Mechanisms of *Bacillus anthracis* spore cortex degradation

by

Jonathan Daniel Giebel

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in The University of Michigan
2011

Doctoral Committee:

Associate Professor Philip C. Hanna, Chair
Professor Vernon B. Carruthers
Professor Victor DiRita
Professor Harry L.T. Mobley
Associate Professor Matt R. Chapman

Dedication

Susan Marie Giebel

Acknowledgements

First and foremost I would like to say thank you to Phil Hanna and all of the members of the Hanna lab that I have had the pleasure of crossing paths with over the past four years. Phil, you have truly been a mentor to me and I am grateful for all of your advice, both scientific and personal. I could not have asked for a better advisor.

I would also like to thank my friends and family who have supported me through this process. Thank you especially to my wife for her love and support in everything I do.

Table of Contents

List of Figures

List of Tables

Abstract

Bacillus anthracis exists in two morphologically distinct states, the spore and the vegetative cell. The deadly animal and human disease anthrax results from *B. anthracis* spores entering a suitable host and transitioning to a toxin producing vegetative cell through germination. The spore cortex is a modified peptidoglycan structure that is critical for spore stability and dormancy and must be hydrolyzed during germination to allow vegetative-cell outgrowth. In *Bacillus anthracis,* this process was thought to involve the enzymatic action of a group of proteins called germination-specific lytic enzymes (GSLEs). I have shown that the GSLEs SleB, CwlJ1 and CwlJ2 are all involved in cortex hydrolysis and that a second pathway, host-lysozyme induced germination, is capable of degrading the cortex in vivo.

Traditional nutrient based germination requires either SleB or CwlJ1, both in vitro and in vivo. In the absence of these two primary GSLEs, CwlJ2 provides minimal cortex hydrolyzing activity and germination rates are severely decreased. Mutant spores lacking all three GSLEs (*ΔsleB/cwlJ1/cwlJ2*) are unable to complete germination in nutrient rich medium in vitro. Although *ΔsleB/cwlJ1/cwlJ2* spores are incapable of germinating in response to nutrients, these mutants are able to germinate at a low rate when incubated in serum, whole blood, or a solution containing purified lysozyme.

Spores lacking SleB and CwlJ1 as well as *ΔsleB/cwlJ1/cwlJ2* spores are highly attenuated in a mouse model of inhalation anthrax. Host lysozyme in the lungs causes germination of *ΔsleB/cwlJ1/cwlJ2* spores in vivo. Due to this host factor dependent germination, *ΔsleB/cwlJ1/cwlJ2* spores remain virulent at high infectious doses. When lysozyme $M^{-/-}$ knockout mice were challenged with *ΔsleB/cwlJ1/cwlJ2* spores, the mutant was significantly more attenuated compared to a wild-type mouse infection. Also, serum and bone marrow-derived macrophages from lysozyme deficient mice were unable to induce germination of *ΔsleB/cwlJ1/cwlJ2* spores.

Cortex hydrolysis is a critical barrier in the process of spore germination. This work provides a more complete understanding of this process for *B. anthracis* spores both in vitro and in vivo.

Chapter I

Introduction

The bacterial spore, and its concomitant sporulation and germination capabilities, are critical to the life cycles of the *Bacillus* and *Clostridium* genera. For all firmicute species capable of forming spores, the developmental processes of spore formation and germination appear conserved at both genetic and mechanistic levels. The resistance properties and extreme longevities of dormant bacterial spores allow bacteria to survive, in a dormant form, long periods of nutrient deprivation and challenging physical and chemical stresses (Nicholson, Munakata et al. 2000). Within the large group of spore-forming species there are several that are severe human and animal pathogens and, as such, are the focus of biomedical research efforts.

