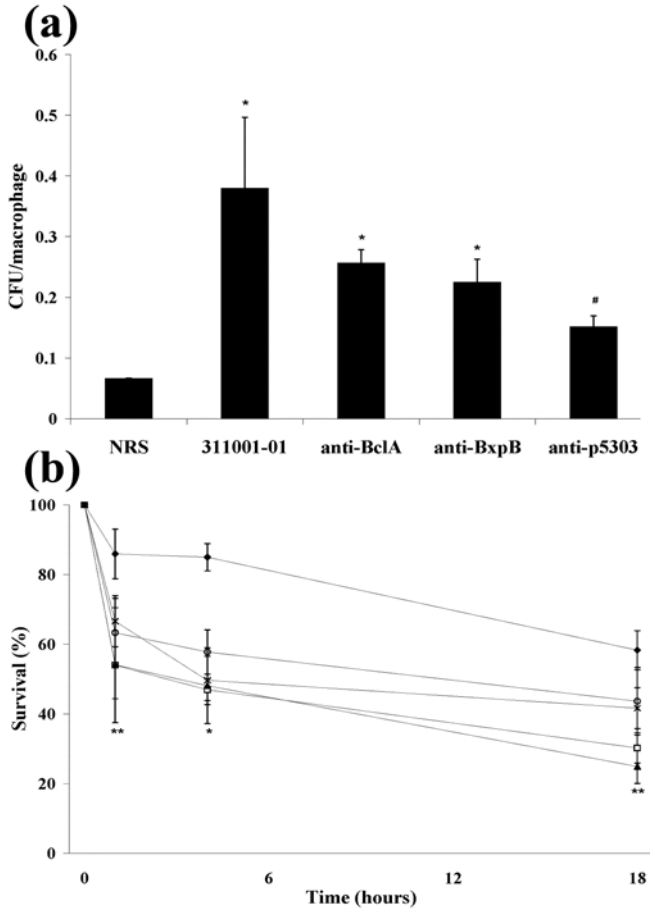

0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
Log CFU (heated)
0
5
10
15
20
25
30
Time (minutes)
*
*

Figure 9. Antisera against individual spore proteins enhance phagocytosis and killing of spores.

Heat-inactivated Sterne spores (1×10^8) were pre-incubated with anti-spore serum 311001-01, anti-BclA, anti-BxpB, anti-p5303, or NRS then diluted to 5×10^6 cfu/ml and added to $(0.8\text{-}1.2) \times 10^6$ RAW264.7 cells at ~5 cfu/cell. Colony counts following heat-treatment represent ungerminated spores. (a) Opsonization of Sterne spores at time 0. * Phagocytosis of spores was significantly greater after treatment with anti-spore serum 311001-01, anti-BclA, or anti-BxpB than with NRS ($p<0.001$). # Phagocytosis of spores was not significantly greater after treatment with anti-p5303 ($p=0.272$). (b) Intracellular survival of spores after treatment with anti-spore serum 311001-01 (□), anti-BclA (○), anti-BxpB (▲), anti-p5303 (×), or NRS (♦).* Pre-incubation of spores with 311001-01, anti-BclA, anti-BxpB or anti-p5303 lead to increased killing inside macrophages after 4 hours compared to pre-incubation with NRS ($P \leq 0.004$). ** Pre-incubation of spores with anti-spore pAb and anti-BxpB led to increased macrophage killing ($P \leq 0.002$) at 1 and 18 hours. Pre-incubation with anti-BclA or anti-p5303 does not lead to a significant increase in killing ($P \geq 0.058$) at 1 and 18 hours.


(a)
CFU/macrophage
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
#
NRS
311001-01
anti-BclA
anti-BxpB
anti-p5303
(b)
Survival (%)
100
80
60
40
20
0
**
*
**
0
6
12
18
Time (hours)

Discussion

In this study, we sought to identify spore antigens capable of enhancing the protective immune response conferred by recombinant protective antigen (rPA) against anthrax in an A/J mouse model. *B. anthracis* proteins for which there was evidence of exosporium localization were individually cloned, expressed in recombinant form, and screened for recognition by rabbit polyclonal antiserum generated against inactivated whole spores (311001-01). Recognition-positive candidates were shown to be present on the basal surface of the exosporium, where they were partially obscured by the presence of the immunodominant antigen BclA. Two of these antigens, the exosporium structural component BxpB and a previously hypothetical protein p5303 for which there is no assigned putative function, enhanced the survival of mice previously immunized with suboptimal amounts of rPA. The mechanism of enhanced protection afforded by anti-BxpB or anti-p5303 IgG was likely revealed by the observations that while these antibodies failed to reduce spore germination they did enhance the uptake of spores by RAW264.6 macrophages as well as the subsequent killing of phagocytosed spores. In the case of both enhanced protection and enhanced phagocytic uptake, the effects seen in targeting p5303 were intriguing but not statistically significant.

A growing body of evidence suggests that spores and spore antigens are potential contributors to a protective immune response against *B. anthracis* challenge. Attenuated live spore vaccines are capable of generating antibodies against the spore body as well as the toxins they elaborate upon germination (42,137,199,234), thereby eliciting a multi-dimensional immune response against different phases of anthrax infection. The

existence of a live spore anthrax vaccine for humans, developed by scientists in the former Soviet Union, provides an ultimate confirmation of the utility of this concept (199). Vaccines based upon inactivated spores, which are incapable of germinating and expressing toxin, can achieve similar responses when combined with the administration of exogenous PA (30,63,82). However, concerns about safety preclude the use of viable spores in future vaccines. Therefore, any spore-based vaccine strategies that seek to recapitulate the protective benefit of whole spore immunization will require the use of acellular formulations comprised of spore subcomponents.

While the anti-PA antibodies generated by the current AVA vaccine are thought to confer protection primarily via prevention of anthrax intoxication, conflicting reports exist regarding the anti-spore capacity of antibodies directed against PA. Certain studies indicate a role for anti-PA antibodies in facilitating phagocytosis and inhibiting germination (45,231), while other studies fail to see such effects (27). On the other hand, the protective benefit of an anti-spore immune response is presumably dependent on an impairment of spore-centric steps essential to the onset of infection. Recent studies employing a variety of approaches suggest that by targeting individual proteins localized to the spore it is possible to achieve a delay in the onset of disease and/or protection against infection (27,45,92,231-233). Antibodies directed against spore components hamper germination (27,45,63,232,233), increase phagocytic uptake (43,45,232) and/or accelerate phagocyte-mediated spore destruction (45,231,232). Each of these actions would result in the effective reduction of the spore inoculum and could delay the establishment of infection. Combined with a spore-induced activation of the cell-mediated immune response that leads to heightened host immune defenses (82), a delay

in vegetative outgrowth would allow the host to limit or eliminate infection prior to the successful elaboration of essential anthrax virulence factors. The use of BclA as a partially-protective immunogen in prior studies (27,92) served as a proof of concept for this strategy. The capacity of BxpB, and to a lesser extent p5303, to provide partial protection against anthrax spore challenge provides a further demonstration of the principle.

Antibodies to BxpB and p5303 enhanced phagocytic uptake of spores despite the fact that we concluded that each antigen was localized beneath the "hair-like" structures of BclA. Indeed, immunoelectron microscopy with anti-BxpB and anti-p5303 antibodies (Fig. 5B) would argue for the complete obscuration of these antigens by the projection of BclA from the surface of the exosporium, a presence that might sterically hinder access of antibodies to the structures below. However, recognition of BxpB and p5303 by antigen-specific antibodies on intact wild type spores in an ELISA format (Fig. 5A), as well as by antiserum generated against whole spores (Fig. 4), support the notion that antibodies might successfully bind to spore targets despite the presence of BclA. The most likely explanation for the contradiction in these data involves the techniques involved. Specifically, while the labeling with anti-BxpB and anti-p5303 antibodies for immunoelectron microscopy occurred against intact spores prior to embedding, and therefore should reflect the results seen in the ELISA protocol, the subsequent thin-sectioning of spores actually resulted in visualization of only of a single ring around the surface of the spore. Such a small "sampling" of the spore surface is more likely to miss targets present in low concentrations across the entirety of the spore than an ELISA directed against the whole spore body. Additionally, whereas the ELISA data suggest

that the presence of BclA only partially obstructs antibody access, the additional size of the immunogold particle conjugated to antibodies used in the electron microscopy study could have reduced the likelihood of recognizing targets on the basal surface of the exosporium in the presence of BclA. Furthermore, recent evidence that a spore antigen localized to the spore cortex plays a role in antibody-directed spore opsonization (43) supports the contention that the spore ultrastructure possesses dynamic characteristics even before the onset of germination. Taken together, these observations suggest that the question of antigen visibility in the presence of BclA is more complex that one might first intuit.

Despite the protective benefits of immunization against spore components, the potentially-lethal outcome of intoxication attributable to those spores that succeed in establishing infection necessitates the inclusion of PA in any anthrax vaccine formulation. Given that a sufficiently high dose of PA alone is adequate to protect A/J mice against challenge with Sterne strain spores, we assessed the protective benefits of BxpB and p5303 by immunizing with suboptimal doses of rPA. By this strategy, we were able to discern that BxpB and p5303 enhanced protection only when administered subsequent to PA, and not when given on the same schedule as PA. In fact, simultaneous immunization of rPA and spore antigens actually reduced protection (Table 2), an outcome undoubtedly related to the diminished antibody response to PA seen in the group that received simultaneous immunization when compared to the group that received rPA alone. This dampening of the anti-PA response is likely due to the difference in the relative amounts of rPA (50 ng) and spore antigen (10 μg) administered. Such a disparate ratio of antigen may have induced “antigenic competition”, a

phenomenon that has been observed previously with combinatorial vaccines and which can result in the diminution of the humoral response generated against one or more of the immunogens administered (16,48,192). Given the essentiality of an anti-PA antibody response in effective protection, interference with this response caused by the disproportionate presence of a second immunogen abrogated any potential added benefit of the spore antigens. When spore antigens were instead used as a second immunogen and enhancement was seen, the deleterious effect on the anti-PA response was not observed (Table 5). In fact, an examination of the anti-PA responses among mice in the different immunization groups (Fig. 7D) suggests that the presence of antibodies against spore antigens helped to protect individual mice that developed comparatively low anti-PA responses. That is, an immune response to spore components that confers the benefits discussed above appears to lower the threshold for the response to PA that is necessary to provide protection from intoxication. Given mouse-to-mouse variations in the antibody response generated upon immunization with rPA, the inclusion of spore components as vaccine additives protected mice that would otherwise have been susceptible upon infection. Alternative immunization regimens, to include varying the relative amounts of rPA and spore antigen in a simultaneous administration or administering simultaneously at different sites, might overcome the interference seen in our first attempt, thereby alleviating the need for sequential immunization.

The primary limitation of this study involves the use of the attenuated, non-encapsulated *Bacillus anthracis* Sterne strain, and the C5 deficient A/J mouse strain (214). The absence of the pXO2-encoded antiphagocytic capsule, a virulence factor that is expressed only in the vegetative stage and likely has no bearing on the mechanism of

an immune response generated against the spore form of the bacteria, necessitates the use of an inbred mouse strain with an increases susceptibility to infection (235). The compromised immune response of A/J mice makes the strain an imperfect model system, and perhaps partially explains the interference in the humoral response generated to antigens administered simultaneously. Indeed, subsequent vaccine studies with PA and spore antigens might be best undertaken with the fully-virulence Ames strain and applicable mouse models. Nevertheless, we contend that the previously described strategy of reducing the effective infectious dose and providing partial protection through the targeting of spore antigens (27,45,92,231-233) was strengthened by the work reported here with the A/J mouse model system.

While prospective anthrax vaccines must always address the need to protect against intoxication, a multivalent vaccine with antigens expressed in various stages of anthrax pathogenesis offers the possibility of improvement over the current PA-based formulation. A vaccine targeting multiple surface-expressed antigens in conjunction with PA provides a strategy analogous to the acellular pertussis vaccine formulation, which elicits an immune response both to the pertussis toxin and to an array of additional bacterial proteins. Given the dual life stages of *B. anthracis* seen by the host during infection, this strategy might incorporate immunogenic antigens from both the infectious spore and the vegetative bacillus. In this investigation we identified a pair of spore antigens capable of inducing a partially protective humoral immune response. A combination of these and other recently-identified spore-bound immunogens may provide viable candidates for a future multi-stage anthrax vaccine.

CHAPTER 3: FOUR SUPEROXIDE DISMUTASES CONTRIBUTE TO *BACILLUS ANTHRACIS* VIRULENCE AND PROVIDE SPORES WITH REDUNDANT PROTECTION FROM OXIDATIVE STRESS

Submitted for publication as: Robert J. Cybulski Jr., Patrick Sanz, Farhang Alem, Scott Stibitz, Robert L. Bull and Alison D. O'Brien. 2008. Four superoxide dismutases contribute to *Bacillus anthracis* virulence and provide spores with redundant protection from oxidative stress. Infection and Immunity. XX(X):XX-XX.

Note: The figures and tables shown reflect the work of Robert Cybulski, with several exceptions. Drs. Scott Stibitz and Patrick Sanz performed the cloning and construction of the plasmid constructs listed in Tables 2-3. Dr. Robert Bull generated the polyclonal antibodies specific to SOD15 and SODA1. Mr. Farhang Alem assisted with several experiments. Additionally, Drs. Sanz and O'Brien contributed to the experimental design and interpretation of the data as well as preparation of the manuscript.

ABSTRACT

The *Bacillus anthracis* genome encodes four superoxide dismutases (SODs), enzymes capable of detoxifying oxygen radicals. That two of these SODs, SOD15 and SODA1, are present in the outer-most layers of the *B. anthracis* spore, is indicated by previous proteomic analyses of the exosporium. Given the requirement that spores must survive interactions with reactive oxygen species generated by cells such as macrophages during infection, we hypothesized that SOD15 and SODA1 protect the spore from oxidative stress and contribute to the pathogenicity of *B. anthracis.* To test these theories, we constructed a double knockout (Δ*sod15*Δ*sodA1*) of *B. anthracis* Sterne strain 34F2 and assessed its lethality in an A/J mouse intranasal infection model. The 50% lethal dose of Δ*sod15*Δ*sodA1* was similar to that of wild-type (34F2), but, surprisingly, measurable whole spore SOD activity was greater than 34F2. A quadruple knockout strain (Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2*) was then generated, and, as anticipated, spore-associated SOD activity was diminished. Moreover, the quadruple knockout strain, when compared to wild-type, was attenuated more than 40-fold upon intranasal challenge of mice. Spore resistance to exogenously-generated oxidative stress and to macrophage-mediated killing correlated with virulence in A/J mice. Allelic exchange that restored *sod15* and *sodA1* to their wild-type state restored wild-type characteristics. We conclude that SOD molecules within the spore afford *B .anthracis* protection against oxidative stress and enhance the pathogenicity of *B. anthracis* in the lung. We also surmise that the presence of four SOD alleles within the genome provides functional redundancy for this key enzyme.

INTRODUCTION

Bacillus anthracis, the etiological agent of anthrax, is a Gram-positive, nonmotile, spore-forming, rod-shaped, facultatively anaerobic bacterium. The versatility provided by sporulation and facultative vegetative growth demands that *B. anthracis* be equipped to protect both life stages from oxygen radicals, such as those encountered by spores in the environment and those generated endogenously in vegetative cells during the course of aerobic vegetative growth. Furthermore, as a pathogen, *B. anthracis* confronts hostile conditions during infection, such as the oxidative burst of professional phagocytes that take up spores and the varying oxidative environments encountered by vegetative cells. Antioxidant enzymes such as superoxide dismutases, catalases and peroxidases are primary defense mechanisms utilized by bacteria for preventing oxidative damage (211), and are all present in multiple copies in the *B. anthracis* genome. Along with the small acid-soluble proteins (SASPs) that protect spore-encased DNA from oxygen radicals and other forms of stress (195), some or all of these enzymes may play a role in combating the stresses faced by of the two distinct lifecycles of *B. anthracis*. Here we investigate the contribution made by spore-bound SODs to *B. anthracis* oxidative stress defenses.

Superoxide dismutases (SODs) are enzymes that catalyze the dismutation of the reactive oxygen species (ROS) superoxide anion ($O_2^{\cdot -}$) to hydrogen peroxide and molecular oxygen (146). These enzymes effectively scavenge $O_2^{\cdot -}$ anions before they are able to cause cellular damage, either directly or through the generation of more reactive species such as hydroxyl radical or peroxynitrite (65). SOD was first discovered and characterized in the 1960s by McCord and Fridovich (146). Since their discovery,

SODs have been found in almost all aerobes studied, as well as many anaerobes. SODs have been shown to exhibit high levels of conservation and to fall within three main structural classes based upon metal specificity: copper-zinc, manganese or iron, or nickel. Whereas the documented distribution of nickel SODs within the bacterial community is thus far relatively limited (162), many bacterial species possess SODs of both Cu-Zn and Mn/Fe classes, perhaps due to specialized roles filled by each class. The fact that superoxide anions do not cross nonpolar lipid membranes, and the observation that Cu-Zn SODs typically localize to the periplasm whereas Mn/Fe SODs localize to the cytoplasm, suggest that different SODs have distinct roles in combating exogenously- and endogenously-produced oxygen radicals, respectively (142,208). SODs of each class have been implicated as contributors to virulence in multiple pathogens, including *Salmonella enterica* serovar Typhimurium (66,68), *Mycobacterium tuberculosis* (62,168), *Staphylococcus aureus* (114), *Streptococcus agalactiae* (177), *Francisella tularensis* (12), *Neisseria meningitidis* (236), *Brucella abortus* (79) and *Enterococcus faecalis* (225). A common element in the infectious course of many of these pathogens is that they are capable of surviving interactions with ROS-generating phagocytic cells such as macrophages (10,49,173).

The *B. anthracis* genome contains four genes that encode proteins with conserved SOD domains, including two of putative manganese specificity [BAS4177 (*sodA1*) and BAS5300 (*sodA2*)], one of putative copper-zinc specificity [BAS4777 (*sodC*)], and one of likely iron and/or manganese specificity [BAS1378 (*sod15*)] (164,180). Amino acid sequence identity between the manganese SODs is approximately 54%, with 75% similarity, whereas comparisons between the manganese-bound SODA1 and the iron-

bound SOD15 indicate an identity of 45% and a similarity of 60%. A 135 amino acid stretch at the N-terminus of SOD15 distinguishes the protein from other *B. anthracis* SODs, and BLAST searches do not yield clues regarding possible roles for this unique domain beyond a likely helical secondary structure. Inter-class comparisons between the Mn/Fe SODs and the Cu-Zn SODC indicate comparatively little homology, though short regions of similarity do exist.

Previous studies (139,206) indicated that two SODs, SOD15 and SODA1, are present within the *B. anthracis* exosporium. This redundancy suggests an important role for SODs in the spore, possibly in defending against the macrophage, and thus contributing to *B. anthracis* virulence. Passalacqua *et al*.. (164) constructed single deletions of each of the four SOD genes, and demonstrated that none of the deletions led to significant attenuation of *B. anthracis* when DBA/2 mice were infected via the intratracheal route, though the deletion of *sodA1* did lead to reduced growth under oxidative stress and increased sensitivity to endogenously-produced superoxide anion in the vegetative state. This report did not investigate SOD activity on the surface of the spore, or sensitivity to oxidative stress among spores made from deletion strains. Recently, studies with related *Bacillus* species report the sensitivity of spores to high oxidative stress of multiple forms (50,51,186,241). These observations raise the possibility that multiple chromosomally-encoded SODs provide redundant and combinatorial protection of the spore from oxidative stress and suggest that the protective role for SODs might be fully apparent only upon the deletion of multiple *sod* genes from the *Bacillus* genome. Because of this possibility, we constructed a *B. anthracis* Sterne strain 34F2 with multiple *sod* deletions to assess the importance of SODs in *B. anthracis*

pathogenicity. We found that spores of a *Bacillus* strain in which all four SOD genes were deleted exhibited enhanced sensitivity to agents of oxidative stress, reduced survival in macrophages, and attenuation on intranasal challenge of mice.

MATERIALS AND METHODS

Bacterial strains. *B. anthracis* Sterne strain (toxigenic, unencapsulated, Naval Medical Research Center, Silver Spring, MD) and *sod* gene deletion strains listed in Table 6 were used for *in vitro* and *in vivo* experiments.

Preparation of recombinant proteins. Our procedures for the construction of a recombinant *Escherichia coli* strain that expressed recombinant SOD15, SODA1, SODC, SODA2, BxpB or BclA with an N-terminal six-histidine tag and for purification of that protein by nickel affinity chromatography are described in detail elsewhere (28). Briefly, genomic DNA was extracted from *B. anthracis* strains with the Easy-DNA kit (Invitrogen, Carlsbad, CA, USA). NdeI-BamHI or XhoI-BamHI fragments that contained each gene of interest were amplified from the Sterne genome by polymerase chain reactions (PCR) using the Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, IN, USA) in a PTC200 Peltier Thermal Cycler (MJ Research, Bio-Rad, Hercules, CA, USA). Primer sets designed from the flanking sequences of the targeted gene [National Center for Biotechnology Information (NCBI) at website http://www.ncbi.nlm.nih.gov.lrc1.usuhs.edu/entrez/viewer.fcgi?db=nucleotide&val=AE0

17225] are listed in Table 7. PCR products were purified with the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA 91355, USA), and the DNA fragments were ligated into the expression vector pET15b (Novagen, San Diego, CA). The resulting expression plasmids are listed in Table 8. DNA sequences were verified with the ABI Prism Big Dye method (Applied Biosystems, Forest City, CA, USA) by the Biomedical Instrumentation Center at the Uniformed Services University, then recombinant plasmids were transformed into *E. coli* BL21(DE3) pLysS according to the pET system manual (Novagen, San Diego, CA). His-tagged proteins were expressed from transformants and subjected to His-Trap nickel affinity column chromatography with the AKTA fast protein liquid chromatography system (GE Healthcare, Piscataway, NJ).

Immune sera and antibody preparation. Polyclonal antibodies were generated against each recombinant SOD by methods previously described (28). Briefly, rabbits were vaccinated monthly with 50 μg of purified recombinant protein in Freund's complete adjuvant for the first inoculation and Freund's incomplete adjuvant for all subsequent immunizations. Production bleeds were taken and analyzed for specific antibodies beginning at month 5, with final bleeds taken at month 12. Immunoglobulin G was purified from each final serum bleed by passage of the sample over a protein G column (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined by a microtiter BCA assay (Pierce Biotechnology, Rockford, IL).

Construction of the SOD knockout and restored strains. Construction of the Δ*bclA* deletion strain was described previously (28). Deletion constructs of the *sod15, sodA1,*

sodC and *sodA2* genes suitable for allelic exchange were created by cloning two NotI-XmaI DNA fragments that contained locus-specific flanking regions into the NotI site of pBKJ236 (109). These fragments were created by PCR with primers listed in Table 7. Ligation of the two fragments created a precise deletion of the respective open reading frame from the start to the stop codon, inclusive, with an XmaI site generated at the site of the locus. A complete list of plasmid constructs created and used in this study is provided in Table 8. The resulting constructs were used to create *sod* null mutants in *B. anthracis* Sterne strain 34F2 by an allelic exchange procedure described previously (109). In brief, the pBKJ236-based plasmid constructs were transferred into strain 34F2 by conjugation, and integrants were isolated by a shift to a replication-nonpermissive temperature followed by growth at the replication-nonpermissive temperature with continuous selection for erythromycin resistance. A second plasmid, pSS4332, was then introduced into each integrant by conjugative transfer and selection for kanamycin resistance. The pSS4332 plasmid, which represents a modification to previously published methods (109) and performs the functions of the previously-described pBKJ233, mediates cleavage within the chromosomally-inserted pBKJ236-derived construct, an event that stimulates recombination with loss of the integrated plasmid and gene replacement in a portion of the erythromycin-sensitive candidates. The resulting knockout strains are summarized in Table 6. The absence of the appropriate SOD loci in the various knockout constructs was demonstrated by PCR (as shown in Figure 2) with primers as listed in Table 7. These primers were designed to bind to sequences flanking the region included in each deletion construct described above.

Strains hereafter referred to as “restorants” were constructed with the 34F2Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* quadruple knockout strain as the parent and represent restoration of *sod* genes by allelic exchange. Constructs of the intact *sod15* and *sodA1* loci suitable for allelic exchange were created by the generation of DNA fragments that contained the intact locus, inclusive of flanking regions, from *B. anthracis* Sterne strain 34F2 by PCR with primers listed in Table 7. Each PCR-amplified fragment was then cloned into the NotI site of pBKJ236. The allelic exchange was then conducted in two sequential steps as described above for generation of knockout strains. The restoration of the two *sod* loci was demonstrated by PCR (Figure 2) with primers listed in Table 7.

Generation and purification of B. anthracis spores. Single colonies of *B. anthracis* Sterne strain 34F2 or mutant strains were taken from brain heart infusion (BHI) agar plates and inoculated into BHI broth for culture overnight at 37°C. Each culture was spread onto modified germination (G) medium (120) agar plates (0.2% yeast extract, 0.2% (NH4)2SO_4, 1.5% Bacto agar, 0.0025% $CaCl_2$ dihydrate, 0.05% K2HPO_4, 0.02% $MgSO_4$ heptahydrate, 0.005% $MnSO_4$ quatrahydrate, 0.0005% $ZnSO_4$ dihydrate, 0.0005% $CuSO_4$ pentahydrate, 0.00005% FeSO4 heptahydrate). The plates were incubated at 30°C for 10 to 14 days in the dark. Colonies scraped from the surface of the agar were re-suspended in distilled water, washed twice in distilled water, and heat-treated at 65°C for 1 hour to kill any viable vegetative cells. Purification of spores was done with 58% (vol/vol) Renografin (Renocal-76 diluted in dH_2O; Bracco Diagnostics, Princeton, N.J., USA). Spores were layered onto the 58% Renografin and centrifuged at 6,000 g for 60 min in a swinging bucket rotor. The sedimented spores were washed twice with distilled water.

After the final sedimentation, the spores were re-suspended in distilled water to yield a final concentration of 10^9 - 10^{10} colony-forming units (cfu)/ml, as determined by vegetative outgrowth on BHI plates.

Preparation of spore surface protein extract (SSPE). Analysis of the proteins contained within the exosporium was achieved by removal of the exosporium from whole spores via chemical extraction according to a method previously described (8). Briefly, 0.5 ml of heat-activated spores (10^9-10^{10} cfu/ml) were pelleted at 7000 g for 15 minutes at 4°C. Spores were washed twice with water and resuspended in 0.5 ml of extraction buffer that contained 0.1M DTT, 0.1M NaCl, 0.5% SDS, pH 10. Samples were incubated in a 37°C shaking water bath for 2.5 hours and pelleted, and the resulting supernatant was collected. Supernatants were then filtered through a 0.2 μm Corning filter and dialyzed against PBS.

Western blot analysis of SSPE. The presence of individual SODs within SSPE generated from various *B. anthracis* strains was detected by Western Blot analysis. 20 μl of SSPE was loaded onto and separated on 4-20% Tris-Glycine sodium dodecyl sulfate (SDS) polyacrylamide gels (Invitrogen, Carlsbad, CA), transferred onto Optitran BA-S 83 reinforced nitrocellulose (Whatman GmbH, Dassel, GE), and blocked overnight at 4ºC in PBST (PBS with 0.5% Tween-20) with 5% nonfat dry milk (PBSTM). Purified His-tagged recombinant proteins were used as positive controls for antibody recognition, with 80 ng of SOD15, 4 ng of SODA1, 100 ng of SODA2, 25 ng of SODC or 100 ng of BclA loaded where appropriate. The nitrocellulose was then incubated with polyclonal anti-

spore protein IgG diluted 1:500 in PBSTM for 1 hour at room temperature. Following three, 5 minute washes at room temperature with PBST, the blot was incubated for 1 hour at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) diluted 1:10,000 in PBST. Blot were then washed 3 times for 5 minutes each in PBST, and developed with Lumigen PS-3 Acridan (GE Healthcare UK, Buchinghamshire, UK) and Kodak BioMax XAR film (Kodak, Rochester, NY).

Characterization of the *B. anthracis sod* mutants and restored strains. Vegetative growth patterns of the parent *B. anthracis* Sterne strain, knockout strains and restorant strains were determined by inoculation of BHI with overnight cultures and measurement of the optical density (600nm) at various times over eight to ten hours. For all strains starting inocula registered an OD_{600} between 0.065 and 0.070, values that corresponded to between 1.00×10^7 and 1.25×10^7 cfu/ml. These data are available in Table 9. The growth rate of each strain was measured in three independent experiments. Alternatively, 5×10^8 heat-inactivated spores of each strain were inoculated into 73 ml of pre-heated modified G medium in a 250 ml Erlenmeyer flask. This inoculation yielded a starting OD_{600} between 0.040 and 0.051, readings that corresponded to between 3.11×10^6 and 3.90×10^6 cfu/ml. These data are also presented in Table 9. The cultures were then grown at 37°C with shaking at 250 rpm, and the optical density (600 nm) was measured at various times over eight to ten hours. The growth rate of each strain was measured in three independent experiments. Germination efficiencies of the strains were compared by culturing spores in 5% BHI broth, taking samples from the broth cultures at various times, and heat-inactivating the vegetative cells at 68°C for 1 hour, as previously

described (28). The heat-treated broth cultures were then sub-cultured onto BHI agar to enumerate the residual heat-resistant spores. The decline in heat-resistant CFU over time was tested for each strain in three independent experiments. Within each experiment three samples of each strain were heat-treated and counted, and the geometric mean CFU for like samples was determined. For each of these assays, error bars represent variation among three like samples at each time point ±1 standard deviation.

Measurement of SOD activity *in vitro*. The superoxide dismutase activity of each of the four recombinant SODs or recombinant spores was determined with the SOD Assay Kit-WST (Dojindo Molecular Technologies, Gaithersburg, MD, USA) (55). In this assay, superoxide anions generated from oxidation of xanthine by xanthine oxidase (XO) react with colorless water-soluble tetrazolium salt (WST) to generate a yellow compound, WST-1 formazan (observed spectrophotometrically at 450 nm). This reaction is inhibited in a competitive fashion when superoxide anions are degraded by active SOD. We measured SOD activity as the percent inhibition of WST-1 formazan formation when 2 μg of recombinant SOD protein or between 1.0×10^8 and 1.0×10^9 heat-activated spores in a total volume of 30 μl were mixed with 200 μl of WST working solution (containing WST and xanthine) and 20 μl of XO working solution. Samples were incubated for 20 minutes at 37ºC. To measure the SOD activity associated with whole spores, samples were clarified by centrifugation at 13,200g for 5 min at 4ºC, and the OD_{450} of supernatant was determined. The percent inhibition of the reaction was calculated by comparing the values to positive (no oxidation of WST in the absence of xanthine oxidase) and negative (no inhibition of WST oxidation in the absence of sample or the use of an irrelevant

protein sample) controls. Percent inhibition values were then converted to units of activity from a standard curve (Figure 10A) that was generated from a recombinant SOD of known specific activity (manganese-containing *E.coli* superoxide dismutase, Sigma-Aldrich, St. Louis, MO). For this mathematical conversion, an equation that describes the steady-state region of the standard curve (i.e., the slope of the dashed line depicted in Figure 1A) was used. Results represent the mean of three assays, and the error bars indicate ±1 standard deviation.

Test of the inhibitory effect of anti-SOD antibodies on spore-associated SOD activity. The impact of polyclonal anti-SOD antibodies on spore-associated SOD activity was evaluated by means of the SOD Assay Kit-WST. For that purpose, samples of 2.5×10^8 heat-activated spores were sedimented by centrifugation and resuspended in 50 µl of PBS that contained 100 µg of appropriate anti-SOD polyclonal IgG. Spore::antibody mixtures were incubated for 24 hours at 4ºC while shaking. Spores were then washed once in PBS to remove unbound antibody and suspended in a final volume of 30 µl. SOD activity was determined with the kit as described above.

Assessment of sensitivity of vegetative bacilli to endogenously-generated oxidative stress. The superoxide dismutase activity of vegetative bacilli was determined by an agar plate assay as previously described (164), with modifications. Briefly, 200µl aliquots of 2.5×10^7 cfu/ml heat-activated spores were added to 2.8 ml of 0.7% sterile soft BHI agar. The 3 ml suspension was then spread onto BHI plates. Sterile paper disks (6 mm in diameter) permeated with 10 µl of 5% paraquat (Ultra Scientific PST-740)

dissolved in water were placed atop the solidified soft agar layer. Paraquat is a commercially-used herbicide which interacts with the redox pathway of respiring cells and undergoes cyclic reduction-oxidation, a process which generates superoxide anion as a byproduct. Given that superoxide anions do not diffuse across lipid membranes, the use of paraquat enables an assessment of the level of SOD protection available within vegetative bacilli. The plates were then incubated overnight at 37ºC. Sensitivity of bacilli to endogenous superoxide anions generated from paraquat was measured as area (in mm^2) of growth inhibition around the disk. For each experiment 10 disks (2 disks per plate) were used.

Evaluation of spore sensitivity to exogenously-generated oxidative stress. Spore survival in the presence of exogenously-generated superoxide anion was tested by methods previously described (79). Briefly, heat-activated spores were washed twice with PBS (pH 7.5) and resuspended at a final concentration of 5×10^6 cfu/ml in PBS with xanthine at a final concentration of 1mM, along with 0.5 units of xanthine oxidase and 1000 units of catalase dissolved in 50mM potassium phosphate, pH 7.5. Spores were incubated at 37ºC, and spore viability assessed at 0, 15, 30 and 60 minutes by colony counts of serial ten-fold dilutions of samples plated on BHI agar. Results were expressed as percent spore survival measured by comparing spore counts at a given time (t_x) with those present at the initial time (t_0).

Macrophage infection model. The interaction of spores with macrophages of the RAW264.7 cell line (American Type Culture Collection, ATCC, Rockville, MD, USA)

was studied by methods detailed earlier (113,231), with modifications. Briefly, the cell line was grown in Dulbecco's Minimal Essential Medium (DMEM) with 10% fetal bovine serum (FBS, Lonza, Walkersville, MD, USA). Seed cultures were subcultured onto 6-well plates and incubated at 37ºC in air with 5% CO_2 for 3-4 days, until cells reached a density of (0.8-1.2) $\times 10^6$ cells/well. Cells were washed three times with PBS and infected at 5×10^6 cfu/well with spores suspended in serum-free DMEM that contained 100ng/ml phorbol 12-myristate 13-acetate (PMA) (38). Cells were incubated at 37ºC in air with 5% CO_2 for 45 minutes. Unphagocytosed spores were then removed by washing the wells three times with PBS. Serum-free DMEM that contained 100ng/ml PMA and 50 μg/ml gentamicin was added to each well, and cells were incubated at 37ºC in air with 5% CO_2 for 45 minutes to kill extracellular bacteria. The cells were then washed three times with PBS and fresh serum-free DMEM was added. Cells were incubated at 37ºC in air with 5% CO_2 for 0, 2, 4 and 6 h, after which bacterial viability was assessed as follows. At each harvest time, cells were washed three times with PBS and incubated for 5 minutes in 1 ml of 0.01% BSA in water to lyse macrophages. Wells were scraped with a sterile rubber syringe stopper and pipetted into tubes. Lysates were heated at 65ºC for 1 hour to kill germinated spores before 10-fold serial dilutions were prepared for colony counts on trypticase soy agar plates. Viability of the bacteria was expressed as percent survival at a given time point (t_x) compared to a sample taken at an initial time point (t_0).

Mouse challenge model. The 50% lethal doses (LD_{50}s) of 34F2, mutant strain and restorant strain spores were determined for both the intranasal and subcutaneous routes of

infection. For intranasal spore challenge, mice were sedated with isoflorane with an XGI-8 Gas Anesthesia System (Xenogen Corporation). Heat-activated spore suspensions of varying concentrations (see below) in 25 µl were placed on the nares of anesthetized mice, and the mice were held upright until the suspension was inhaled (191). Treatment groups of 10 mice received doses of spores that ranged from ~10^3 to 10^6 for 34F2 spores and ~10^3 to 10^7 for mutant and restorant strain spores. For subcutaneous spore challenge, 200 µl of each dose of heat-activated spores in suspension was delivered through a tuberculin syringe fitted with a 29-guage needle into the loose skin beneath the right foreleg of the mouse. Treatment groups of 10 mice for 34F2 challenge and 5 mice for mutant and restorant strain challenges received doses ranging from ~10^2 to 10^5 spores.

Statistical evaluations. The vegetative growth curves of the wild type and mutant strains were generated by plotting the average outcome (OD_{600}) of three experiments per strain. SOD activity differences were analyzed by one-way ANOVA followed by Tukey's pairwise post-hoc comparisons. Differences in spore germination rates or percent survival of spores exposed to exogenously-generated oxygen radicals or macrophages were appraised by repeated measures ANOVA followed by one-way ANOVA and Tukey's pairwise post-hoc comparisons. The LD_{50} of each selected strain used in these experiments, along with 95% confidence limits, was calculated by probit analysis. Differences in survival and mean-time-to-death (MTD) for an intranasal challenge at 10^6 spores were calculated by Fisher's Exact Test and Kaplan-Meier analysis, respectively.

Table 6. Bacterial strains used or constructed in Chapter 3.

Strain Name	**Description**	**Shorthand Name**	**Source**
34F2	Wild-type *Bacillus anthracis*	34F2	Sterne (1937)
ΔBclA	34F2Δ*bclA*	ΔBclA	(28)
RJC010	34F2Δ*sod15*	Δ*15*	This study
RJC020	34F2Δ*sodA1*	Δ*A1*	This study
RJC050	34F2Δ*sod15*Δ*sodA1*	Δ*15*Δ*A1*	This study
RJC060	34F2Δ*sod15*Δ*sodA1*Δ*sodC*	Δ*15*Δ*A1*Δ*A2*	This study
RJC070	34F2Δ*sod15*Δ*sodA1*Δ*sodA2*	Δ*15*Δ*A1*Δ*C*	This study
RJC080	34F2Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2*	Δ*15*Δ*A1*Δ*A2*Δ*C*	This study
RJC011	34F2Δ*bclA*Δ*sod15*	Δ*bclA*Δ*15*	This study
RJC021	34F2Δ*bclA*Δ*sodA1*	Δ*bclA*Δ*A1*	This study
RJC051	34F2Δ*bclA*Δ*sod15*Δ*sodA1*	Δ*bclA*Δ*15*Δ*A1*	This study
RJC110	34F2Δ*sodA1*Δ*sodC*Δ*sodA2*	Res15	This study
RJC120	34F2Δ*sod15*Δ*sodC*Δ*sodA2*	ResA1	This study
RJC150	34F2Δ*sodC*Δ*sodA2*	Res15,A1	This study

Table 7. PCR primers and reactions used in Chapter 3. Restriction sites underlined.