Bacillus anthracis, the causative agent of anthrax, has been the subject of renewed research interest in the last decade. Ten years ago, following the September and October 2001 terrorist acts in the United States, *B. anthracis* was identified as a Select Agent by the Centers for Disease Control and Prevention (http://www.selectagents.gov) and the United Stated Department of Agriculture (http://www.aphis.usda.gov/) because of the potential use of this bacterium as an agent of bioterrorism. Like other spore-forming species, *B. anthracis* spores are very stable in the environment, remaining viable for hundreds of years (Gest and Mandelstam 1987; Cano and Borucki 1995). This longevity

causes decontamination efforts to be extremely difficult and costly (Turnbull 1996; WHO 2008). In addition to their extreme persistence in nature, *B. anthracis* spores are highly infectious and are the etiologic agents for all forms of anthrax, with each variation dictated by their point of entry into the host (Druett, Henderson et al. 1953; Navacharoen, Sirisanthana et al. 1985; Sirisanthana, Nelson et al. 1988; Dixon, Meselson et al. 1999; Friedlander 1999; Sirisanthana and Brown 2002).

In the early 20th century, *B. anthracis* was used as a weapon of war, perhaps its first well-documented use as a bioweapon. During World War I, Germany intentionally infected the horses of neutral countries that were aiding the allied powers (Wheelis 1999). In the years following World War II, *B. anthracis* research interest increased with several countries including the United States, the United Kingdom and the USSR conducting research into the defensive measures against, and offensive capabilities of, this bacterium (Hammond and Carter 2002). The 1979 accidental release of *B. anthracis* spores from a Soviet weapons plant in Sverdlovsk and the Amerithrax attacks through the US mail in 2001 each prompted intense periods of research interest into this pathogen (Meselson, Guillemin et al. 1994; Jernigan, Stephens et al. 2001; Jernigan, Raghunathan et al. 2002).

This introduction will outline unique traits of *B. anthracis,* with additional focus on the spore, which contribute to pathogenicity. I will also discuss closely related pathogens, anthrax pathology, recognition of spores by host immune cells, role of redundant germinant receptors in host infection, and the array of germination-specific lytic enzymes used to breakdown spore cortex during the breaking of dormancy.

Taxonomy

The ability to form endospores, and the subsequent transition to vegetative cells through germination, are traits of a large group of bacteria known as spore-forming bacteria. Among spore-forming bacteria, the genera *Bacillus* and *Clostridium* are the most actively studied. The mechanistic steps of spore germination and many of the proteins that are involved, such as germinant receptor proteins and germination-specific lytic enzymes, are shared between species of these two genera.

There are at least 25 genera that include species identified as aerobic endospore-forming bacteria (AEFB) (Fritze 2004). Among AEFB, the genera *Bacillus* and *Paenibacillus* contain the vast majority of spore-forming species. The *Bacillus* genus contains two well-defined taxa, the non-pathogenic *B. subtilis* group, and the *B. cereus* group to which *B. anthracis* is assigned (Fritze 2004). *B. subtilis,* and other closely related species within this group, have received a very high level of research attention due to their relative ease of genetic manipulation and lack of pathogenicity. In its role as a well-studied model organism for spore forming bacteria, *B. subtilis* has provided the foundation for much of our understanding of spore structure and spore germination (Driks 1999; Popham 2002; Setlow 2003). The *B. cereus* group contains pathogenic species such as *B. anthracis, B. cereus,* and *B. thuringiensis,* as well as non-pathogenic species including, *B. mycoides, B. psudomycoides, B. weihenstephanensis,* and *B. medusa*. Among the pathogenic species of this group, there is extremely low sequence diversity across chromosomal DNA (Ivanova, Sorokin et al. 2003; Read, Peterson et al. 2003). In fact, some studies have suggested that these three bacteria should be