Reaction: product/purpose	**Template**	**Primer**	**Primer sequence**
PCR1: 624 bp / truncated *sod15* (a.a. 97-304) loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS125	CACACACATATGGCTGTTCCAATTGGAG GACATACACTCCC
		PS110	CACACAGGATCCTACTAATACGGTGTCC ATTCAATTTGTTTTGCAC
PCR2: 612 bp / *sodA1* loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS037	CACACACATATGGCAAAACACGAATTAC CAAATTTACC
		PS038	CACACAGGATCCTATTATTTTGCTTCTTG GTAACGTTTTTCAGC
PCR3: 540 bp / *sodC* loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	RC075	CACACACATATGAAAAAACGGCTTTTTT TCAGTTGTTGTTTACTATTTC
		RC076	CACACAGGATCCTTACTTTTTCTTCATAT CCGACGCTTTTTTTACAAT
PCR4: 627 bp / *sodA2* loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	RC078	CACACAGGATCCCTAATGTTTTTGTGATT GAATTGCTTGTAAATACTTTC
		RC079	CACACACTCGAGTCTTCATTTCAATTGCC AAAACTTTCATATGAC
PCR5: 504 bp / *bxpB* loci for cloning into pET15b	*B. anthracis* Sterne genomic DNA	PS0102	CACACA<u>CATATG</u>AGTTGTAACGAAAATA AACACCATGGC
		PS0103	CACACA<u>GGATCC</u>TATTAGATAGTAACGT CGCGTAAGC
PCR6: 617 bp / 5' flanking region of *sod15* loci for pSS4091 K.O. construct	*B. anthracis* Sterne genomic DNA	1489Los	CGC<u>GCGGCCGC</u>ATGGTTGAGAATCAAAC AGAACAAG
		1489Lis	GCG<u>CCCGGG</u>ACCTTGCTGACTCATGTTA CACCTCCTT

PCR7: 619 bp / 3' flanking region of *sod15* loci for pSS4091 K.O. construct	*B. anthracis* Sterne genomic DNA	1489Ros	GCGGCGGCCGCAAACCATGTGGGTTTGAAAAGTGAGTA
		1489Ris	GCGCCCGGGGAATGGACACCGTATTAGACGCAGGTT
PCR8: 622 bp / 5' flanking region of *sodA1* loci for pSS4093 K.O. construct	*B. anthracis* Sterne genomic DNA	4499Los	CGCGCGGCCGCCCAATCATGCCAGAGAAAGATGTAAG
		4499Lis	GCGCCCGGGTACCAAGAAGCAAAATAATTGTAATA
PCR9: 619 bp / 3' flanking region of *sodA1* loci for pSS4093 K.O. construct	*B. anthracis* Sterne genomic DNA	4499Ros	GCGGCGGCCGCATCTTTAGGCGAATATACGCTTTTG
		4499Ris	GCGCCCGGGTTCGTGTTTTGCCATTGTTATATTC
PCR10: 516 bp / 5' flanking region of *sodC* loci for pSS4095 K.O. construct	*B. anthracis* Sterne genomic DNA	5139DSos	CGCGGATCCTGAAATTCGATTCTATATAGAAGAT
		5139DSis	CTTCCCGGGGATATGAAGAAAAAGTAAAAGCTAC
PCR11: 518 bp / 3' flanking region of *sodC* loci for pSS4095 K.O. construct	*B. anthracis* Sterne genomic DNA	5139USos	CGCGCGGCCGCTGTAACTTCGCCAATGTATTTTCCT
		5139USis	ATCCCCGGGAAGCCGTTTTTTCATTTCATTCCCT
PCR12: 517 bp / 5' flanking region of *sodA2* loci for pSS4097 K.O. construct	*B. anthracis* Sterne genomic DNA	5696DSos	CGCGGATCCAGAAAACGATTATACGAAAAGGTTA
		5696DSis	CAACCCGGGCAATCACAAAAACATTAGTCGTGCGTAT
PCR13: 519 bp / 3' flanking region of *sodA2* loci for pSS4097 K.O. construct	*B. anthracis* Sterne genomic DNA	5696USos	CGCGCGGCCGCTATACACCTTCCTCATCGTTTACTT
		5696USis	TTGCCCGGGTTGAAATGAAGACATGACATTCCCT

PCR14: 2103 bp / *sod15* loci plus flanking regions for pSS4092 reversion construct	*B. anthracis* Sterne genomic DNA	1489Ros 1489Los	GCGGCGGCCGCAAACCATGTGGGTTTGAAAAGTGAGTA CGCGCGGCCGCCCAATCATGCCAGAGAAAGATGTAAG
PCR15: 1800 bp / *sodA1* loci plus flanking regions for pSS4092 reversion construct	*B. anthracis* Sterne genomic DNA	4499Ros 4499Los	CGCGCGGCCGCCCAATCATGCCAGAGAAAGATGTAAG GCGGCGGCCGCATCTTTAGGCGAATATACGCTTTTG
PCR16: 1522 bp / *sodC* loci plus flanking regions for pSS4094 reversion construct	*B. anthracis* Sterne genomic DNA	5139DSos 5139USos	CGCGGATCCTGAAATTCGATTCTATATAGAAGAT CGCGCGGCCGCTGTAACTTCGCCAATGTATTTTCCT
PCR17: 1611 bp / *sodA2* loci plus flanking regions for pSS4094 reversion construct	*B. anthracis* Sterne genomic DNA	5696USos 5696DSos	CGCGCGGCCGCTATACACCTTCCTCATCGTTTACTT CGCGGATCCAGAAAACGATTATACGAAAAGGTTA
PCR18: 3172 bp / confirmation of wild-type length *sod15* loci	*B. anthracis* Sterne genomic DNA	RC0060 RC0061	GGTTATGCGGGATATGAGTTAGAG GACTGCCATCGCTACCCATACGAG
PCR19: 2872 bp / confirmation of wild-type length *sodA1* loci	*B. anthracis* Sterne genomic DNA	RC0058 RC0059	GTCATCGATCCATACGGAACGAG GTGTAGCTGTAAGCAGTTAACG

PCR20: 1622 bp / confirmation of wild-type length *sodC* loci	*B. anthracis* Sterne genomic DNA	RC0071 RC0072	GCGATGCAAAGTGCCTATACAGAATG GTTATGTAAGTCACCTTGCACCGGATG
PCR21: 1682 bp / confirmation of wild-type length *sodA2* loci	*B. anthracis* Sterne genomic DNA	RC0073 RC0074	CACTTTTCCGTCGTTCGTTGCATA CATTCCCAGTCGTTGCAGTTCGT
PCR22: 2257 bp / confirmation of knockout length *sod15* loci	*B. anthracis* Sterne genomic DNA	RC0060 RC0061	GGTTATGCGGGATATGAGTTAGAG GACTGCCATCGCTACCCATACGAG
PCR23: 2338 bp / confirmation of knockout length *sodA1* loci	*B. anthracis* Sterne genomic DNA	RC0058 RC0059	GTCATCGATCCATACGGAACGAG GTGTAGCTGTAAGCAGTTAACG
PCR24: 1082 bp / confirmation of knockout length *sodC* loci	*B. anthracis* Sterne genomic DNA	RC0071 RC0072	GCGATGCAAAGTGCCTATACAGAATG GTTATGTAAGTCACCTTGCACCGGATG
PCR25: 1055 bp / confirmation of knockout length *sodA2* loci	*B. anthracis* Sterne genomic DNA	RC0073 RC0074	CACTTTTCCGTCGTTCGTTGCATA CATTCCCAGTCGTTGCAGTTCGT

Table 8. Plasmids used or constructed in Chapter 3.

Plasmid	Description	Source/Reference
pET15b-*sod15*	*sod15* expression vector. 617 bp PCR fragment of *sod15* loci in pET15b.	This study
pSS1827	Conjugation facilitator plasmid	(22)
pET15b-*sodA1*	*sodA1* expression vector. 540 bp PCR fragment of *sodA1* loci in pET15b.	This study
pBKJ236	Integration plasmid backbone	(22)
pSS4223	Recombination facilitator plasmid	Unpublished results
pET15b-*sodC*	*sodC* expression vector. 612 bp PCR fragment of *sodC* loci in pET15b.	This study
pSS4091	*sod15* knockout plasmid. 1194 bp PCR fragment of *sod15* flanking regions in pBKJ236.	This study
pET15b-*sodA2*	*sodA2* expression vector. 534 bp PCR fragment of *sodA2* loci in pET15b.	This study
pSS4093	*sodA1* knockout plasmid. 1194 bp PCR fragment of *sodA1* flanking regions in pBKJ236.	This study
pET15b-*bxpB*	*bxpB* expression vector. 504 bp PCR fragment of *bxpB* loci in pET15b.	This study
pSS4095	*sodC* knockout plasmid. 988 bp PCR fragment of *sodC* flanking regions in pBKJ236.	This study
pSS4097	*sodA2* knockout plasmid. 990 bp PCR fragment of *sodA2* flanking regions in pBKJ236.	This study
pSS4092	*sod15* reversion plasmid. 2103 bp PCR fragment of *sod15* plus flanking regions in pBKJ236.	This study
pSS4094	*sodA1* reversion plasmid. 1800 bp PCR fragment of *sodA2* plus flanking regions in pBKJ236.	This study
pSS4096	*sodC* reversion plasmid. 1522 bp PCR fragment of *sodC* plus flanking regions in pBKJ236.	This study
pSS4098	*sodA2* reversion plasmid. 1611 bp PCR fragment of *sodA2* plus flanking regions in pBKJ236.	This study
pET15b	N-terminal His Tag expression vector.	Novagen
pET15b-*sod15*	*sod15* expression vector. 617 bp PCR fragment	This study

Table 9. Comparisons of optical density (OD_{600}) and corresponding colony-forming units (CFUs) in starting inocula taken from overnight cultures or heat-inactivated spores

	O/N Culture			Spores		
	Avg OD_{600}	**Avg cfu/ml**	**Ratio**	**Avg OD_{600}**	**Avg cfu/ml**	**Ratio**
34F2	0.065	8.22×10^5	1.27×10^7	0.049	3.90×10^6	7.91×10^7
Δ15ΔA1	0.068	8.56×10^5	1.26×10^7	0.040	3.11×10^6	7.83×10^7
Δ15ΔA1ΔA2ΔC	0.067	6.89×10^5	1.03×10^7	0.046	3.73×10^6	8.05×10^7
Res15,A1	0.067	7.00×10^5	1.05×10^7	0.051	3.90×10^6	7.70×10^7

RESULTS

Detection of SOD activity within the spore structure. Heat-inactivated whole spores of the *B. anthracis* Sterne strain 34F2 were tested for the presence of SOD activity by a commercially-available *in vitro* assay that spectrophotometrically measures the dismutation of superoxide anions. A standard curve (Fig. 10A) generated using a well-characterized *E.coli* SOD of known specific activity allowed the values generated by this assay to be converted into units of enzymatic activity (55). Using these methods spore-associated SOD activity was detectable (Fig. 10B), as was activity associated with recombinant forms of each of the four SODs encoded by the *B. anthracis* genome (data not shown). While expression of these recombinant proteins in an artificial system makes it difficult to ensure proper folding and metallation and therefore to make statements about relative levels of activity, confirmation of some level of activity above background in the presence of each recombinant form indicates that each protein is in fact a functional SOD. In combination with the detection of spore-associated SOD activity and previous data that localized SODs to the *B. anthracis* spore (139,206), these data support the assertion that SOD15 and SODA1 are active within the spore.

We next tested whether polyclonal IgG raised against SOD15 and SODA1 could inhibit the activity of SODs on the surface of the wild-type 34F2 spores and spores of an isogenic *bclA* mutant from which the outer hair-like nap was no longer expressed [see ref (28) for a description of the properties of that strain]. Although these anti-SOD antibodies had no effect on SOD activity found on the surface of wild-type 34F2 spores, most likely due to the barrier effect of BclA on the outer surface of the exosporium, they

were capable of reducing activity in the presence of Δ*bclA* spores (Fig. 10C). Moreover, the inhibitory effect of each individual anti-SOD antibody was additive, as indicated by the greater total inhibition noted on simultaneous incubation of Δ*bclA* spores with both anti-SOD15 and anti-SODA1 IgG. These effects were specific, since antibodies against BxpB [a major component of the exosporium, exposed in the absence of BclA (47)] did not affect spore-associated SOD activity. These data agree with ELISA and immunoelectron microscopy results indicating that anti-SOD antibodies bind specifically to the surface of whole spores in the absence, but not in the presence, of BclA (47), and suggest that the SOD activity associated with the spore is primarily attributable to the spore-bound SOD protein that is localized to the outer layers of the spore and is available for recognition and inhibition.

A *B. anthracis* Δ*sod15*Δ*sodA1* mutant is unattenuated in an A/J mouse model. To examine the contribution of spore-associated SOD activity to the virulence of *B. anthracis*, a Δ*sod15*Δ*sodA1* double mutant with markerless deletions of *sod15* and *sodA1* was generated via homologous recombination by a modified version of a previously described method (109) (Fig. 11). This technique replaced the open reading frame in question with an XmaI site (CCCGGG) and would not be expected to exert polarity on downstream genes. Mutants were compared to wild type for defects in growth from either overnight cultures or heat-inactivated spores (Fig. 12A and 12B). The Δ*sod15*Δ*sodA1* mutant replicated like the wild-type strain 34F2 from an overnight culture but showed a slight delay in vegetative cell outgrowth from spores. We speculated that this delay in bacilli growth from Δ*sod15*Δ*sodA1* spores might be attributable to a delay in

germination of those spores. We then tested this hypothesis and found that the Δ*sod15*Δ*sodA1* mutant spores did in fact germinate more slowly than did 34F2 spores (Fig. 12C). Additionally, growth of the Δ*sod15*Δ*sodA1* mutant was more sensitive to oxidative stress, as shown by increased zones of inhibition surrounding disks impregnated with the redox cycling compound paraquat when compared to 34F2 (Fig. 13A). This increased sensitivity of vegetative cells to endogenously-produced superoxide anion is consistent with the idea that SODA1 is the predominant protective enzyme during aerobic growth, as has been previously reported (164). However, spores made from the Δ*sod15*Δ*sodA1* mutant unexpectedly showed increased levels of SOD activity when compared to 34F2 spores (Fig. 13B). Analysis of single Δ*sod15* and Δ*sodA1* spores suggested that the increase in activity was primarily attributable to the deletion of *sod15*, as Δ*sod15* showed an increase in activity similar to the double knockout whereas Δ*sodA1* possessed resembled wild-type levels of activity (data not shown). Evaluation of the virulence of the Δ*sod15*Δ*sodA1* mutant in the Sterne strain-sensitive A/J mouse model indicated no attenuation when compared to wild-type as assessed by both intranasal and subcutaneous LD_{50} values in spore-challenged A/J mice (Table 10, Figure 14).

Demonstration of functional redundancy among SOD genes in the *B. anthracis* genome. Western blot analyses of the spore-surface protein extracts (SSPE) taken from the 34F2 and Δ*sod15*Δ*sodA1* strains indicated that, upon deletion of *sod15* and *sodA1*, at least one of the remaining SODs (SODA2) encoded by the *B. anthracis* genome was incorporated into the spore (Fig. 15). To better understand the nature of the compensation in spore-

associated SOD activity seen with Δ*sod15*Δ*sodA1*, triple and quadruple deletion mutations were generated from the Δ*sod15*Δ*sodA1* mutant background (Fig. 11). Analysis of the various deletion strains indicated that whereas deletion of *sod15* and *sodA1* results in the incorporation of SODA2 into the spore, SODC is incorporated at a detectable level only when all three other *sod* genes have been (Fig. 15). Further analysis demonstrated that each genotype possessed a different amount of spore-bound SOD activity (Fig. 13B). Only upon removal of all four *sod* genes from the genome was all detectable SOD cleared from the SSPE (Fig. 15), with a corresponding complete elimination of spore-associated SOD activity (Fig. 13B). In addition to being without spore-bound SOD activity, the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* strain displayed a delay in spore germination, as well as a delay in vegetative outgrowth both from heat-inactivated spores and overnight culture (Fig. 12). Furthermore, vegetative bacilli of the quadruple knockout strain showed greater sensitivity to paraquat than did the 34F2 bacilli (Fig. 14A), although the increase in the area of inhibition beyond that of Δ*sod15*Δ*sodA1* was not statistically significant.

Attenuation of Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* in the mouse model of intranasal infection.

Upon successful elimination of all spore-bound SOD activity, we attempted to assess the importance of SOD's to *B. anthracis* virulence by challenging A/J mice with Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* spores (191). Mice were infected by both the subcutaneous and intranasal routes, to examine possible differences in the relative importance of oxidative stress resistance for each infectious route (Table 10). Subcutaneous infection revealed a non-significant difference in LD_{50} between 34F2 (1.13

$\times 10^3$ cfu) and Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* (5.04×10^3 cfu) of less than 5-fold, whereas intranasal challenge showed a variation between 34F2 (5.57×10^4 cfu) and Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* (2.51×10^6 cfu) of more than 40-fold. While Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* appeared to be attenuated by both routes, only the difference in LD_{50}s seen by intranasal inoculation was statistically significant ($P < 0.005$) as assessed by probit analyses.

Enhanced susceptibility of *B. anthracis* Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* spores to exogenous oxidative stress. The attenuation of the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* strain in mice is suggestive of a role for spore-associated SODs in *B. anthracis* pathogenesis. The most obvious role for SODs bound within the spore structure would be to protect against sources of oxidative stress during the early stages of infection. To better understand the relationship between spore-associated SOD activity and resistance to oxidative stress, spores were subjected to exposure to exogenously-generated superoxide anions (Fig. 16). These superoxide anions were supplied from the oxidation of the compound xanthine by xanthine oxidase. The Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* mutant spores displayed greater sensitivity to oxidative stress than did 34F2 spores, as demonstrated by the decreased percent survival over time (Fig. 16A). The Δ*sod15*Δ*sodA1* mutant spores, which previously demonstrated increased SOD activity compared to 34F2, showed a degree of resistance no different from that of 34F2.

Macrophages kill Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* spores more rapidly than they kill 34F2 spores. Given the observed differences in sensitivity to artificially-generated exogenous

oxidative stress, and the potential role of reactive oxygen species as contributors to the destruction of *B. anthracis* in macrophages, we next investigated comparative levels of sensitivity to macrophage killing. 34F2 and *sod* mutant spores were tested for their ability to survive phagocytosis by RAW 264.7 macrophages. As shown in Fig. 17, phagocytosed Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* spores were more sensitive to killing by macrophages than either 34F2 or Δ*sod15*Δ*sodA1* spores. The increased level of SOD activity resident in Δ*sod15*Δ*sodA1* spores in comparison to 34F2 spores did not translate into greater survival, as spores of both strains were killed at the same rate.

Restoration of wild-type SOD activity and virulence by restoration of *sod15* and *sodA1*. In order to further demonstrate that the phenotypes associated with Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* were directly related to the absence of SODs in the spore, we next attempted to restore *sod15* and *sodA1* to the quadruple knockout strain. The "restorants" were generated in a Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* background by the same markerless replacement techniques used in the *sod* gene deletion strategy (outlined in Materials and Methods). The *sod15*$^{+}$*sodA1*$^{+}$ double restorant (Res15,A1) grew as well as did 34F2, either from overnight culture or from heat-inactivated spores (Fig. 12A and 12B). The growth kinetics following inoculation of heat-inactivated spores into growth media suggested that any initial delay in germination (Fig. 12C) was overcome at later time points and did not significantly hinder vegetative outgrowth. Additionally, Res15,A1 demonstrated wild-type levels of SOD activity, both in the vegetative bacilli (Fig. 13A) and on the surface of the spore (Fig. 13B). The LD_{50} of spores of the double restorant introduced both subcutaneously and intranasally into A/J mice was equivalent to

34F2 (Table 10), as was the kinetics of infection after an intranasal inoculum of ~10^6 spores (Fig. 14). Finally, *in vitro* assessments of the sensitivity of Res15,A1 to oxidative stress (Fig. 16) and macrophage killing (Fig. 17) indicated that the restoration of *sod15* and *sodA1* enabled levels of resistance similar to that of 34F2.

Figure 10. Demonstration of spore-associated SOD activity.

(A.) SOD activity was measured spectrophotometrically with the Dojindo SOD Activity Kit-WST. Activity in that kit is expressed as percent inhibition of the xanthine oxidase mediated conversion of colorless WST-1 to yellow WST-formazan, as described in Materials and Methods. We then used an *E.coli* manganese SOD (eSOD) of known specific activity to generate a standard curve from which to convert percent inhibition to units of SOD activity. For this mathematical conversion, an equation that describes the steady-state region of the standard curve (i.e., the slope of the dashed line depicted in the figure) was used and is presented. (B.) Spore-associated SOD activity was assessed using 2.5×10^9 or 2.5×10^8 34F2 spores. eSOD of known specific activity used as a positive control. An irrelevant *B. anthracis* exosporium protein (2µg of His-tagged, purified BxpB) was included as a negative control. (C.) 34F2 and Δ*bclA* spore-bound SOD activity (% inhibition) in the presence of anti-SOD or control (anti-BxpB) antibodies. * Incubation with α-SOD15 plus α-SODA1 pAbs yielded a statistically significant decline in measurable SOD activity ($p = 0.004$). # Incubation with α-SOD15 or α-SODA1 alone failed to yielded a statistically significant decreases ($p \geq 0.087$).

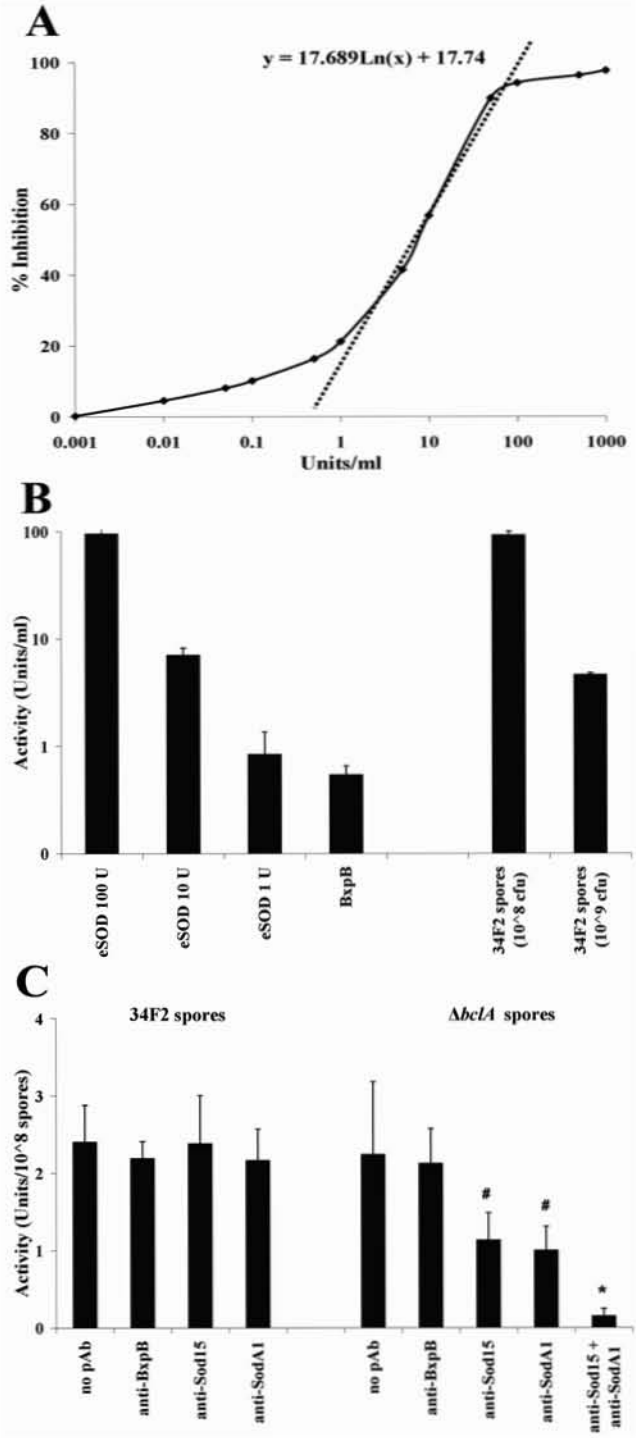

A
y = 17.689Ln(x) + 17.74
% Inhibition
Units/ml
B
Activity (Units/ml)
eSOD 100 U
eSOD 10 U
eSOD 1 U
BxpB
34F2 spores (10^8 cfu)
34F2 spores (10^9 cfu)
C
34F2 spores
ΔbclA spores
Activity (Units/10^8 spores)
no pAb
anti-BxpB
anti-Sod15
anti-SodA1
anti-Sod15 + anti-SodA1

Figure 11. Deletion of the *B. anthracis* SOD genes.