reclassified as a single species (Helgason, Caugant et al. 2000; Helgason, Okstad et al. 2000). Despite their similarities, *B. anthracis, B. cereus* and *B. thuringiensis* have very different virulence characteristics and a diverse host tropism (Jensen, Hansen et al. 2003; Rasko, Altherr et al. 2005). The genetic basis for this diversity has mostly been attributed to variances within a group of plasmids carried by these bacteria that are typically species specific (Rasko, Rosovitz et al. 2007). *B. anthracis* carries two such plasmids, pXO1 and pXO2, which contain genes encoding a tripartite toxin and biosynthetic genes required for production of a poly-D-glutamic acid capsule respectively (Okinaka, Cloud et al. 1999; Pannucci, Okinaka et al. 2002). These two components are essential virulence factors for *B. anthracis* and are also the most reliable means of species identification (Mock and Fouet 2001). Similarly, *B. thuringiensis* specific plasmids encode genes for crystal proteins, a group of δ-endotoxins that have been extensively studied by the agricultural industry for use as biopesticides (Hofte and Whiteley 1989). *B. cereus* strains contain a wide array of different plasmids, many of which encode specific toxins that dictate disease, but none of which are universally conserved across this species (Rasko, Rosovitz et al. 2007). The plasmid-based nature of virulence factors within this group of closely related species is cause for concern over the potential of horizontal gene transfer leading to new host specificity (Hoffmaster, Ravel et al. 2004).

Anthrax

As initially described by Robert Koch, *B. anthracis* is the etiological agent of the disease anthrax (Koch 1877). It exists in two morphologically distinct forms, the spore and the vegetative bacillus. Koch went on to show that the spore form of the bacterium initiates disease once it has entered a suitable host (Koch 1881). Although grazing ruminants, such as cattle and sheep, are the most common hosts of *B. anthracis* infection it is believed that most mammals are susceptible to the bacterium (Dixon, Meselson et al. 1999). The most common form of the disease in natural veterinary outbreaks is oropharyngeal anthrax. This occurs when spores in the soil enter the body of grazing herbivores (domesticated or wild) through abrasions in the mouth or pharynx (Navacharoen, Sirisanthana et al. 1985; Quinn and Turnbull 1998; Glomski, Corre et al. 2007). A second form of the disease, gastrointestinal anthrax, occurs when spores are ingested, usually from the consumption of contaminated meat (Sirisanthana, Navachareon et al. 1984; Dixon, Meselson et al. 1999; Sirisanthana and Brown 2002; Watts, Hahn et al. 2008; Watts, Hahn et al. 2009). The most common form of the disease in human cases is cutaneous anthrax. This occurs when spores cross the epidermis through small cuts or abrasions, and results in a self-contained black eschar at the site of infection (Dixon, Meselson et al. 1999; Friedlander 1999). This is the least severe form of anthrax and is often resolved without medical intervention (Smego, Gebrian et al. 1998). However, in some cases of cutaneous anthrax, bacilli can enter the blood stream causing toxemia and death (Dixon, Meselson et al. 1999). The final and most severe form of the disease is inhalational anthrax. This occurs when spores are taken up into the

lungs and is often fatal (Druett, Henderson et al. 1953; Dixon, Meselson et al. 1999). Because this form of anthrax is the most severe and is therefore the most likely targeted pathway for bioterrorism applications of *B. anthracis,* it has received the most attention from researchers in the community and will be the focus of the remainder of this chapter.

Spore-phagocyte interactions in vivo

It has been a long held belief in the field that spores entering the lungs associate with phagocytes and are trafficked to nearby lymph nodes where the bacteria escape causing a widespread infection. This model was first suggested in a study published by Ross in 1957 that used guinea pigs as a model organism to study inhalation anthrax (Figure 1.1) (Ross 1957). Since then, several groups have attempted to confirm this hypothesis and to determine the exact point in the internalization process where spore germination occurs. In her initial study, Ross relied on two new methods for accurately determining the fate of *B. anthracis* spores entering the lung. The first was a differential staining technique that distinguished dormant spores from those that had begun germination. Vegetative cells were identified by both their morphology as well as their staining properties. Second, a new method for rapid fixation of pulmonary tissue in formaldehyde was developed. Following infection, animals were anesthetized then euthanized by removing the blood and perfusing the tissue with 4% formaldehyde. This effectively halted spore germination immediately providing a snapshot of the infection. Using these techniques, Ross found that *B. anthracis* spores in the lungs are rapidly taken up (less than 35 minutes) by alveolar phagocytes that she believed were macrophages. Although no vegetative growth or spore germination was observed in the extracellular