PCR reactions show the deletion and restoration of genes from the *sod15* (BAS1378), *sodA1* (BAS4177), *sodC* (BAS4777) and *sodA2* (BAS5300) loci. Lower (larger) bands represent intact loci, whereas higher (smaller) bands depict deleted loci in which the gene of interest is deleted and flanking regions left intact. Primers were designed to bind to sites outside the flanking regions of the targeted loci. Primers used and sizes of PCR fragments generated in these reactions are provided in Supplemental Materials, Table 1. Strain shorthand reflects the affected loci of the strain. Example: Δ*15*Δ*A1* possesses deletions (smaller PCR products) at *sod15* and *sodA1* and wild-type loci (larger PCR products) at *sodA2* and *sodC*, whereas the Res15,A1 strain constructed from the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* background possesses restored loci at *sod15* and *sodA1* and deletions at *sodA2* and *sodC*..

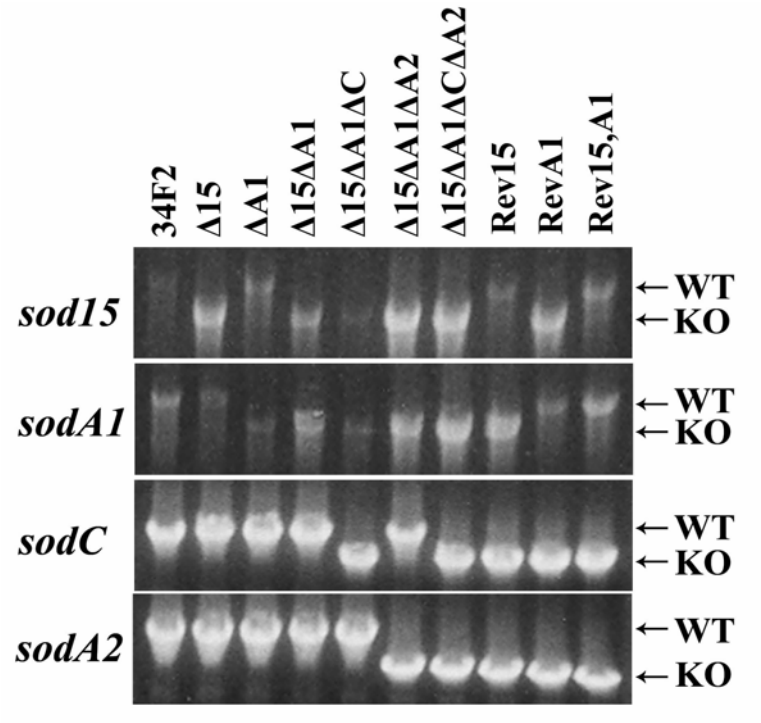

34F2
Δ15
ΔA1
Δ15ΔA1
Δ15ΔA1ΔC
Δ15ΔA1ΔA2
Δ15ΔA1ΔCΔA2
Rev15
RevA1
Rev15,A1
sod15
sodA1
sodC
sodA2
←WT
←KO
←WT
←KO
←WT
←KO
←WT
←KO

Figure. 12. Growth and germination of *B. anthracis* wild-type and mutant strains. (A.) Vegetative growth of the wild type Sterne strain and SOD deletion mutants in BHI broth over time after inoculation with overnight cultures, (B.) Vegetative outgrowth of the wild type Sterne strain and SOD deletion mutants in Modified G broth over time after inoculation with 5×10^8 heat-activated spores, (C.) Kinetics of germination of spores from the parent and mutant strains. Spores were inoculated in 5% BHI broth, growth at 37ºC and 225 rpm and samples were heat-treated to inactivate vegetative cells at the time points shown. Colony counts following heat treatment represent the numbers of heat-resistant spores. Percent germination at test points (t_x) indicate the numbers of spores that germinated at each time point relative to the spore count at the starting point (t_0). * The level of Δ*sod15*Δ*sodA1* and Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* germination was statistically lower than wild type at each time point ($p < 0.001$). ** The level of Res15,A1 germination was lower than that of wild-type at the 5 min ($p < 0.001$), 10 min ($p = 0.002$) and 30 min ($p = 0.007$), but by 60 min the differences were not significant ($p = 0.10$).

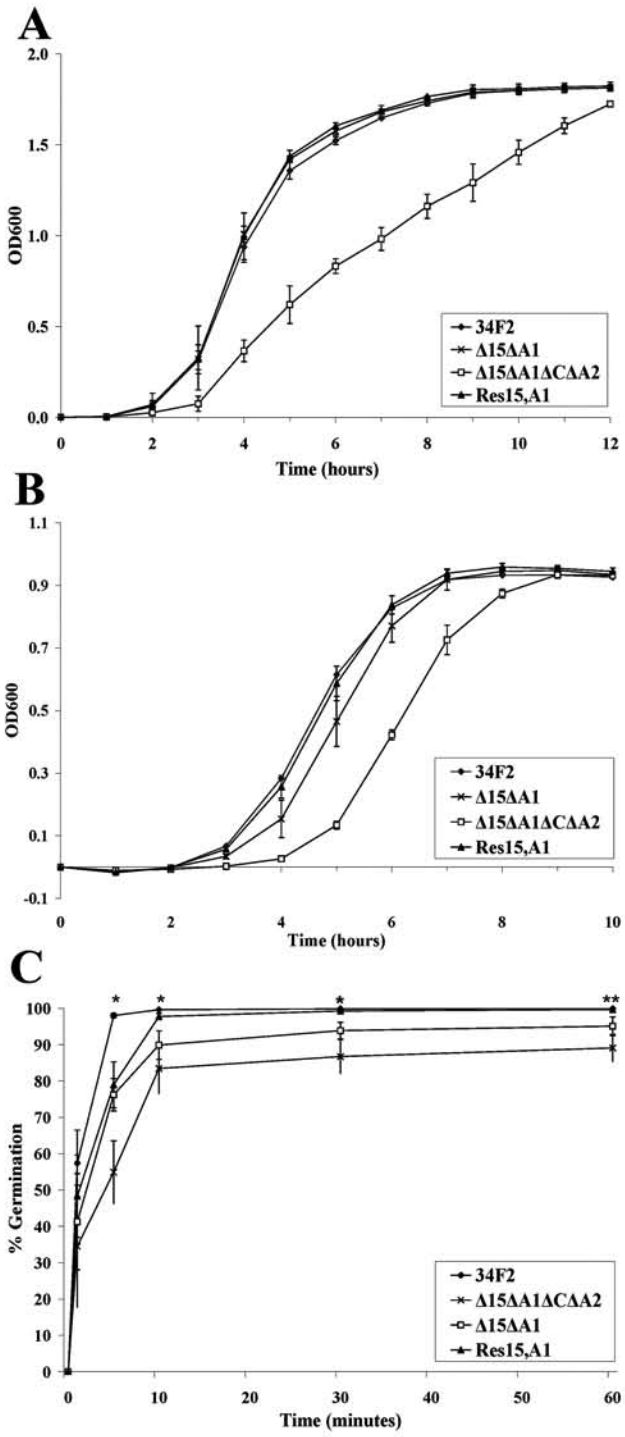

A
OD600
Time (hours)
34F2
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1
B
OD600
Time (hours)
34F2
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1
C
% Germination
Time (minutes)
34F2
Δ15ΔA1ΔCΔA2
Δ15ΔA1
Res15,A1

Figure 13. Comparison of SOD activity for wild type Sterne strain and mutants. (A.) 2.5×10^7 heat-activated spores were mixed with soft agar, spread on BHI agar, and plates were overlaid with 6 mm disks soaked with paraquat. After overnight incubation of the plates at 37ºC, zones of inhibition of bacterial growth around the disk were calculated as the area (mm^2) area the disk in which bacterial growth was interrupted; n = 10 disks per strain per experiment. * Zones of inhibition were higher than for the wild-type ($P < 0.001$), (B.) Spore-associated SOD activity was measured from 2.5×10^8 heat-activated spores. * SOD activity was higher than that of wild type spores ($P < 0.001$). ** SOD Activity was lower than that of wild type spores ($P < 0.001$).

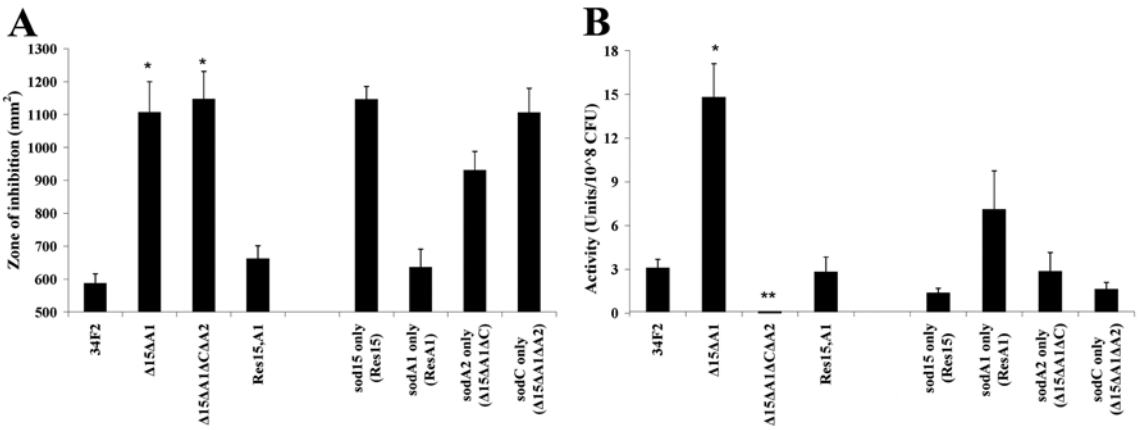

A
Zone of inhibition (mm²)
1300
1200
1100
1000
900
800
700
600
500
*
*
34F2
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1
sod15 only (Res15)
sodA1 only (ResA1)
sodA2 only (Δ15ΔA1ΔC)
sodC only (Δ15ΔA1ΔA2)
B
Activity (Units/10^8 CFU)
18
15
12
9
6
3
0
*
**
34F2
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1
sod15 only (Res15)
sodA1 only (ResA1)
sodA2 only (Δ15ΔA1ΔC)
sodC only (Δ15ΔA1ΔA2)

Table 10. Fifty percent lethal dose (LD_{50}) and mean-time-to-death (MTD) for intranasal and subcutaneous infection of A/J mice with 34F2 and mutant strains.

Strain	**LD_{50}[a]**		**10^6 CFU i.n. challenge**	
	Intranasal	**Subcutaneous**	**Survival**	**MTD**
34F2	5.57×10^4	1.13×10^3	0/10	3.80
Δ*sod15*Δ*sodA1* mutant	4.47×10^4	2.47×10^3	0/10	4.90
Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* mutant	2.51×10^6 *	5.04×10^3	9/10**	6.00***
sod15$^+$*sodA1*$^+$ restorant	1.28×10^5	3.21×10^3	0/10	4.50

[a] 50% lethal doses were calculated and compared by probit analysis.

* A statistically significant difference from wild type with P value < 0.005. Relative median potency of Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* compared to 34F2 was 0.7419 with 95% confidence limits of 0.5103 and 0.9050.

** A statistically significant difference from wild type with P value = 0.0001.

*** A statistically significant difference from wild type with P value < 0.005. 95% confidence limits for wild type MTD were 3.04 to 4.56 days.

Figure 14. Percent survival of A/J mice challenged intranasally with 10^6 spores of 34F2, Δ*sod* mutants and *sod* restorant strains.

Treatment groups of 10 mice received 25 µl of a ~4×10^7 cfu/ml heat-activated spore suspension (1×10^6 cfu). Spores were placed on the nares of anesthetized mice, and the mice were held upright until the suspension was inhaled. Survival of the mice was followed for a period of 14 days. Mean time to death was significantly different for Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* (6.0 days) compared to wild type (3.80 days), $P < 0.005$.

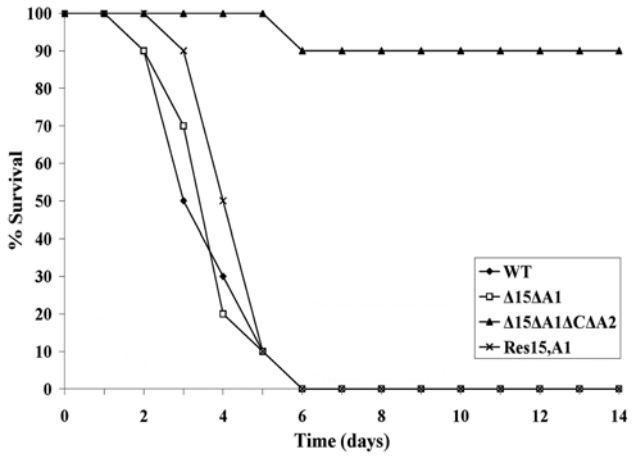

100
90
80
70
60
50
40
30
20
10
0
% Survival
0
2
4
6
8
10
12
14
Time (days)
WT
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1

Figure 15. Detection of individual SODs within spore extracts by Western Blot analysis.

Incubation of SSPE (20 µl/lane) prepared from 34F2, mutant or restorant strain spores with anti-SOD polyclonal IgG. His-tagged recombinant proteins were included as controls for antibody recognition (see Materials and Methods).

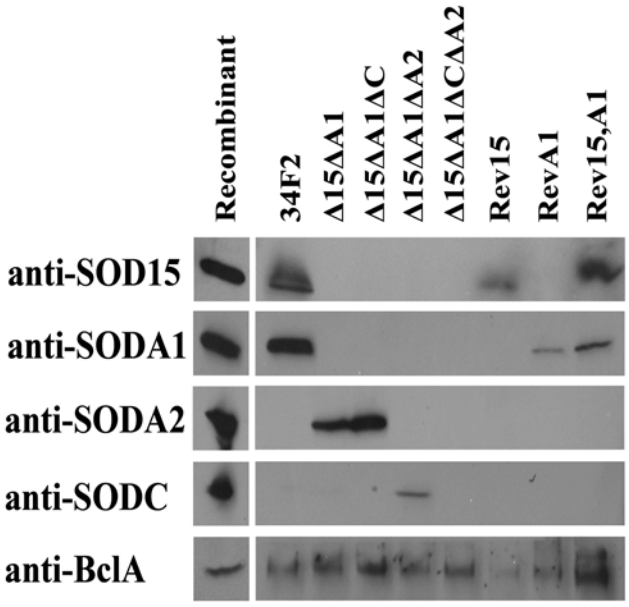

Recombinant
34F2
Δ15ΔA1
Δ15ΔA1ΔC
Δ15ΔA1ΔA2
Δ15ΔA1ΔCΔA2
Rev15
RevA1
Rev15,A1
anti-SOD15
anti-SODA1
anti-SODA2
anti-SODC
anti-BclA

Figure 16. Resistance of wild type Sterne strain and SOD deletion mutant spores to oxidative stress.

A sample of 5×10^6 heat-activated spores was incubated with 1 mM xanthine, 0.5 units xanthine oxidase and 1000 units of catalase at 37ºC. Samples were taken at 0, 15, 30 and 60 minutes. * Percent survival for Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* spores was less than that for wild type spores at all timepoints ($P \leq 0.023$).

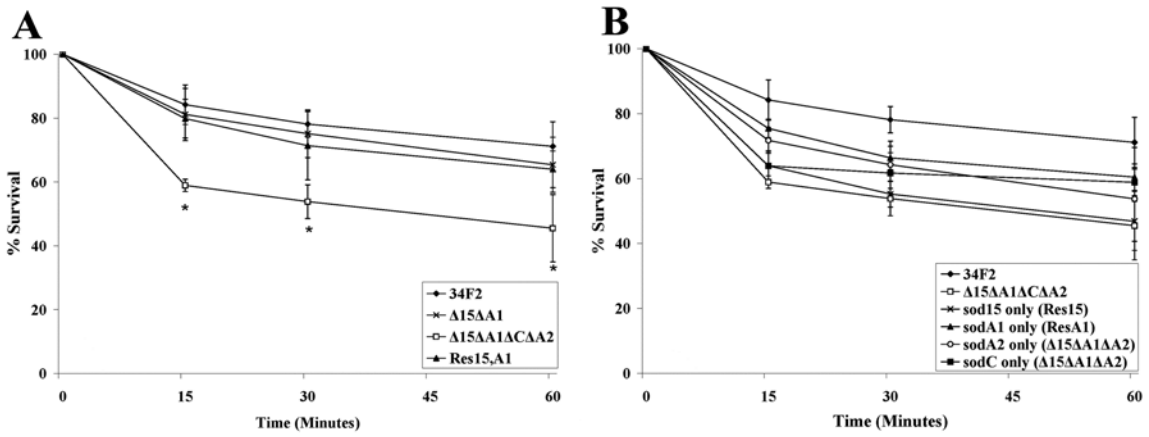
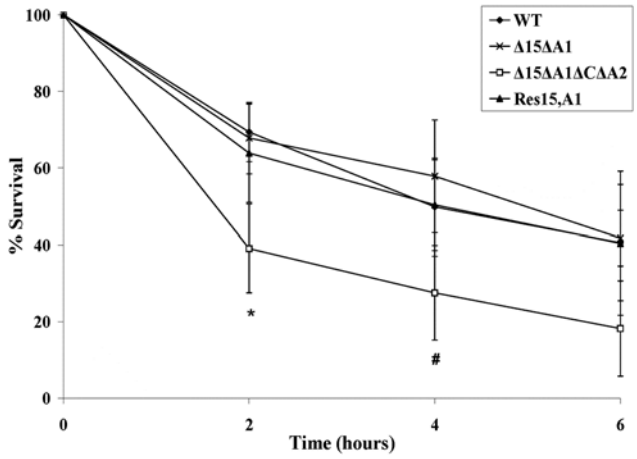

A
% Survival
Time (Minutes)
34F2
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1
B
% Survival
Time (Minutes)
34F2
Δ15ΔA1ΔCΔA2
sod15 only (Res15)
sodA1 only (ResA1)
sodA2 only (Δ15ΔA1ΔA2)
sodC only (Δ15ΔA1ΔA2)

Figure 17. Resistance of wild type Sterne strain and SOD deletion mutant spores to macrophage killing.

A sample of 5×10^6 heat-activated spores was added to $(0.8\text{-}1.2) \times 10^6$ RAW264.7 macrophages in serum-free DMEM plus 100ng/ml PMA. Spore counts were measured at 0, 2, 4 and 6 h by plating on TSA following procedures described in Materials and Methods. * Percent survival for Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* spores was less than that for wild type spores at 2 hours (P = 0.03). # At 4 hours the difference is not statistically significant (P = 0.08).


WT
Δ15ΔA1
Δ15ΔA1ΔCΔA2
Res15,A1
% Survival
Time (hours)
100
80
60
40
20
0
0
2
4
6
*
#

DISCUSSION

Here we describe the construction of a series of *sod* gene deletions in *B. anthracis* that allow examination of the role played by superoxide dismutases in the organism's pathogenesis. Proteomic analyses (139,206) of the *B. anthracis* spore indicated that at least two of the four SODs encoded by the bacterial genome were present in the endospore. These findings raised the possibility of combinatorial protection from oxidative stress. To test this theory, we constructed a strain that lacked both spore-associated SODs. The Δ*sod15*Δ*sodA1* mutant showed a decrease in resistance to oxidative stress in the vegetative form compared to wild type 34F2, findings that are consistent with results from Passalacqua *et al.* (164). However, spores generated from Δ*sod15*Δ*sodA1* showed an increase in spore-associated SOD activity. This unanticipated compensation for the loss of *sod15* and *sodA1* was accompanied by the appearance of an alternate SOD species within the spore structure. Furthermore, Δ*sod15*Δ*sodA1* was not attenuated in the A/J mouse model. Additional deletion of the *sodA2* and *sodC* genes produced a Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* quadruple knockout that displayed a loss of SOD activity associated with the spore. This Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* strain was attenuated when inoculated intranasally into mice. Additionally, virulence of the strains correlated with sensitivity of the organisms to exogenously-produced superoxide anion or activated macrophages. Restoration of *sod15* and *sodA1* within the quadruple knockout background generated a strain (Res15,A1) that essentially mimicked wild-type phenotypes in all aspects related to SOD activity, oxidative stress resistance and virulence.

This study was founded on the hypothesis that SODs incorporated into the spore structure afford the spore with increased resistance to oxidative stress that translates into enhanced virulence through an increased capacity of spores to survive interaction with ROS-producing phagocytes. The data presented in this paper strongly support the notion that the protection from oxidative stress afforded by spore-bound SODs is necessary for the organism to achieve peak virulence, at least within the A/J mouse model. However, alternative explanations for the attenuation seen in the absence of *sod* genes remain possible. One such explanation involves a role for SODs in proper spore formation. Work done by Henriques and colleagues (97) with the related species *B.subtilis* showed that deletion of the primary bacterial SOD led directly to a change in the spore ultrastructure. These authors speculated that such structural changes might indicate a role for SODs in promoting the appropriate chemistry necessary for proper spore maturation, i.e. the construction of outer spore layers might be dependent on a local redox environment that is affected by the presence or absence of SODs. While the authors reported no discernible effects on spore resistance properties in the face of these ultrastructural differences, and recent studies by Passalacqua *et al.* (164) similarly failed to find profound structural changes upon deletion of any of the SODs encoded by *B. anthracis*, it remains formally possible that the deletion of the four *sod* genes of *B. anthracis* may result in structural changes that are relevant to the resistance properties of the spores.

In this investigation, we observed definite changes in both *B. anthracis* growth and germination kinetics that appeared to be directly attributable to the absence of *sod* genes. Perhaps not surprisingly, a delay in the vegetative growth rate of certain mutants

correlated with increased sensitivity of the vegetative bacilli to endogenous oxidative stress. Intermediate knockout and restorant strains that carried *sodA1* or *sodA2* alone exhibited a certain degree of resistance to the effects of paraquat compared to Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* (Fig. 13A) and displayed wild-type growth characteristics (Fig. 12A). Conversely, strains that carried *sod15* or *sodC* alone showed little capacity to grow vegetatively in the face of endogenous oxidative stress and had growth rates similar to Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2*. These differences indicate a specific role for SOD-mediated protection from internal oxidative stress in achieving maximum growth rates.

Germination was also affected by the SODs available to the organism. Both Δ*sod15*Δ*sodA1* and Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* demonstrated delayed kinetics compared to 34F2 (Fig. 12C), though the total extent of germination was ultimately the same for all strains (data not shown). Restoration of the *sod15* and *sodA1* loci within the quadruple knockout background restored wild-type germination. These results are notable in light of a previous study (11) that suggested the possibility of an interplay between the local redox environment and optimal germination. The findings in that study raise the possibility that the attenuation of our SOD quadruple mutant might be attributable to a delayed germination phenotype, rather than to the role of SODs in oxidative stress resistance. However, several pieces of evidence suggest that slower germination cannot fully explain the reduced virulence of Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2*. The fact that the Δ*sod15*Δ*sodA1* double deletion strain exhibited wild-type virulence despite slower germination kinetics indicates that a retardation in spore germination does not apriori lead to a diminution in virulence. Furthermore, a delay in germination (or growth) capable of causing attenuation would presumably affect pathogenesis regardless

of the route of infection. To the contrary, the LD_{50} data (Table 10) indicates a major difference in the degree of attenuation depending upon the route of infection, with Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* possessing a statistically insignificant 4.5-fold decrease in virulence via subcutaneous inoculation and more than a 40-fold attenuation via intranasal entry. This dramatic difference seems to argue against delayed germination (or growth) as the central determinant of the attenuation of Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2,* and favors the importance of a factor whose role in pathogenicity is site-specific. Of note, available information on the effects of germination kinetics on virulence are mixed, as certain reports indicate an increase in virulence associated with a delay in germination (147) whereas other reports show that delayed germination attenuates virulence (141,191)

Whereas numerous studies indicate the central role of macrophages in spore germination and dissemination in the pulmonary infectious process (53,89,187,189,191,231), recent evidence suggests that spores inoculated subcutaneously are capable of extracellular germination and vegetative outgrowth independent of macrophages (19,44,46). Dormant and/or germinating spores introduced by the pulmonary route must survive interaction with ROS-generating alveolar macrophages, while macrophage-independent germination via the cutaneous route of entry might provide a less oxidatively stressful milieu. Under the conditions found in the phagolysosomal compartment of the alveolar macrophage, the attenuation of a strain which lacks the ability to resist oxidative stress would likely be exacerbated. Seen in this way, the severe attenuation of the *sod* mutant seen via the intranasal model is explainable by the loss of a capability that cannot be properly exploited by the host when bacteria invade subcutaneously.

The oxidative stress protection afforded by spore-bound SODs could potentially benefit either the dormant spore or the newly-germinated bacillus. While the *in vitro* data described in Figures 16 and 17 measure ungerminated spores and suggest a real increase in oxidative stress sensitivity among spores of the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* strain, it remains possible that the spore is capable of surviving oxygen radical concentrations encountered *in vivo* without the benefit of SOD. In this situation, the protection afforded by SODs bound to the spore might become more important as *B. anthracis* undergoes germination within the stressful confines of the phagocytic vacuole. A previous microscopic examination of germinating spores demonstrated that the outer layers of the spore remain present and continue to envelop newly-formed vegetative bacillus (91). Furthermore, it has also been shown that components of the spore are capable of conferring resistance properties to phagolysosome-bound organisms, even if the two are no longer tightly associated (229). The greatest benefit of incorporating SODs into the spore may come only after *B. anthracis* begins to germinate and sheds the protective shield afforded by the total spore structure.

Available expression data (164) indicate that *w*hile *sod15* appears to undergo a sporulation-specific regulation program, *sodA1* demonstrates a high level of constitutive expression throughout all phases of the growth cycle. Thus, through distinct regulatory mechanisms, both SODs detectable within 34F2 spores appear to achieve high levels of expression within the mother cell at the time of sporulation. According to prevailing models on spore construction (36,56,98,224), these high concentrations would make the proteins available for inclusion in the growing endospore. This inclusion may come about through distinct and specific protein-protein interactions occurring under the

overall direction of the spore's critical morphogenetic proteins (56), or by random, non-specific mechanics in which the highly-abundant proteins are entangled in the rapidly-assembling spore. Either mechanism could explain the increased spore-associated SOD activity in the Δ*sod15*Δ*sodA1* double mutant. In the event that SODs are incorporated into the spore via specific protein-protein interaction competencies, the amino-acid similarity between SODs might allow for the inclusion of the remaining SODs in spaces normally occupied by SOD15 and/or SODA1. SODA2, which bears far greater similarity to both SODA1 and SOD15 than does SODC, would be better suited for such inclusion. An examination of SODs present in the SSPE generated from the various knockouts constructions in this study (Fig. 15) support this concept; deletion of *sod15* and *sodA1* led directly to the appearance of SODA2 in the spore extract, whereas SODC was detectable only after all other SOD species had been deleted. Additionally, while both *sodA2* and *sodC* are expressed throughout the vegetative life cycle of 34F2 at relatively low levels (164), one or both genes may undergo increased levels of expression as part of a general stress response needed to cope with the loss of the primary protection against oxidative stress. In this scenario, increased concentrations of SODs in the mother cell would likely facilitate greater non-specific incorporation into the spore.

For many bacteria, redundancy among key components is important to maintaining fitness. Isozymes can provide distinct yet overlapping capabilities that allow the bacteria to thrive in multiple environmental conditions. *B. anthracis* harbors four *sod* genes, including one gene (*sodC*) putatively encoding a Cu-Zn SOD and three genes putatively encoding Mn/Fe SODs. While our work and the work of Passalacqua *et al.* (164) fail to find a critical role for any one SOD in an A/J or DBA/J mouse model, it is

also true that mice (and humans) represent incidental hosts that make no significant contribution to the evolution and fitness of *B .anthracis.* Given the need for *B. anthracis* to survive a range of diverse hosts and environments, it is perhaps not surprising that the organism has evolved to retain such a diverse array of SODs. At the same time, maintenance of these four genes may provide the bacteria with secondary benefits. Functional redundancy and compensation through homologous replacement is supported by previous observations with structural proteins in other *Bacillus* species (56). Regardless of the reason(s) for the persistence of multiple *sod* loci in the *B. anthracis* genome, our data suggest that the presence of four *sod* genes in the anthrax chromosome allows for compensation in the absence of one or more species. The spore-associated SOD activity (Fig. 13B) and resistance characteristics (Fig. 16B) of intermediate knockout and restorant strains carrying only a single SOD species, combined with the evidence suggesting that all SODs are capable of inclusion in the spore under the appropriate circumstances (Fig. 15), illustrates that each SOD can contribute to the properties of the spore. However, care must be taken in drawing conclusions about the relative importance of any one SOD to spore activity from data based on an evaluation of its activity in isolation because the composite level of SOD activity resident in the wild-type 34F2 spore is likely dependent on several factors. These factors include, but are not limited to, the inherent specific activity of each SOD, synergistic properties between and among SODs , the concentration of each SOD within the mother cell during sporulation, and competence for inclusion of available SOD proteins into the spore.

In conclusion, this study revealed an important role for superoxide dismutases in the full virulence of *B. anthracis* Sterne strain in the intranasally-challenged A/J mouse

model. The contribution of SODs to virulence appears to reflect the protection against oxidative stress afforded the spore during the early stages of infection. We theorize that functional redundancy created by the existence of four *sod* genes within the *B. anthracis* genome ensures that significant attenuation of the bacteria is only seen with deletion of all four genes and the loss of spore-associated SOD activity. The sensitivity of spores to macrophages in the absence of SODs suggests the potential therapeutic utility of small molecular inhibitors that would be capable of blocking the activity of spore-associated SOD molecules. Finally, enhanced attenuation of the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* strain seen via intranasal challenge as compared to subcutaneous challenge indicates an important role for phagocyte-generated reactive oxygen species in controlling anthrax infections in the lung.

ACKNOWLEDGEMENTS

We thank Cara Olson for expert statistical analysis, Michael Flora for primer synthesis and DNA sequencing and Jill Czarnecki for polyclonal antibody generation. This work was supported by NIH/NIAID Middle Atlantic Regional Center of Excellence grant U54 AI057168 and research funds from the United States Navy through Uniformed Services University grant # G173HS. Special thanks also to the United States Army Long-Term Health Education and Training Program for support of one of us (R. Cybulski). The opinions and assertions in this paper are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Defense. This research was conducted in compliance with the Animal Welfare Act. All animal use

protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Uniformed Services University.

Chapter 4

Discussion and Future Directions

Summary of results in the context of dissertation objectives and specific aims

The goal of this work was to investigate individual components of the *B. anthracis* exosporium so as to better understand the contribution of these factors to the pathogenic mechanisms of the organism and to evaluate them as potential contributors to a PA-based anthrax vaccine. The work was guided by two distinct hypotheses. To investigate the first hypothesis, which asserted that antibodies to individual components of the exosporium might be immunogenic and contribute to a protective immune response against *B. anthracis* spore challenge, we first identified candidate proteins for which there was indication of inclusion in the exosporium. Genes for these candidate proteins were cloned and expressed in *E.coli*, and those that showed reactivity to sera raised against whole, inactivated spores were further pursued. Experiments in which A/J mice were immunized with individual exosporium proteins and subsequently challenged subcutaneously with *B. anthracis* Sterne strain spores showed that all candidate proteins failed to generate a protective immune response. However, when the proteins were administered as a boost following immunization with a suboptimal dose of protective antigen (PA), two proteins (BxpB and p5303) conveyed enhanced protection over PA alone. Rabbit polyclonal antibodies raised against BxpB and p5303 failed to delay spore germination but did enhance opsonophagocytosis and subsequent killing of spore by RAW264.7 macrophages.

Among the subset of exosporium proteins recognized by the anti-spore sera were two putative superoxide dismutase (SOD) proteins, SOD15 and SODA1. Given the known contribution of SODs to the virulence of certain intracellular pathogens, our second

hypothesis was that SODs present in the *B. anthracis* spore were important for anthrax pathogenesis. Our first aim in pursuit of this theory was to demonstrate that the putative SOD proteins did in fact possess superoxide dismutase activity and that this activity could be detected in association with intact spores. SOD activity was demonstrated for each protein by analyzing recombinant forms of the proteins with a commercially-available *in vitro* assay. This same assay was then used to demonstrate SOD activity in the presence of whole spores. To achieve our next specific aim, which was the identification of any contributions made by spore-bound SODs to *B. anthracis* virulence, we constructed isogenic Sterne strains that lacked one or both of the *sod* genes in question. The elimination of SOD15 and SODA1 by genetic means failed to attenuate the bacteria, and in fact led to increased levels of spore-associated SOD activity. Assuming that these observations were the result of compensation attributable to the remaining *sod* genes present in the bacterial genome (*sodC* and *sodA2*), we constructed additional knockouts to eliminate all four *sod* loci . The resulting quadruple knockout was in fact attenuated. This mutant showed a loss of measurable SOD activity on the spore and an increased sensitivity to oxidative stress and macrophage killing. Wild-type spore characteristics could be largely regained by genetic restoration of the *sod15* and *sodA1* genes in the Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* background.

Conclusions

B. anthracis exosporium proteins

Location, immunogenicity and protective efficacy. An initial list of proteins believed to be present in the exosporium was obtained by searching the available literature (37,131,139,181,182,206,213,217). In fact, numerous proteomic studies with *B. anthracis*, the closely-related *B. cereus* and *B. thuringiensis*, or the more distantly-related *B. subtilis* has generated a growing list of proteins that are located either in the exosporium or the outer spore coat (available methods do not allow one to claim inclusion of exosporium components alone). Our catalog of more than 30 candidate exosporium proteins (or glycoproteins) contained proteins identified in previous studies with *B. anthracis* as well as homologs of *B. anthracis* proteins found in related *Bacillus* species. An initial screen of each protein identified eleven proteins that were recognized by antiserum generated against whole spores. Given that this serum represents the composite immune response to the collection of antigens visible to the host in the context of whole spores, recognition of a candidate protein suggested localization of that protein to the exterior of the spore (see below). This observation was further explored with polyclonal antibodies generated against five of the screen-positive candidates. Probing of intact spores with these antigen-specific polyclonal antibodies in either an ELISA or immunoelectron microscopy format indicated that all five proteins were present on the basal surface of the exosporium, beneath the immunodominant antigen BclA that protrudes like hairs from the surface of the spore. BxpB and p5303 appeared most abundant in the exosporium among the five candidates, based on the semiquantitative

measurement of spore labeling via each method. For the most part, BclA was an obstructive presence, and only when BclA was effectively removed through the use of spores generated with a Δ*bclA* Sterne strain could the underlying antigens be observed. Immunoelectron microscopy following post-embed labeling, in which spores are fixed and sectioned prior to labeling with antibodies and epitopes deep within the spore are thereby exposed, revealed a more complex picture of spore antigen localization (Figure 6). Although the known exosporium structural component BxpB was restricted almost entirely to the basal surface of the exosporium, the remaining four candidates (p5303, YwdL, SOD15 and SODA1) appeared to be distributed evenly across the different layers of the spore structure. While it is formally possible that these results are an artifact of epitope "smearing" from the exosporium across the spore during the sectioning process, the data are consistent with prevailing models of spore construction (36,56,98,224) that suggest a dynamic deposition of proteins on the growing spore rather than a targeting of particular proteins to specific locations.

Since the five proteins discussed above localized to the exosporium and elicited an immune response in the context of intact spores, we considered them to be potential candidates for generating protective antibodies. However, the reality concerning using exosporium proteins as immunogens proved complex. As had been observed previously with BclA (27), none of the five proteins in question were capable of protecting A/J mice against subsequent subcutaneous spore challenge when administered alone, despite generating strong antigen-specific antibody titers. Furthermore, simultaneous immunization of mice with spore antigens and PA, the critical element in the current AVA vaccine, also failed to protect. Decreased survival of mice immunized with spore

antigen plus PA, as compared to those receiving PA alone, was likely a result of reduced anti-PA titers seen in mice receiving the simultaneous immunization. This cross-antigen interference may be a phenomenon unique to the A/J mouse model used in this study, as similar difficulties have not been reported in other models. Regardless, these results reaffirm the essentiality of PA and anti-PA antibodies in anthrax vaccinology. When spore antigens were administered separately following the elicitation of a suboptimal anti-PA response, the result was enhanced protection with the use of BxpB and p5303. This additive effect, in which high titers to BxpB or p5303 appeared to increase the odds of survival in mice that produced poor responses to PA (Figure 7), highlights the utility of a multi-faceted immune response to anthrax. This is particularly germane given the available evidence that the AVA vaccine generates variable responses to PA (69,106-108,234). Simultaneous immunization with BxpB and p5303 led to reduced titers achieved against each individual spore component, and negated the beneficial effects of each spore antigen. These recurrent problems regarding apparent interference between immunogens upon simultaneous immunization are worth considering in the event of further investigations into multi-component anthrax vaccines (see below).

Mechanisms of host protection through targeting of spore antigens. Enhanced protection from spore challenge afforded by antibodies directed at individual spore components required an investigation into the possible mechanisms by which those antibodies might act. The most obvious rationale for targeting the spore with the immune system is that by responding to spores as they enter into the body, the host might be able to clear some or all of the spores before they germinate and undergo vegetative

outgrowth. A clearance of even a portion of the total inoculum would effectively reduce that inoculum, thereby lowering the population of spores competent to initiate an infection. Given the tremendous challenge that vegetative bacilli present to the host, any reduction in the effective infectious dose of such bacilli would increase the host's chances to control the infection and survive. In terms of mechanisms by which an antibody directed against an exosporium protein might facilitate such clearance, two possibilities have previously been demonstrated with different antibodies: reduced germination (27,45,63,232,233) and increased opsonophagocytosis (27,45,232).

Multiple studies indicate that spores possessing delayed germination kinetics are less virulent in the host (141,191), a finding most likely due to the increased opportunity given to the host immune response to destroy spores before they successfully germinate and become vegetative cells. Similar germination inhibition and consequent protection can be elicited from the host's humoral immune response by generating antibodies against whole spores (63,232,233) and PA (45,232). Though no mechanism for this effect has been established, the most likely explanations involve antibodies complexed to the spore surface stearically hindering germinant:germinant receptor interaction or interfering with the swelling of the spore that results from rehydration. Attempts on our part to achieve the same results with antibodies to BxpB and p5303 failed to yield significant delays in germination. These findings are most likely due to the relatively low abundance of these antigens as compared to those proteins that proved more effective targets, such as the highly-abundant BclA. This low abundance is made more problematic by the obstructive presence of BclA on the surface of the spore, which may effectively lower the available quantity of the non-BclA target in question. Under these

constraints, the level of binding of antibody to the spore surface is likely insufficient to mediate the effects described above.

An alternative mechanism of spore clearance is an enhancement of opsonophagocytosis mediated by antibodies bound to the spore surface. As with germination inhibition, the capacity of an antibody to facilitate opsonization and phagocytic uptake by professional phagocytes is dependent on that antibody recognizing its target on the spore. Most antibodies that successfully enhance opsonophagocytosis are directed at the surface-bound proteins BclA (27) and PA (45,232). However, the discovery of a previously-unknown protein SoaA that is localized to the spore cortex yet can be successfully targeted by antibodies for opsonization (43), demands a reexamination of the traditional view of impenetrable spore layers. One possible explanation for these findings of Cote and colleagues (43) involves a more dynamic situation within the spore, one that allows for opening and closing of the outermost layers and access to otherwise-secluded targets. Alternatively, within a population of spores extensively exposed to the environment (or repeatedly vortexed in the lab) there may be a significant subset for which disruption of segments of BclA, the exosporium and/or spore coat uncovers inner epitopes. Finally, a redistribution of some or all of the antigen in question from one location within the spore to another at or near the spore surface during the early steps of germination, a potential scenario posited by the group that discovered SoaA (43), might allow for recognition by relevant antibodies. Any or all of these possible scenarios might explain our observation that antibodies directed against BxpB and p5303, as well as BclA, enhance opsonophagocytosis. However, a simpler explanation involves relative levels of target abundance. While less abundant than BclA,

the protective proteins BxpB and p5303 are apparently still more numerous in the exosporium than the non-protective proteins YwdL, SOD15 or SODA1. Therefore, these antigens offer the best opportunity for antibody recognition amidst the relatively limited gaps in the BclA nap. Unlike in the case of germination reduction, low levels of recognition may in fact be sufficient to mediate antibody-directed opsonophagocytosis.