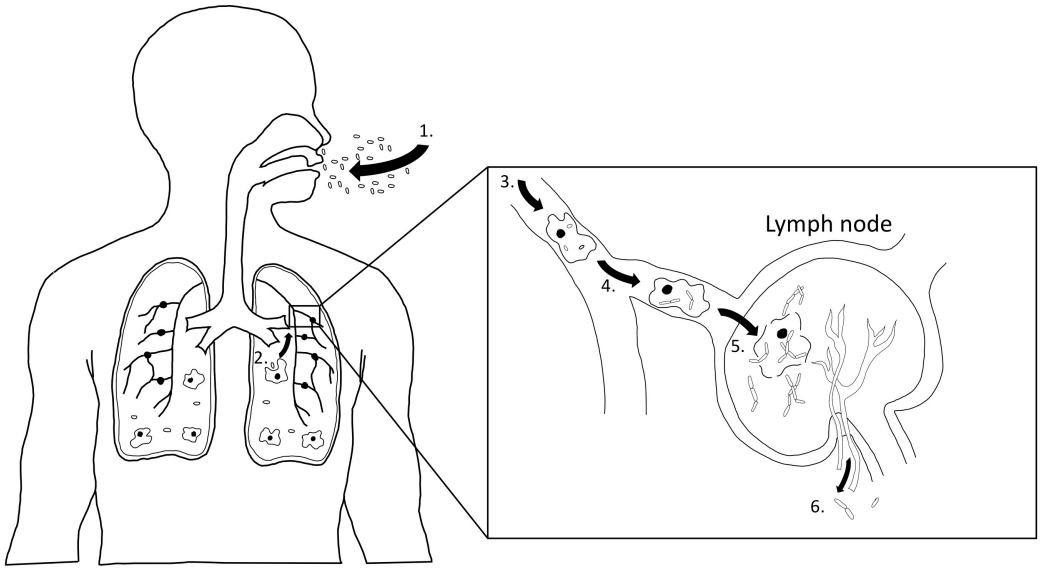

Figure 1.1: Model of inhalation anthrax progression based on the 1957 study by Ross.
(1) Aerosolized spores are inhaled. (2) Alveolar macrophages phagocytose spores in the lungs. (3) Macrophages containing spores are trafficked through the lymphatic system to a mediastinal lymph node. (4) Spores germinate within the macrophage while en route and vegetative cells begin to divide. (5) Once inside the lymph node, vegetative bacilli are released and bacterial growth continues. (6) Vegetative cells escape the lymph node into the circulatory system causing bacteremia, toxemia and eventually death. Artwork by Nicole Biltz.

lung tissue (there is typically no acute pneumonia during inhalation anthrax until very late in the disease, if ever), macrophages were seen to contain dormant spores, germinating spores and bacilli at various time points between 0 and 18 hours post infection. After 18 hours, vegetative cells were predominantly present and by 24 hours vegetative cell division was seen in the lymph nodes, free of the macrophages. *B. subtilis* spores did not show signs of germination following infection and no vegetative cells were found. Ross concluded from this study that anthrax was not a traditional pneumonia, but that spores were engulfed by lung phagocytes, and only once bacteria were trafficked to lymph nodes, and released, did they cause a general bacteremia. The model established by Ross in 1957 has been further studied and refined by many groups using the newest technologies available.