The opsonic effect of anti-BxpB and anti-p5303 antibodies is made more intriguing by the fact that, similar to effects seen with anti-PA antibody (45,231,232), these antibodies also seem to promote greater killing of spores following phagocytosis. It is possible that this effect is due to some microbicidal capacity of the antibodies. However, a more likely explanation would be the targeting of spores to more efficient, highly-activated phagolysosomal compartments following F_c-mediated phagocytosis. The combined capacities of anti-BxpB and anti-p5303 antibodies to promote greater phagocytic uptake and greater killing illustrate a viable mechanism by which they might promote spore clearance and provide protection from anthrax infection.

Potential additives to protective antigen for future vaccines. The demonstrated facility for BxpB, and to a lesser extent p5303, to enhance PA-mediated protective immunity provides further evidence for the value of targeting spore components as potential vaccine additives. Combined with the previously-published data regarding the benefits of boosting with BclA (27), our data suggest that combinatorial vaccines encompassing both protection from intoxication and targeting of the spore should be pursued. A starting point might be to determine what benefit can be achieved by boosting PA immunizations with both BclA and BxpB. Such a strategy, which

theoretically would combine the protective capacities of anti-BclA and anti-BxpB antibodies, must take into account certain considerations. First, the achievement of 100% protection of A/J mice following a PA-BclA prime-boost strategy would require that conditions be made less favorable to the mouse in order to facilitate the observation of any added benefit from the introduction of a third immunogen. This could be achieved either by a further reduction in the amount of PA or BclA administered or an increase in the spore challenge dose. Alternatively, the use of the more virulent Ames strain, while necessitating animal experimentation in a Biosafety Level 3 facility, would be a more stringent test of vaccine efficacy. Also requiring consideration is the evidence of interference in the humoral response generated against individual immunogens when those immunogens are administered simultaneously. Immunization of different antigens into different sites might alleviate the problem. So might an alteration in the relative amounts of each antigen. Another strategy might be the administration of the three proteins in a series of three shots. While such a protracted scheme is not desirable, an immunization scheme that requires three shots would still represent an improvement over the current six-shot regimen applied with AVA. Finally, attempts to use both BclA and BxpB as vaccine additives would be best served by utilizing native, glycosylated forms of the proteins, which can theoretically be obtained by expressing the *bclA* and *bxpB* genes in a *Bacillus* expression vector, rather than *E. coli.* Glycosylation would create an immunogen more like the protein form found on the spore surface, and perhaps would improve the protection afforded by immunization.

Superoxide dismutases and the *B. anthracis* spore

Contributions of SODs to *B. anthracis* pathogenicity. The work described in this dissertation supports the assertion that SODs, particularly spore-bound SODs, are important contributors to the pathogenicity of *B. anthracis*. The elimination of spore-bound SOD activity led directly to an increased sensitivity of spores to killing by exposure to superoxide anion produced exogenously in PBS and exposure to the killing actions of macrophages upon phagocytosis. These increased sensitivites correlated well with an attenuation of the bacteria when administered to A/J mice intranasally. Though the approximately 40-fold attenuation attributable to SODs is not as great as the degree of attenuation seen with the loss of certain major virulence factors such as the capsule or the full complement of toxins (223), it nonetheless represents a significant contribution to overall pathogenic make-up of wild-type *B. anthracis*. While it is clear that macrophages and phagocytes employ numerous strategies to destroy pathogens, the capacity of SODs to detoxify the phagocyte-generated superoxide anions that play a direct role in the generation of reactive oxygen species and an indirect role in the generation of reactive nitrogen species is vital to the survival of the ingested organism. Recent reports indicate that, in contrast to the traditional view that spores are impervious to stress until germination is initiated, exposure to high levels of oxidative stress is capable of inflicting damage on dormant spores (50,51,186,241). What's more, depending on the site of spore germination, SODs present in the exosporium or spore coat may be capable of providing protection to the infant vegetative cell while it remains in the phagolysosome. Findings that the early vegetative cell remains bound by the outer spore structure (91) and that spore remnants contained in the phagolysosomal compartment may still impart

protective benefit (229) suggest that benefits of spore-bound SODs may extend beyond the spore itself.

Functional redundancy of SODs encoded by the *B. anthracis* genome. The virulence retained by *B. anthracis* in spite of the elimination of both *sod15* and *sodA1* (the two SODs known to be present within the wild-type spore structure) could either mean that the bacteria is capable of compensating for the absence of these SODs or that the protection afforded by SODs from oxidative stress is not critical to spore survival and virulence. However, the evidence outlined above, which clearly demonstrates that the eventual elimination of SOD activity leads to attenuation, favors the former explanation over the latter. The mechanism of such compensation remains in open question. Given the current model for spore construction (36,56,98,224) and the established precedent within the *Bacillus* family whereby structurally similar proteins are capable of substitution within the spore structure [see (56) and the Chapter 3 *Discussion*], it is possible that in the absence of one or more *sod* genes the remaining SODs are capable of inclusion within the growing spore structure by virtue of amino acid similarity with the absent SOD species. Such compensation, facilitated by broad stretches of homology allowing for the necessary protein-protein interactions that govern inclusion in the spore, might occur regardless of any active up-regulation of remaining *sod* genes. Conversely, the absence of certain *sod* genes, particularly the *sodA1* gene that encodes for the primary source of oxidative stress protection in the vegetative cell, might place the bacteria in a state of heightened oxidative stress, thereby initiating an up-regulation of the remaining *sod* genes as part of a general stress response. Such a situation would elevate the

concentration of each of the remaining SODs within the cytoplasm of the vegetative cell and increase the chance for random incorporation those SODs into the endospore. A model in which a combination of the first two scenarios coalesces to elevate the levels of certain SODs within the spore is depicted in Figure 18.

While the compensation described above was not anticipated at the start of this dissertation work, such a phenomenon should not be surprising given the existence of four *sod* genes within the *B. anthracis* genome. However, assuming that the genes for *sod15* and *sodA1* are stably and reliably expressed, and not typically lost in nature as they were under targeted allelic exchange, the bacteria is not likely to be called upon to utilize this functional redundancy in nature. Furthermore, a previous investigation (164) into the contribution of individual SODs suggested that neither *sodC* nor *sodA2* (or, for that matter, *sod15*) were significant contributors to the capacity of the vegetative bacilli to protect itself from endogenous stress. Bearing in mind these observations, why are all four *sod* genes maintained? The most likely explanation lies in as-yet uncharacterized requirements found in the typical hosts for *B. anthracis*, herbivores such as cattle and sheep. Given that the infection of humans and mice are insignificant to the natural maintenance cycle and evolutionary pressures experienced by *B. anthracis*, the continued persistence of all four *sod* genes is likely an indication of differing requirements in different hosts. SODC is classified as a Cu-Zn SOD, meaning that according to available evidence from other bacterial species (74,208), it is likely membrane-bound and responsible for combating exogenous forms of oxidative stress. While the absence of SODC is not attenuating in a mouse model (164), the need to protect vegetative bacilli from exogenous oxidizers may be more critical in other systems. SODA2, a Mn SOD

with a high degree of similarity to SODA1, has previously (164) been shown to interact with SODA1 to form heterodimeric complexes which may possess subtle characteristics of unrealized importance in certain environments. *B. anthracis* presumably encodes four SOD isozymes because the subtly distinct capabilities of each isozyme somehow contributes to greater overall fitness by allowing the organism to widen the range of hosts and environments in which it may persist. In so doing, the ability to substitute alternate SODs within the spore structure likely reflects an ancillary, if interesting, benefit.

While it is true that our evidence indicates that composite spore-bound SOD activity, and not activity attributable to any one SOD species, is important to *B. anthracis* virulence, there is one spore characteristic for which the various SODs do not seem to demonstrate functional redundancy. The Δ*sod15*Δ*sodA1* spores show a delay in germination compared to wild-type, and this slower initiation of germination is somewhat exacerbated upon further deletion of the remaining *sod* genes (Figure 12). That this delay in germination was specifically attributable to the loss of SODs bound to the wild-type spore was demonstrated by restoration of *sod15* and *sodA1* in the quadruple SOD knockout background, a restoration that almost completely restored wild-type germination kinetics (Figure 12). Preliminary experiments in which optical density measurements were taken to assess the germination kinetics of certain deletion intermediates (data not shown) suggest an important germination-related role for SOD15. Germination experiments with single *sod* knockout strains and single *sod* revertant strains revealed that Δ*sod15* spores exhibited a greater delay compared to wild type than did Δ*sodA1* spores. Moreover, Res15 spores showed a greater relative restoration of germination kinetics compared to Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* than did RevA1 spores.

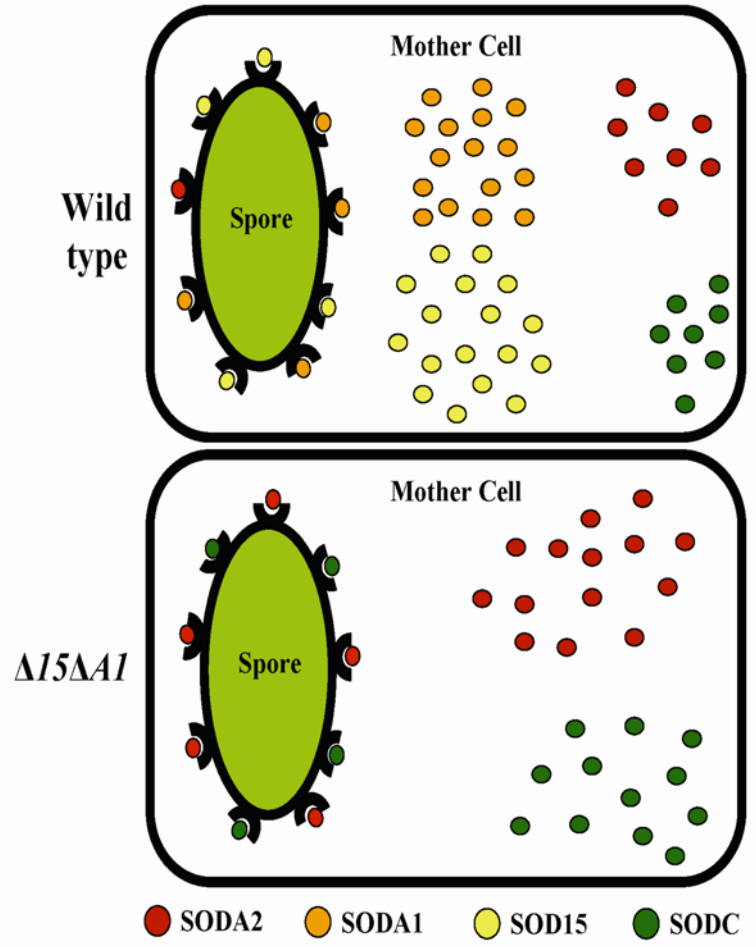
These observations, while certainly indirect, suggest that SOD15 contributes disproportionately to optimal germination. SOD15 has limited amino acid similarity to fellow Mn/Fe superoxide dismutases SODA1 and SODA2 and possesses a 135 amino acid domain at its N-terminus that has no ascribed function. Additionally, previous reports suggest the coregulation of *sod15* with an operon believed to be involved in aspects of spore ultrastructure (164). Taken in their entirety, these data suggest that *sod15* has undergone divergent evolution relative to fellow SODs. While the persistence of some level of activity indicates that SOD15 retains SOD function, it is possible that this species has evolved over time to acquire additional, "non-SOD" activities that contribute to the spore in novel ways. The existence of three other *sod* genes within the genome could have provided the bacteria with the flexibility to tolerate mutations in *sod15* that ultimately led to the divergence of this locus.

Relevance of *B. anthracis* SODs in different routes of infection. While the available data indicate a role for SODs in *B. anthracis* virulence, they also appear to imply that there are different challenges to the organisms engendered in different routes of infection. Whereas elimination of the four *sod* genes led to an approximately 40-fold attenuation when spores were administered to A/J mice intranasally, the subcutaneous inoculation of the same strain resulted in a measured LD_{50} not significantly different from that of wild type. This difference in degree of attenuation likely says something about the level of oxidative stress seen by a pathogen via the two routes. These data indirectly contribute to the ongoing discussion regarding spore:host interactions as they relate to phagocytosis, spore migration and spore germination. Attenuation of Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2*

upon intranasal inoculation supports the notion that spores introduced to the alveolar spaces interact with host macrophages. Whether they germinate and escape macrophages while still in the lung or transit back to the regional lymph nodes before germination and escape, spores would be required to resist the harsh, oxidatively-stressed environment of the macrophage phagolysosome. Conversely, the relatively unaffected virulence of these spores when introduced subcutaneously indicates that the same requirements are not met along this route. The most probable explanation for this is that subcutaneously-inoculated spores escape macrophage interaction. If so, the quadruple SOD mutant would not be "penalized" for its compromised resistance to oxidative pressures. This scenario would necessitate extracellular spore germination, an idea that has long been rejected but which has recently acquired experimental support (19,44,46,191). In the event of germination outside the phagocyte, the capacity of the spore to rapidly germinate and seed infection would be of prime importance. Seen in this light, the mild attenuation of Δ*sod15*Δ*sodA1*Δ*sodC*Δ*sodA2* via the subcutaneous route might be attributable to a slower onset of infection explained by the delay in germination and growth seen with this strain. Of note, the experiments described in this dissertation were done with A/J mice, a murine strain that is known to be deficient in the C5 molecule (214) that is critical to the host's innate immune response. Given the role of C5 and the breakdown product C5a in effective activation of phagocytes and the phagocytic oxidative burst (49), the relative disadvantage attributable to the loss of spore-bound SODs, and by extension the differences in the rigors of the different routes of infection, may actually be underrepresented in our system.

Figure 18. Model of SOD incorporation during sporulation.

Wild-type 34F2 spores accomplish deposition of SOD15 and SODA1 in the spore structure due to the high levels of expression of *sod15* and *sodA1* as the mother cell undergoes sporulation (164). Deletion of *sod15* and/or *sodA1* results in open spaces within the growing spore structure that are normally occupied by these gene products. SODC and/or SODA2, which are typically expressed at lower levels within the cell but may be upregulated as the vegetative bacilli experiences greater oxidative stress, occupy these open spaces. Inherently different levels of activity among the different SODs as well as relative abundance within the spore structure, help determine the total spore-associated SOD activity.


Wild type
Mother Cell
Spore
Δ15ΔA1
Mother Cell
Spore
SODA2
SODA1
SOD15
SODC

Future Directions

The findings and conclusions described in the first half of this dissertation support the idea that individual components of the spore structure may be effectively targeted to generate a protective immune response, provided that the anti-spore response is generated in combination with an anti-PA response. The logical extension of this work, and the work of Brahmbhatt *et al.* (27), would be an attempt to design a combinatorial vaccination regimen that incorporates PA and multiple spore targets. Variations in the relative amounts of each immunogen, the site(s) of immunization and the sequence of immunization could all be pursued. A higher challenge dose would provide a more stringent test of protective efficacy and better illustrate the value of the vaccination strategy. The primary limitation of this study, which involves the deficient immune response of A/J mice stemming from the absence of C5 and the consequent impairment of immune activation, might also be addressed. The use of fully-virulent Ames strain spores in an appropriate preliminary model system such as BALB/c or C3H/HeN mice would provide a better test of the potential value of this vaccination approach in humans.

Regarding the examination of the role of SODs in *B. anthracis* virulence discussed in the second half of this thesis, the primary question remaining entails the mechanism by which the bacteria integrate SODA2 and/or SODC in the absence of the SOD15 and/or SODA1 molecules bound to the spore in the wild type. The favored model for this process is outlined in Figure 18 . It remains to be determined whether or not the inclusion of SODA2 and SODC involves upregulation of *sodC* and/or *sodA2*, perhaps in the face of increased oxidative stress brought about by the absence of SOD15 and/or

SODA1. To test this possibility, qRT-PCR could be employed to compare the levels of expression of *sodC* and *sodA2* in both the wild-type and Δ*sod15*Δ*sodA1* strains as they enter sporulation. The absence of specific upregulation of either remaining *sod* gene would favor the notion of a more passive process of achieving compensatory SOD integration in the double knockout. Additionally, examination of the germination and growth characteristics of 34F2 and *sod* mutant strains in an anaerobic environment might help to determine if the germination and growth defects seen in the quadruple *sod* knockout strain are specifically attributable to differences in how the strain responds to oxygen and oxygen-related stress. Finally, an examination of the interaction of our SOD knockout strains with PMNs (which generate a robust respiratory burst) or *phox*$^{-/-}$ mice (which fail to generate an respiratory burst) would provide a more detailed assessment of the importance of spore-bound SOD activity to spore survival and *B. anthracis* virulence.

Reference List

1. 2000. Anthrax: The Investigation of a Deadly Outbreak. Nat. Med. 6:245.

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

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

4. Agrawal, A., J. Lingappa, S. H. Leppla, S. Agrawal, A. Jabbar, C. Quinn, and B. Pulendran. 2003. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. Nature 424:329-334.

5. Ahuja, N., P. Kumar, and R. Bhatnagar. 2004. The adenylate cyclase toxins. Crit Rev. Microbiol. 30:187-196.

6. Albrink, W. S. 1961. Pathogenesis of inhalation anthrax. Bacteriol. Rev. 25:268-273.

7. Alexeyev, O. A., V. G. Morozov, T. V. Suzdaltseva, A. S. Mishukov, and L. A. Steinberg. 1994. Impaired neutrophil function in the cutaneous form of anthrax. Infection 22:281-282.

8. Aronson, A. I. and P. Fitz-James. 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol Rev. 40:360-402.

9. Ascenzi, P., P. Visca, G. Ippolito, A. Spallarossa, M. Bolognesi, and C. Montecucco. 2002. Anthrax toxin: a tripartite lethal combination. FEBS Lett. 531:384-388.

10. Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin. Invest 52:741-744.

11. Baillie, L., S. Hibbs, P. Tsai, G. L. Cao, and G. M. Rosen. 2005. Role of superoxide in the germination of *Bacillus anthracis* endospores. FEMS Microbiol Lett. 245:33-38.

12. Bakshi, C. S., M. Malik, K. Regan, J. A. Melendez, D. W. Metzger, V. M. Pavlov, and T. J. Sellati. 2006. Superoxide dismutase B gene (*sodB*)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. J Bacteriol. 188:6443-6448.

13. Basu, S., T. J. Kang, W. H. Chen, M. J. Fenton, L. Baillie, S. Hibbs, and A. S. Cross. 2007. Role of *Bacillus anthracis* Spore Structures in Macrophage Cytokine Responses. Infect Immun 75:2351-2358.

14. Beall, F. A., M. J. Taylor, and C. B. Thorne. 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis.* J Bacteriol. 83:1274-1280.

15. Beaman, T. C., H. S. Pankratz, and P. Gerhardt. 1971. Paracrystalline sheets reaggregated from solubilized exosporium of *Bacillus cereus.* J Bacteriol 107:320-324.

16. Ben-Efraim, S. and P. Liacopoulos. 1967. Inhibition, no-effect or enhancement of immune responses following injection of mixtures of immunogenic and non-immunogenic synthetic polypeptides. Immunology 12:517-524.

17. Benov, L. T. and I. Fridovich. 1994. *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. J Biol Chem. 269:25310-25314.

18. Bhalla, D. K. and D. B. Warheit. 2004. Biological agents with potential for misuse: a historical perspective and defensive measures. Toxicol. Appl. Pharmacol. 199:71-84.

19. Bischof, T. S., B. L. Hahn, and P. G. Sohnle. 2007. Characteristics of spore germination in a mouse model of cutaneous anthrax. J Infect Dis 195:888-894.

20. Boydston, J. A., P. Chen, C. T. Steichen, and C. L. Turnbough, Jr. 2005. Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis.* J Bacteriol. 187:5310-5317.

21. Boydston, J. A., L. Yue, J. F. Kearney, and C. L. Turnbough, Jr. 2006. The ExsY protein is required for complete formation of the exosporium of *Bacillus anthracis.* J Bacteriol. 188:7440-7448.

22. Bozue, J., C. K. Cote, K. L. Moody, and S. L. Welkos. 2007. Fully Virulent *Bacillus anthracis* Does Not Require the Immunodominant Protein BclA for Pathogenesis. Infect Immun 75:508-511.

23. Bozue, J., K. L. Moody, C. K. Cote, B. G. Stiles, A. M. Friedlander, S. L. Welkos, and M. L. Hale. 2007. *Bacillus anthracis* Spores of the *bclA* Mutant Exhibit Increased Adherence to Epithelial Cells, Fibroblasts, and Endothelial Cells but Not to Macrophages. Infect. Immun. 75:4498-4505.

24. Brachman, P. S., H. Gold, S. A. Plotkin, F. R. Fekety, M. Werrin, and N. R. Ingraham. 1962. Field Evaluation of a Human Anthrax Vaccine. Am. J Public Health Nations. Health 52:632-645.

25. Bradley, K. A., J. Mogridge, M. Mourez, R. J. Collier, and J. A. Young. 2001. Identification of the cellular receptor for anthrax toxin. Nature 414:225-229.

26. Brahmbhatt, T. N. 2007. *Bacillus* Collagen Like Protein of Anthracis: Immunological and Functional Analyses. Ph.D. Dissertation. Uniformed Services University of the Health Sciences.

27. Brahmbhatt, T. N., S. C. Darnell, H. M. Carvalho, P. Sanz, T. J. Kang, R. L. Bull, S. B. Rasmussen, A. S. Cross, and A. D. O'Brien. 2007. Recombinant exosporium protein BclA of *Bacillus anthracis* is effective as a booster for mice primed with suboptimal amounts of protective antigen. Infect. Immun. 75:5240-5247.

28. Brahmbhatt, T. N., B. K. Janes, E. S. Stibitz, S. C. Darnell, P. Sanz, S. B. Rasmussen, and A. D. O'Brien. 2007. *Bacillus anthracis* exosporium protein BclA affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. Infect. Immun. 75:5233-5239.

29. Brittingham, K. C., G. Ruthel, R. G. Panchal, C. L. Fuller, W. J. Ribot, T. A. Hoover, H. A. Young, A. O. Anderson, and S. Bavari. 2005. Dendritic cells endocytose *Bacillus anthracis* spores: implications for anthrax pathogenesis. J Immunol 174:5545-5552.

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

31. Brossier, F., M. Mock, and J. C. Sirard. 1999. Antigen delivery by attenuated *Bacillus anthracis*: new prospects in veterinary vaccines. J Appl. Microbiol. 87:298-302.

32. Cabrera-Martinez, R. M., F. Tovar-Rojo, V. R. Vepachedu, and P. Setlow. 2003. Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. J Bacteriol. 185:2457-2464.

33. Castro, L., M. Rodriguez, and R. Radi. 1994. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J Biol Chem. 269:29409-29415.

34. Centers for Disease Control and Prevention. 2001. MMWR Update: Investigation of bioterrorism-related anthrax and interim guidelines for exposure management and antimicrobial therapy, October 2001. MMWR Morb. Mortal. Wkly. Rep. 50:909-919.

35. Centers for Disease Control and Prevention. 2008. Fact Sheet: Anthrax Information for Health Care Providers, *In* www.bt.cdc.gov/agent/anthrax/anthrax-hcp-factsheet.asp.

36. Chada, V. G., E. A. Sanstad, R. Wang, and A. Driks. 2003. Morphogenesis of *Bacillus* spore surfaces. J Bacteriol. 185:6255-6261.

37. Charlton, S., A. J. Moir, L. Baillie, and A. Moir. 1999. Characterization of the exosporium of *Bacillus cereus.* J Appl. Microbiol. 87:241-245.

38. Chiara, M. D., F. Bedoya, and F. Sobrino. 1989. Cyclosporin A inhibits phorbol ester-induced activation of superoxide production in resident mouse peritoneal macrophages. Biochem. J 264:21-26.

39. Christopher, G. W., T. J. Cieslak, J. A. Pavlin, and E. M. Eitzen, Jr. 1997. Biological warfare. A historical perspective. JAMA 278:412-417.

40. Cleret, A., A. Quesnel-Hellmann, J. Mathieu, D. Vidal, and J. N. Tournier. 2006. Resident CD11c+ lung cells are impaired by anthrax toxins after spore infection. J. Infect. Dis. 194:86-94.

41. Cleret, A., A. Quesnel-Hellmann, A. Vallon-Eberhard, B. Verrier, S. Jung, D. Vidal, J. Mathieu, and J. N. Tournier. 2007. Lung dendritic cells rapidly mediate anthrax spore entry through the pulmonary route. J. Immunol. 178:7994-8001.

42. Cohen, S., I. Mendelson, Z. Altboum, D. Kobiler, E. Elhanany, T. Bino, M. Leitner, I. Inbar, H. Rosenberg, Y. Gozes, R. Barak, M. Fisher, C. Kronman, B. Velan, and A. Shafferman. 2000. Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. Infect. Immun. 68:4549-4558.

43. Cote, C. K., J. Bozue, K. L. Moody, T. L. Dimezzo, C. E. Chapman, and S. L. Welkos. 2008. Analysis of a novel spore antigen in Bacillus anthracis that contributes to spore opsonization. Microbiology 154:619-632.

44. Cote, C. K., K. M. Rea, S. L. Norris, R. N. Van, and S. L. Welkos. 2004. The use of a model of in vivo macrophage depletion to study the role of macrophages during infection with *Bacillus anthracis* spores. Microb. Pathog. 37:169-175.

45. Cote, C. K., C. A. Rossi, A. S. Kang, P. R. Morrow, J. S. Lee, and S. L. Welkos. 2005. The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb. Pathog. 38:209-225.

46. Cote, C. K., R. N. Van, and S. L. Welkos. 2006. Roles of macrophages and neutrophils in the early host response to *Bacillus anthracis* spores in a mouse model of infection. Infect Immun 74:469-480.

47. Cybulski, R. J., P. Sanz, D. McDaniel, S. C. Darnell, R. L. Bull, and A. D. O'Brien. 2008. Recombinant *Bacillus anthracis* spore proteins enhance protection of mice primed with suboptimal amounts of protective antigen. Vaccine In press.

48. Dagan, R., D. Goldblatt, J. R. Maleckar, M. Yaich, and J. Eskola. 2004. Reduction of antibody response to an 11-valent pneumococcal vaccine

coadministered with a vaccine containing acellular pertussis components. Infect. Immun. 72:5383-5391.

49. Daniel, D. S., G. Dai, C. R. Singh, D. R. Lindsey, A. K. Smith, S. Dhandayuthapani, R. L. Hunter, Jr., and C. Jagannath. 2006. The reduced bactericidal function of complement C5-deficient murine macrophages is associated with defects in the synthesis and delivery of reactive oxygen radicals to mycobacterial phagosomes. J Immunol. 177:4688-4698.

50. de Benito, A. A., N. L. Padula, B. Setlow, and P. Setlow. 2008. Sensitization of *Bacillus subtilis* spores to dry heat and desiccation by pretreatment with oxidizing agents. Lett. Appl. Microbiol. 46:492-497.

51. Dequeiroz, G. A. and D. F. Day. 2008. Disinfection of *Bacillus subtilis* spore-contaminated surface materials with a sodium hypochlorite and a hydrogen peroxide-based sanitizer. Lett. Appl. Microbiol. 46:176-180.

52. Desrosier, J. P. and J. Cano Lara. 1984. Synthesis of the Exosporium During Sporulation of *Bacillus cereus.* J. Gen. Microbiol. 130:935-940.

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

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

55. Dojindo Molecular Technologies. 2004. SOD Assay Kit-WST: technical manual. Dojindo Molecular Technologies, Gaithersburg, MD.

56. Driks, A. 1999. *Bacillus subtilis* spore coat. Microbiol. Mol. Biol Rev. 63:1-20.

57. Driks, A. 2002. Proteins of the spore core and coat, p. 527-536. *In* A. Sonenshein, J. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C.

58. Driks, A. 2004. From Rings to Layers: Surprising Patterns of Protein Deposition during Bacterial Spore Assembly. J Bacteriol. 186:4423-4426.

59. Driks, A., S. Roels, B. Beall, C. P. Moran, Jr., and R. Losick. 1994. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Genes Dev. 8:234-244.

60. Duesbery, N. S. and G. F. Vande Woude. 1999. Anthrax lethal factor causes proteolytic inactivation of mitogen-activated protein kinase kinase. J Appl. Microbiol. 87:289-293.

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

62. Edwards, K. M., M. H. Cynamon, R. K. Voladri, C. C. Hager, M. S. DeStefano, K. T. Tham, D. L. Lakey, M. R. Bochan, and D. S. Kernodle. 2001. Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis*. Am. J Respir. Crit Care Med. 164:2213-2219.

63. Enkhtuya, J., K. Kawamoto, Y. Kobayashi, I. Uchida, N. Rana, and S. Makino. 2006. Significant passive protective effect against anthrax by antibody to *Bacillus anthracis* inactivated spores that lack two virulence plasmids. Microbiology 152:3103-3110.

64. Ezzell, J. W. and S. L. Welkos. 1999. The capsule of *Bacillus anthracis*, a review. J. Appl. Microbiol. 87:250.

65. Fang, F. C. 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. 2:820-832.

66. Fang, F. C., M. A. DeGroote, J. W. Foster, A. J. Bäumler, U. Ochsner, T. Testerman, S. Bearson, J. C. Giárd, Y. Xu, G. Campbell, and T. Laessig. 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc. Natl. Acad. Sci. U. S. A 96:7502-7507.

67. Farchaus, J. W., W. J. Ribot, S. Jendrek, and S. F. Little. 1998. Fermentation, purification, and characterization of protective antigen from a recombinant, avirulent strain of *Bacillus anthracis.* Appl. Environ. Microbiol. 64:982-991.

68. Farrant, J. L., A. Sansone, J. R. Canvin, M. J. Pallen, P. R. Langford, T. S. Wallis, G. Dougan, and J. S. Kroll. 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. Mol. Microbiol. 25:785-796.

69. Fellows, P. F., M. K. Linscott, B. E. Ivins, M. L. Pitt, C. A. Rossi, P. H. Gibbs, and A. M. Friedlander. 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine 19:3241-3247.

70. Flint, D. H., J. F. Tuminello, and M. H. Emptage. 1993. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J Biol Chem. 268:22369-22376.

71. Foster, S. J. and K. Johnstone. 1990. Pulling the trigger: the mechanism of bacterial spore germination. Mol. Microbiol. 4:137-141.

72. Franz, D. R., P. B. Jahrling, D. J. McClain, D. L. Hoover, W. R. Byrne, J. A. Pavlin, G. W. Christopher, T. J. Cieslak, A. M. Friedlander, and E. M. Eitzen, Jr. 2001. Clinical recognition and management of patients exposed to biological warfare agents. Clin. Lab Med. 21:435-473.

73. Franzon, V. L., J. Arondel, and P. J. Sansonetti. 1990. Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. Infect. Immun. 58:529-535.

74. Fridovich, I. 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. 64:97-112.

75. Friedlander, A. M., P. R. Pittman, and G. W. Parker. 1999. Anthrax vaccine: evidence for safety and efficacy against inhalational anthrax. JAMA 282:2104-2106.

76. Friedlander, A. M., S. L. Welkos, and B. E. Ivins. 2002. Anthrax vaccines. Curr. Top. Microbiol. Immunol. 271:33-60.

77. Friedlander, A. M., S. L. Welkos, M. L. Pitt, J. W. Ezzell, P. L. Worsham, K. J. Rose, B. E. Ivins, J. R. Lowe, G. B. Howe, P. Mikesell, and . 1993. Postexposure prophylaxis against experimental inhalation anthrax. J Infect. Dis. 167:1239-1243.

78. Gardner, P. R., G. Costantino, C. Szabo, and A. L. Salzman. 1997. Nitric oxide sensitivity of the aconitases. J Biol Chem. 272:25071-25076.

79. Gee, J. M., M. W. Valderas, M. E. Kovach, V. K. Grippe, G. T. Robertson, W. L. Ng, J. M. Richardson, M. E. Winkler, and R. M. Roop. 2005. The *Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. Infect Immun 73:2873-2880.

80. Gerhardt, P. and E. Ribi. 1964. Ultrastructure of the exosporium enveloping spores of *Bacillus cereus.* J Bacteriol 88:1774-1789.

81. Giorno, R., J. Bozue, C. Cote, T. Wenzel, K. S. Moody, M. mallozzi, M. Ryan, R. Wang, R. Zielke, J. R. Maddock, A. Friedlander, S. Welkos, and A. Driks. 2007. Morphogenesis of the *Bacillus anthracis* spore. J Bacteriol 189:691-705.

82. Glomski, I. J., J. P. Corre, M. Mock, and P. L. Goossens. 2007. Cutting Edge: IFN-γ-producing CD4 T lymphocytes mediate spore-induced immunity to capsulated *Bacillus anthracis.* J Immunol 178:2646-2650.

83. Gort, A. S. and J. A. Imlay. 1998. Balance between endogenous superoxide stress and antioxidant defenses. J Bacteriol. 180:1402-1410.

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

85. Guarner, J., J. A. Jernigan, W. J. Shieh, K. Tatti, L. M. Flannagan, D. S. Stephens, T. Popovic, D. A. Ashford, B. A. Perkins, and S. R. Zaki. 2003. Pathology and pathogenesis of bioterrorism-related inhalational anthrax. Am. J Pathol. 163:701-709.

86. Guidi-Rontani, C. 2002. The alveolar macrophage: the Trojan horse of *Bacillus anthracis*. Trends Microbiol. 10:405-409.

87. Guidi-Rontani, C., M. Levy, H. Ohayon, and M. Mock. 2001. Fate of germinated *Bacillus anthracis* spores in primary murine macrophages. Mol. Microbiol. 42:931-938.

88. Guidi-Rontani, C., Y. Pereira, S. Ruffie, J. C. Sirard, M. Weber-Levy, and M. Mock. 1999. Identification and characterization of a germination operon on the virulence plasmid pXO1 of *Bacillus anthracis*. Mol. Microbiol. 33:407-414.

89. Guidi-Rontani, C., M. Weber-Levy, E. Labruyere, and M. Mock. 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. 31:9-17.

90. Guidi-Rontani, C., M. Weber-Levy, M. Mock, and V. Cabiaux. 2000. Translocation of *Bacillus anthracis* lethal and oedema factors across endosome membranes. Cell Microbiol. 2:259-264.

91. Hachisuka, Y., K. Kojima, and T. Sato. 1966. Fine filaments on the outside of the exosporium of *Bacillus anthracis* spores. J Bacteriol. 91:2382-2384.

92. Hahn, U. K., R. Boehm, and W. Beyer. 2005. DNA vaccination against anthrax in mice-combination of anti-spore and anti-toxin components. Vaccine .

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

94. Hanna, P. C. and J. A. Ireland. 1999. Understanding *Bacillus anthracis* pathogenesis. Trends Microbiol. 7:180-182.

95. Hausladen, A. and I. Fridovich. 1994. Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. J Biol Chem. 269:29405-29408.

96. Henriques, A. O., B. W. Beall, K. Roland, and C. P. Moran, Jr. 1995. Characterization of cotJ, a sigma E-controlled operon affecting the polypeptide composition of the coat of *Bacillus subtilis* spores. J Bacteriol. 177:3394-3406.

97. Henriques, A. O., L. R. Melsen, and C. P. Moran, Jr. 1998. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis.* J Bacteriol. 180:2285-2291.

98. Henriques, A. O. and C. P. Moran, Jr. 2000. Structure and assembly of the bacterial endospore coat. Methods 20:95-110.

99. Henriques, A. O. and C. P. Moran, Jr. 2007. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. 61:555-588.

100. Hoffmaster, A. R. and T. M. Koehler. 1999. Autogenous regulation of the *Bacillus anthracis pag* operon. J. Bacteriol. 181:4485-4492.

101. Holt, S. C. and E. R. Leadbetter. 1969. Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. Bacteriol. Rev. 33:346-378.

102. Hoover, D. L., A. M. Friedlander, L. C. Rogers, I. K. Yoon, R. L. Warren, and A. S. Cross. 1994. Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP. Infect. Immun. 62:4432-4439.

103. Hu, H., Q. Sa, T. M. Koehler, A. I. Aronson, and D. Zhou. 2006. Inactivation of *Bacillus anthracis* spores in murine primary macrophages. Cell Microbiol 8:1634-1642.

104. Imlay, J. A. 1995. A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. J Biol Chem. 270:19767-19777.

105. Imlay, J. A. and I. Fridovich. 1991. Assay of metabolic superoxide production in *Escherichia coli*. J Biol Chem. 266:6957-6965.

106. Ivins, B., P. Fellows, L. Pitt, J. Estep, J. Farchaus, A. Friedlander, and P. Gibbs. 1995. Experimental anthrax vaccines: efficacy of adjuvants combined with

protective antigen against an aerosol Bacillus anthracis spore challenge in guinea pigs. Vaccine 13:1779-1784.

107. Ivins, B. E., M. L. Pitt, P. F. Fellows, J. W. Farchaus, G. E. Benner, D. M. Waag, S. F. Little, G. W. Anderson, Jr., P. H. Gibbs, and A. M. Friedlander. 1998. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine 16:1141-1148.

108. Ivins, B. E., S. L. Welkos, S. F. Little, M. H. Crumrine, and G. O. Nelson. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. Infect. Immun. 60:662-668.

109. Janes, B. K. and S. Stibitz. 2006. Routine markerless gene replacement in *Bacillus anthracis*. Infect Immun 74:1949-1953.

110. Jernigan, D. B., P. L. Raghunathan, B. P. Bell, R. Brechner, E. A. Bresnitz, J. C. Butler, M. Cetron, M. Cohen, T. Doyle, M. Fischer, C. Greene, K. S. Griffith, J. Guarner, J. L. Hadler, J. A. Hayslett, R. Meyer, L. R. Petersen, M. Phillips, R. Pinner, T. Popovic, C. P. Quinn, J. Reefhuis, D. Reissman, N. Rosenstein, A. Schuchat, W. J. Shieh, L. Siegal, D. L. Swerdlow, F. C. Tenover, M. Traeger, J. W. Ward, I. Weisfuse, S. Wiersma, K. Yeskey, S. Zaki, D. A. Ashford, B. A. Perkins, S. Ostroff, J. Hughes, D. Fleming, J. P. Koplan, and J. L. Gerberding. 2002. Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings. Emerg. Infect Dis 8:1019-1028.

111. Jernigan, J. A., D. S. Stephens, D. A. Ashford, C. Omenaca, M. S. Topiel, M. Galbraith, M. Tapper, T. L. Fisk, S. Zaki, T. Popovic, R. F. Meyer, C. P. Quinn, S. A. Harper, S. K. Fridkin, J. J. Sejvar, C. W. Shepard, M. McConnell, J. Guarner, W. J. Shieh, J. M. Malecki, J. L. Gerberding, J. M. Hughes, and B. A. Perkins. 2001. Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. Emerg. Infect. Dis. 7:933-944.

112. Kanafani, Z. A., A. Ghossain, A. I. Sharara, J. M. Hatem, and S. S. Kanj. 2003. Endemic gastrointestinal anthrax in 1960s Lebanon: clinical manifestations and surgical findings. Emerg. Infect. Dis. 9:520-525.

113. Kang, T. J., M. J. Fenton, M. A. Weiner, S. Hibbs, S. Basu, L. Baillie, and A. S. Cross. 2005. Murine macrophages kill the vegetative form of *Bacillus anthracis*. Infect Immun 73:7495-7501.

114. Karavolos, M. H., M. J. Horsburgh, E. Ingham, and S. J. Foster. 2003. Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. Microbiology 149:2749-2758.

115. Keim, P. and K. L. Smith. 2002. *Bacillus anthracis* evolution and epidemiology. Curr. Top. Microbiol. Immunol. 271:21-32.

116. Keppie, J., H. Smith, and P. W. Harris-Smith. 1955. The chemical basis of the virulence of *Bacillus anthracis*. III. The role of the terminal bacteraemia in death of guinea-pigs from anthrax. Br. J Exp. Pathol. 36:315-322.

117. Keyer, K. and J. A. Imlay. 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proc. Natl. Acad. Sci. U. S. A 93:13635-13640.

118. Keyer, K. and J. A. Imlay. 1997. Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. J Biol Chem. 272:27652-27659.

119. Khelef, N., D. DeShazer, R. L. Friedman, and N. Guiso. 1996. In vivo and in vitro analysis of *Bordetella pertussis* catalase and Fe-superoxide dismutase mutants. FEMS Microbiol. Lett. 142:231-235.

120. Kim, H. U. and J. M. Goepfert. 1974. A sporulation medium for *Bacillus anthracis*. J Appl. Bacteriol 37:265-267.

121. Kirby, J. E. 2004. Anthrax lethal toxin induces human endothelial cell apoptosis. Infect Immun. 72:430-439.

122. Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. J Exp. Med. 126:1063-1078.

123. Klebanoff, S. J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J Bacteriol 95:2131-2138.

124. Klein, F., I. A. Dearmon, Jr., R. E. Lincoln, B. G. Mahlandt, and A. L. Fernelius. 1962. Immunological studies of anthrox. II. Levels of immunity against *Bacillus anthracis* obtained with protective antigen and live vaccine. J Immunol. 88:15-19.

125. Klimpel, K. R., S. S. Molloy, G. Thomas, and S. H. Leppla. 1992. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc. Natl. Acad. Sci. U. S. A 89:10277-10281.

126. Kochi, S. K., I. Martin, G. Schiavo, M. Mock, and V. Cabiaux. 1994. The effects of pH on the interaction of anthrax toxin lethal and edema factors with phospholipid vesicles. Biochemistry 33:2604-2609.

127. Koehler, T. M. 2002. *Bacillus anthracis* genetics and virulence gene regulation. Curr. Top. Microbiol. Immunol. 271:143-164.

128. Kuwana, R., Y. Kasahara, M. Fujibayashi, H. Takamatsu, N. Ogasawara, and K. Watabe. 2002. Proteomics characterization of novel spore proteins of *Bacillus subtilis*. Microbiology 148:3971-3982.