More recently, fluorescently activated cell sorting and confocal microscopy have been used to further define the interaction between *B. anthracis* spores and lung phagocytes following infection (Cleret, Quesnel-Hellmann et al. 2007). In agreement with Ross, Cleret et al. found that spores were engulfed by alveolar macrophages within 10 minutes of infection. However, these cells were not the phagocytes responsible for transport to the lymph nodes. Within 30 minutes of infection, dendritic cells in the interstitial space of the lungs were able to sample spores without crossing the lung epithelial barrier. These cells then trafficked the spores to the draining lymph nodes. The role of dendritic cells in spore trafficking was confirmed using transgenic mice in which these cells could be transiently depleted (Shetron-Rama, Herring-Palmer et al.

2010). This resolution of lung phagocytic cell type was not available to Ross in the 1950s.

Work done by Guidi-Rontani and colleagues also found that *B. anthracis* spores rapidly associate with mononuclear phagocytes in the lungs (Guidi-Rontani, Weber-Levy et al. 1999). Guidi-Rontani et al. developed polyclonal antibodies against spores and vegetative bacilli to distinguished dormant and germinated spores using immunofluorescence confocal microscopy. Using these tools, they found that germinated spores were associated with phagolysosomes within phagocytes collected from bronchoalveolar lavage (BAL). They also showed that spores only germinated when associated with phagocytes, not in the presence of BAL fluid alone. This further supports the idea that spores do not germinate in the extracellular space of the lungs, but only inside alveolar phagocytes.

Despite the abundance of evidence supporting the theory of macrophage/dendritic cell-associated germination and trafficking to lymph nodes, there remain many studies that find conflicting results. Glomski and colleagues have seen that germination can occur at the initial site of inoculation (Glomski, Corre et al. 2007; Glomski, Dumetz et al. 2008). This was done using a capsulated non-toxigenic strain in which a bioluminescence cassette was expressed under the *pag* promoter. Therefore, only cells that had resumed metabolic activity, or had active expression from the *pag* promoter would be detected. This difference cannot be solely attributed to the new technique employed. Sanz et al. used a similar model in which the bioluminescence cassette was expressed under the *sspB* promoter or the *pag* promoter to produce light in germinating spores or vegetative cells respectively (Sanz, Teel et al. 2008). In this study, spores were

seen to germinate in the lung tissue, but only when associated with macrophages. Additionally, Sanz et al. found no germination within the nearby lymph nodes. This study suggests that spore germination occurs primarily within the phagocyte while in transit to the lymph node. Still other groups have found that spores remain dormant in the lungs for several days or even weeks (Henderson, Peacock et al. 1956; Friedlander, Bhatnagar et al. 1993; Pickering, Osorio et al. 2004; Cote, Van Rooijen et al. 2006; Heine, Bassett et al. 2007; Loving, Kennett et al. 2007).

In addition to *in vivo* studies tracking the movements of *B. anthracis* spores within a host, several groups have used *in vitro* cell co-culture techniques with primary phagocytes as well as transformed cell lines to study the interaction between spores and immune cells (Dixon, Fadl et al. 2000; Ireland and Hanna 2002; Weiner and Hanna 2003; Kang, Fenton et al. 2005; Ribot, Panchal et al. 2006; Basu, Kang et al. 2007; Hu, Emerson et al. 2007; Gut, Tamilselvam et al. 2011). RAW 264.7 cells, a murine macrophage-like cell line, are capable of rapidly phagocytosing spores of several bacillus species including *B. subtilis, B. megaterium,* and *B. anthracis.* Association of *B. anthracis* spores with phagocytic cells leads to germination, while the spores of the closely related but non-pathogenic species are not triggered to germinate (Dixon, Fadl et al. 2000; Ireland and Hanna 2002; Weiner and Hanna 2003). In the germination model originally described by Ross, spores germinate within phagocytes not in the extracellular spaces of the lungs. However it has been shown *in vitro* that macrophages can induce germination without direct contact with the spores (Weiner and Hanna 2003; Gut, Tamilselvam et al. 2011). The ability of cell-free conditioned media to induce germination is heat stable, but is lost following dialysis suggesting that this phenotype

maybe caused by the release of small molecule germinants supplied by the macrophages themselves.