129. Lacy, D. B. and R. J. Collier. 2002. Structure and function of anthrax toxin. Curr. Top. Microbiol. Immunol. 271:61-85.

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

131. Lai, E. M., N. D. Phadke, M. T. Kachman, R. Giorno, S. Vazquez, J. A. Vazquez, J. R. Maddock, and A. Driks. 2003. Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. J. Bacteriol. 185:1443-1454.

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

133. Leppla, S. H. 1984. *Bacillus anthracis* calmodulin-dependent adenylate cyclase: chemical and enzymatic properties and interactions with eucaryotic cells. Adv. Cyclic. Nucleotide. Protein Phosphorylation. Res. 17:189-198.

134. Liochev, S. I. and I. Fridovich. 1994. The role of O2.- in the production of HO.: in vitro and in vivo. Free Radic. Biol Med. 16:29-33.

135. Little, S. F., B. E. Ivins, P. F. Fellows, and A. M. Friedlander. 1997. Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. Infect. Immun. 65:5171-5175.

136. Little, S. F., B. E. Ivins, P. F. Fellows, M. L. Pitt, S. L. Norris, and G. P. Andrews. 2004. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine 22:422-430.

137. Little, S. F. and G. B. Knudson. 1986. Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. Infect. Immun. 52:509-512.

138. Little, S. F., S. H. Leppla, and E. Cora. 1988. Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. Infect Immun. 56:1807-1813.

139. Liu, H., N. H. Bergman, B. Thomason, S. Shallom, A. Hazen, J. Crossno, D. A. Rasko, J. Ravel, T. D. Read, S. N. Peterson, J. Yates, III, and P. C. Hanna. 2004. Formation and Composition of the *Bacillus anthracis* Endospore. J Bacteriol. 186:164-178.

140. Losick, R. and P. Stragier. 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature 355:601-604.

141. Lybarger, S., N. Fisher, and P. Hanna. 2008. Abstr. 5th Natl. RCE Meet., Chicago, IL. Spore Germination: Immediate Early Stage of Anthrax, *In* .

142. Lynch, R. E. and I. Fridovich. 1978. Effects of superoxide on the erythrocyte membrane. J Biol Chem. 253:1838-1845.

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

144. Matz, L. L., T. C. Beaman, and P. Gerhardt. 1970. Chemical composition of exosporium from spores of *Bacillus cereus*. J Bacteriol 101:196-201.

145. Mayer-Scholl, A., R. Hurwitz, V. Brinkmann, M. Schmid, P. Jungblut, Y. Weinrauch, and A. Zychlinsky. 2005. Human neutrophils kill *Bacillus anthracis*. PLoS. Pathog. 1:0179-0186.

146. McCord, J. M. and I. Fridovich. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem. 244:6049-6055.

147. McKevitt, M. T., K. M. Bryant, S. M. Shakir, J. L. Larabee, S. R. Blanke, J. Lovchik, C. R. Lyons, and J. D. Ballard. 2007. Effects of endogenous D-alanine synthesis and autoinhibition of *Bacillus anthracis* germination on in vitro and in vivo infections. Infect. Immun. 75:5726-5734.

148. Meador-Parton, J. and D. L. Popham. 2000. Structural analysis of *Bacillus subtilis* spore peptidoglycan during sporulation. J Bacteriol. 182:4491-4499.

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

150. Mikszta, J. A., V. J. Sullivan, C. Dean, A. M. Waterston, J. B. Alarcon, J. P. Dekker, III, J. M. Brittingham, J. Huang, C. R. Hwang, M. Ferriter, G. Jiang, K. Mar, K. U. Saikh, B. G. Stiles, C. J. Roy, R. G. Ulrich, and N. G. Harvey. 2005. Protective immunization against inhalational anthrax: a comparison of minimally invasive delivery platforms. J. Infect. Dis. 191:278-288.

151. Milne, J. C. and R. J. Collier. 1993. pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. Mol. Microbiol. 10:647-653.

152. Milne, J. C., D. Furlong, P. C. Hanna, J. S. Wall, and R. J. Collier. 1994. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J Biol. Chem. 269:20607-20612.

153. Moayeri, M. and S. H. Leppla. 2004. The roles of anthrax toxin in pathogenesis. Curr. Opin. Microbiol. 7:19-24.

154. Mock, M. and A. Fouet. 2001. Anthrax. Annu. Rev. Microbiol. 55:647-671.

155. Moir, A., B. M. Corfe, and J. Behravan. 2002. Spore germination. Cell Mol. Life Sci. 59:403-409.

156. Molnar, D. M. and R. A. Altenbern. 1963. Alterations in the biologocal activity of protective antigen of *Bacillus anthracis* toxin. Proc. Soc. Exp. Biol. Med. 114:294-297.

157. Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64:548-572.

158. Nunoshiba, T., T. DeRojas-Walker, J. S. Wishnok, S. R. Tannenbaum, and B. Demple. 1993. Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. Proc. Natl. Acad. Sci. U. S. A 90:9993-9997.

159. O'Brien, J., A. Friedlander, T. Dreier, J. Ezzell, and S. Leppla. 1985. Effects of anthrax toxin components on human neutrophils. Infect. Immun. 47:306-310.

160. Ohye, D. F. and W. G. Murrell. 1973. Exosporium and spore coat formation in *Bacillus cereus* T. J Bacteriol. 115:1179-1190.

161. Oliva, C. R., M. K. Swiecki, C. E. Griguer, M. W. Lisanby, D. C. Bullard, C. L. Turnbough, and J. F. Kearney. 2008. The integrin Mac-1 (CR3) mediates

internalization and directs *Bacillus anthracis* spores into professional phagocytes. PNAS 105:1261-1266.

162. Palenik, B., B. Brahamsha, F. W. Larimer, M. Land, L. Hauser, P. Chain, J. Lamerdin, W. Regala, E. E. Allen, J. McCarren, I. Paulsen, A. Dufresne, F. Partensky, E. A. Webb, and J. Waterbury. 2003. The genome of a motile marine Synechococcus. Nature 424:1037-1042.

163. Park, J. M., F. R. Greten, Z. W. Li, and M. Karin. 2002. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science 297:2048-2051.

164. Passalacqua, K. D., N. H. Bergman, A. Herring-Palmer, and P. Hanna. 2006. The superoxide dismutases of *Bacillus anthracis* do not cooperatively protect against endogenous superoxide stress. J Bacteriol 188:3837-3848.

165. Peachman, K. K., M. Rao, C. R. Alving, R. Burge, S. H. Leppla, V. B. Rao, and G. R. Matyas. 2006. Correlation between lethal toxin-neutralizing antibody titers and protection from intranasal challenge with *Bacillus anthracis* Ames strain spores in mice after transcutaneous immunization with recombinant anthrax protective antigen. Infect Immun 74:794-797.

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

167. Pezard, C., P. Berche, and M. Mock. 1991. Contribution of individual toxin components to virulence of *Bacillus anthracis*. Infect. Immun. 59:3472-3477.

168. Piddington, D. L., F. C. Fang, T. Laessig, A. M. Cooper, I. M. Orme, and N. A. Buchmeier. 2001. Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infect. Immun. 69:4980-4987.

169. Piggot, P. and R. Losick. 2002. Sporulation genes and intercompartmental regulation in *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C.

170. Pitt, M. L., S. Little, B. E. Ivins, P. Fellows, J. Boles, J. Barth, J. Hewetson, and A. M. Friedlander. 1999. *In vitro* correlate of immunity in an animal model of inhalational anthrax. J Appl. Microbiol. 87:304.

171. Pitt, M. L., S. F. Little, B. E. Ivins, P. Fellows, J. Barth, J. Hewetson, P. Gibbs, M. Dertzbaugh, and A. M. Friedlander. 2001. *In vitro* correlate of immunity in a rabbit model of inhalational anthrax. Vaccine 19:4768-4773.

172. Pittman, P. R., S. F. Leitman, J. G. Oro, S. L. Norris, N. M. Marano, M. V. Ranadive, B. S. Sink, and K. T. McKee, Jr. 2005. Protective antigen and toxin neutralization antibody patterns in anthrax vaccinees undergoing serial plasmapheresis. Clin. Diagn. Lab Immunol. 12:713-721.

173. Pollock, J. D., D. A. Williams, M. A. Gifford, L. L. Li, X. Du, J. Fisherman, S. H. Orkin, C. M. Doerschuk, and M. C. Dinauer. 1995. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. Nat. Genet. 9:202-209.

174. Popham, D. L. 2002. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cell Mol. Life Sci. 59:426-433.

175. Popham, D. L., M. E. Gilmore, and P. Setlow. 1999. Roles of low-molecular-weight penicillin-binding proteins in *Bacillus subtilis* spore peptidoglycan synthesis and spore properties. J Bacteriol. 181:126-132.

176. Popham, D. L., J. Helin, C. E. Costello, and P. Setlow. 1996. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc. Natl. Acad. Sci. U. S. A 93:15405-15410.

177. Poyart, C., E. Pellegrini, O. Gaillot, C. Boumaila, M. Baptista, and P. Trieu-Cuot. 2001. Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of *Streptococcus agalactiae*. Infect. Immun. 69:5098-5106.

178. Purdy, D., S. Cawthraw, J. H. Dickinson, D. G. Newell, and S. F. Park. 1999. Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter*

coli: evidence for the significance of SOD in Campylobacter survival and colonization. Appl. Environ. Microbiol. 65:2540-2546.

179. Radi, R., J. S. Beckman, K. M. Bush, and B. A. Freeman. 1991. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem. 266:4244-4250.

180. Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A. OKstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. DeBoy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Jiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A. B. Kolsto, and C. M. Fraser. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature 423:81-86.

181. Read, T. D., S. L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J. D. Busch, K. L. Smith, J. M. Schupp, D. Solomon, P. Keim, and C. M. Fraser. 2002. Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis.* Science 296:2028-2033.

182. Redmond, C., L. W. Baillie, S. Hibbs, A. J. Moir, and A. Moir. 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology 150:355-363.

183. Reuveny, S., M. D. White, Y. Y. Adar, Y. Kafri, Z. Altboum, Y. Gozes, D. Kobiler, A. Shafferman, and B. Velan. 2001. Search for correlates of protective immunity conferred by anthrax vaccine. Infect Immun 69:2888-2893.

184. Robinow, C. F. 1951. Observations on the structure of *Bacillus* spores. J Gen. Microbiol. 5:439-457.

185. Roels, S., A. Driks, and R. Losick. 1992. Characterization of spoIVA, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*. J Bacteriol. 174:575-585.

186. Rose, R., B. Setlow, A. Monroe, M. mallozzi, A. Driks, and P. Setlow. 2007. Comparison of the properties of *Bacillus subtilis* spores made in liquid or on agar plates. J Appl. Microbiol 103:691-699.

187. Ross, J. M. 1957. The pathogenesis of anthrax following the administration of spores by the respiratory route. J. Pathol. Bacteriol. 73:485-494.

188. ROTH, N. G. and D. H. LIVELY. 1956. Germination of spores of certain aerobic bacilli under anaerobic conditions. J Bacteriol. 71:162-166.

189. Ruthel, G., W. J. Ribot, S. Bavari, and T. A. Hoover. 2004. Time-lapse confocal imaging of development of *Bacillus anthracis* in macrophages. J. Infect. Dis. 189:1313-1316.

190. Santelli, E., L. A. Bankston, S. H. Leppla, and R. C. Liddington. 2004. Crystal structure of a complex between anthrax toxin and its host cell receptor. Nature 430:905-908.

191. Sanz, P., L. D. Teel, F. Alem, H. M. Carvalho, S. C. Darnell, and A. D. O'Brien. 2008. Detection of *Bacillus anthracis* spore germination in vivo by bioluminescence imaging. Infect. Immun. 76:1036-1047.

192. Schechter, I. 1968. Antigenic competition between polypeptidyl determinants in normal and tolerant rabbits. J Exp. Med. 127:237-250.

193. Scobie, H. M., G. J. Rainey, K. A. Bradley, and J. A. Young. 2003. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc. Natl. Acad. Sci. U. S. A 100:5170-5174.

194. Scott, I. R. and D. J. Ellar. 1978. Metabolism and the triggering of germination of *Bacillus megaterium*. Use of L-[3H]alanine and tritiated water to detect metabolism. Biochem. J 174:635-640.

195. Setlow, P. 1988. Small, acid-soluble spore proteins of *Bacillus* species: structure, synthesis, genetics, function, and degradation. Annu. Rev. Microbiol 42:319-338.

196. Setlow, P. 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annu. Rev. Microbiol. 49:29-54.

197. Setlow, P. 2003. Spore germination. Curr. Opin. Microbiol. 6:550-556.

198. Shafazand, S., R. Doyle, S. Ruoss, A. Weinacker, and T. A. Raffin. 1999. Inhalational anthrax: epidemiology, diagnosis, and management. Chest 116:1369-1376.

199. Shlyakhov, E. N. and E. Rubinstein. 1994. Human live anthrax vaccine in the former USSR. Vaccine 12:727-730.

200. Singh, Y., K. R. Klimpel, S. Goel, P. K. Swain, and S. H. Leppla. 1999. Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. Infect. Immun. 67:1853-1859.

201. Sirisanthana, T., K. E. Nelson, J. W. Ezzell, and T. G. Abshire. 1988. Serological studies of patients with cutaneous and oral-oropharyngeal anthrax from northern Thailand. Am. J Trop. Med. Hyg. 39:575-581.

202. Sloat, B. R. and Z. Cui. 2006. Nasal immunization with anthrax protective antigen protein adjuvanted with polyriboinosinic-polyribocytidylic acid induced strong mucosal and systemic immunities. Pharm. Res. 23:1217-1226.

203. Smith, H., J. Keppie, and J. L. Stanley. 1955. The chemical basis of the virulence of *Bacillus anthracis.* V. The specific toxin produced by *B. Anthracis* in vivo. Br. J Exp. Pathol. 36:460-472.

204. Smith, H., J. Keppie, J. L. Stanley, and P. W. Harris-Smith. 1955. The chemical basis of the virulence of *Bacillus anthracis.* IV. Secondary shock as the major factor in death of guinea-pigs from anthrax. Br. J Exp. Pathol. 36:323-335.

205. Stanley, J. L. and H. Smith. 1961. Purification of factor I and recognition of a third factor of the anthrax toxin. J Gen. Microbiol. 26:49-63.

206. Steichen, C., P. Chen, J. F. Kearney, and C. L. Turnbough, Jr. 2003. Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. J Bacteriol. 185:1903-1910.

207. Steichen, C. T., J. F. Kearney, and C. L. Turnbough, Jr. 2005. Characterization of the exosporium basal layer protein BxpB of *Bacillus anthracis.* J Bacteriol. 187:5868-5876.

208. Steinman, H. M. 1993. Function of periplasmic copper-zinc superoxide dismutase in *Caulobacter crescentus*. J Bacteriol. 175:1198-1202.

209. Sternbach, G. 2003. The history of anthrax. J Emerg. Med. 24:463-467.

210. Stevens, C. M., R. Daniel, N. Illing, and J. Errington. 1992. Characterization of a sporulation gene, spoIVA, involved in spore coat morphogenesis in *Bacillus subtilis*. J Bacteriol. 174:586-594.

211. Storz, G. and J. A. Imlay. 1999. Oxidative stress. Curr. Opin. Microbiol. 2:188-194.

212. Strauch, M. A. and J. A. Hoch. 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol. Microbiol. 7:337-342.

213. Sylvestre, P., E. Couture-Tosi, and M. Mock. 2002. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. Mol. Microbiol. 45:169-178.

214. The Jackson Laboratory. 2008. A/J Strain Information Page, *In* http://jaxmice.jax.org/strain/000646.html.

215. Thorne, C. B. 1993. *Bacillus anthracis*, p. 113-124. *In* A. Sonenshein, J. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. ASM Press, Washington, D.C.

216. Tigertt, W. D. 1980. Anthrax. William Smith Greenfield, M.D., F.R.C.P., Professor Superintendent, the Brown Animal Sanatory Institution (1878-81). Concerning the priority due to him for the production of the first vaccine against anthrax. J Hyg. (Lond) 85:415-420.

217. Todd, S. J., A. J. Moir, M. J. Johnson, and A. Moir. 2003. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. J Bacteriol. 185:3373-3378.

218. Tsolis, R. M., A. J. Bäumler, and F. Heffron. 1995. Role of *Salmonella typhimurium* Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. Infect. Immun. 63:1739-1744.

219. Turnbull, P. C. 2000. Current status of immunization against anthrax: old vaccines may be here to stay for a while. Curr. Opin. Infect Dis 13:113-120.

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

221. Turnbull, P. C., S. H. Leppla, M. G. Broster, C. P. Quinn, and J. Melling. 1988. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Med. Microbiol Immunol 177:293-303.

222. Tutrone, W. D., N. S. Scheinfeld, and J. M. Weinberg. 2002. Cutaneous anthrax: a concise review. Cutis 69:27-33.

223. Uchida, I., K. Hashimoto, and N. Terakado. 1986. Virulence and immunogenicity in experimental animals of *Bacillus anthracis* strains harbouring or lacking 110 MDa and 60 MDa plasmids. J Gen. Microbiol. 132:557-559.

224. van Ooij, C., P. Eichenberger, and R. Losick. 2004. Dynamic patterns of subcellular protein localization during spore coat morphogenesis in *Bacillus subtilis*. J Bacteriol. 186:4441-4448.

225. Verneuil, N., A. Mazé, M. Sanguinetti, J. M. Laplace, A. Benachour, Y. Auffray, J. C. Giard, and A. Hartke. 2006. Implication of (Mn)superoxide dismutase of *Enterococcus faecalis* in oxidative stress responses and survival inside macrophages. Microbiology 152:2579-2589.

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

227. Vreeland, R. H., W. D. Rosenzweig, and D. W. Powers. 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature 407:897-900.

228. Wade, B. H., G. G. Wright, E. L. Hewlett, S. H. Leppla, and G. L. Mandell. 1985. Anthrax toxin components stimulate chemotaxis of human polymorphonuclear neutrophils. Proc. Soc. Exp. Biol Med. 179:159-162.

229. Weaver, J., T. J. Kang, K. W. Raines, G. L. Cao, S. Hibbs, P. Tsai, L. Baillie, G. M. Rosen, and A. S. Cross. 2007. Protective role of *Bacillus anthracis* exosporium in macrophage-mediated killing by nitric oxide. Infect Immun 75:3894-3901.

230. Weiner, M. A. and P. C. Hanna. 2003. Macrophage-mediated germination of *Bacillus anthracis* endospores requires the *gerH* operon. Infect Immun 71:3954-3959.

231. Welkos, S., A. Friedlander, S. Weeks, S. Little, and I. Mendelson. 2002. *In-vitro* characterisation of the phagocytosis and fate of anthrax spores in macrophages and the effects of anti-PA antibody. J Med. Microbiol. 51:821-831.

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

233. Welkos, S. L., C. K. Cote, K. M. Rea, and P. H. Gibbs. 2004. A microtiter fluorometric assay to detect the germination of *Bacillus anthracis* spores and the germination inhibitory effects of antibodies. J Microbiol. Methods 56:253-265.

234. Welkos, S. L. and A. M. Friedlander. 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. Microb. Pathog. 5:127-139.

235. Welkos, S. L. and A. M. Friedlander. 1988. Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. Microb. Pathog. 4:53-69.

236. Wilks, K. E., K. L. Dunn, J. L. Farrant, K. M. Reddin, A. R. Gorringe, P. R. Langford, and J. S. Kroll. 1998. Periplasmic superoxide dismutase in meningococcal pathogenicity. Infect. Immun. 66:213-217.

237. Williamson, E. D., A. M. Bennett, S. D. Perkins, R. J. Beedham, J. Miller, and L. W. Baillie. 2002. Co-immunisation with a plasmid DNA cocktail primes mice against anthrax and plague. Vaccine 20:2933-2941.

238. World Health Organization. 2008. Guidelines for the Surveillance and Control of Anthrax in Humans and Animals, *In* www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/.

239. World Health Organization. 2008. World Anthrax Data Site, *In* www.vetmed.lsu.edu/whocc/mp_world.htm.

240. Wright, G. G. and G. L. Mandell. 1986. Anthrax toxin blocks priming of neutrophils by lipopolysaccharide and by muramyl dipeptide. J Exp. Med. 164:1700-1709.

241. Xu, S., T. P. Labuza, and F. ez-Gonzalez. 2008. Inactivation kinetics of avirulent *Bacillus anthracis* spores in milk with a combination of heat and hydrogen peroxide. J Food Prot. 71:333-338.

242. Zheng, L. B., W. P. Donovan, P. C. Fitz-James, and R. Losick. 1988. Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. Genes Dev. 2:1047-1054.

243. Zilhao, R., M. Serrano, R. Isticato, E. Ricca, C. P. Moran, Jr., and A. O. Henriques. 2004. Interactions among CotB, CotG, and CotH during assembly of the *Bacillus subtilis* spore coat. J Bacteriol. 186:1110-1119.