Virulence Factors

Following germination within a suitable host, *B. anthracis* employs three primary virulence factors, two A/B-type toxins, and a poly-D-glutamic acid capsule. Toxins and capsule are required to initiate and sustain disease (Dixon, Meselson et al. 1999). Genes encoding the subunits of lethal toxin and edema toxin are encoded on the plasmid pXO1 (Okinaka, Cloud et al. 1999). The plasmid pXO2 contains a biosynthetic operon that is responsible for capsule production (Pannucci, Okinaka et al. 2002). Both plasmids are required for full virulence, however the Sterne $34F_2$ strain of *B. anthracis* which lacks pXO2, has been shown to retain the ability to cause disease in several strains of inbred mice (Welkos, Keener et al. 1986; Welkos and Friedlander 1988).

Anthrax toxins are composed of three exotoxin components, lethal factor (LF), edema factor (EF) and protective antigen (PA). These components combine to form two A/B-type toxins, lethal toxin and edema toxin (Ascenzi, Visca et al. 2002). A/B-type toxins have two functional subunits, an A moiety that is active once inside the cell, and a B moiety that is responsible for cellular recognition and binding. For both lethal toxin and edema toxin, PA serves as the B subunit. Once PA is bound to one of two cellular receptors located on the cell surface, it is proteolytically cleaved (Beauregard, Collier et al. 2000). Cleavage of PA induces multimerization of the carboxy-terminal fragment on the cell surface and signals binding of LF, EF, or a combination of the two (Mogridge, Mourez et al. 2001). The final toxin complex of seven cleaved PA fragments and three

LF or EF subunits is then endocytosed. Endosome acidification leads to a conformational change in PA and the release of the LF or EF subunits into the cytosol where they are active (Petosa, Collier et al. 1997; Mogridge, Mourez et al. 2001).

LF is a zinc-metalloprotease that targets mitogen-activated protein kinases (Duesbery, Webb et al. 1998). Mitogen-activated protein kinases are important for signaling between the cell surface and the nucleus (Chang and Karin 2001). In this way, LF alters normal gene expression in the nucleus of host macrophages impairing proper innate and adaptive immune responses and triggering the release of inflammatory cytokines that contribute to pathogenesis in the later stages of infection (Hanna, Kochi et al. 1992; Hanna, Acosta et al. 1993; Hanna, Kruskal et al. 1994; Vitale, Pellizzari et al. 1998). EF is a calmodulin-dependent adenylate cyclase that causes increased levels of cyclic AMP and subsequent edema due to the resulting imbalance of water homeostasis within the cell (Leppla 1982). EF can also disrupt the ability of neutrophils to phagocytose *B. anthracis* cells (O'Brien, Friedlander et al. 1985).

The pXO2 virulence plasmid encodes a biosynthetic operon, *capBCDAE,* required for the synthesis of a poly-D-glutamic acid capsule that surrounds the bacillus (Green, Battisti et al. 1985; Uchida, Sekizaki et al. 1985; Makino, Sasakawa et al. 1988; Makino, Uchida et al. 1989). CapB, CapC, CapA, and CapE are required for capsule synthesis while CapD is responsible for covalently linking the capsule to the cell wall peptidoglycan (Candela and Fouet 2005; Candela, Mock et al. 2005). The primary role of the capsule during infection is to protect the bacillus from phagocytosis (Drysdale, Heninger et al. 2005).

Sporulation and Spore Structure

Sporulation is the process by which vegetative cells return to the dormant spore phase of the bacterium. This process is initiated when bacterial cells recognize that the nutrient supply in their environment has been exhausted, as is the case for *B. anthracis* following host death. These environmental signals trigger a complex phosphorelay system that culminates in the phosphorylation of the master sporulation regulator Spo0A (Stragier and Losick 1996). Phosphorylation of Spo0A activates transcription of several genes required for inducing an asymmetrical cell division (Figure 1.2A). This results in two distinct compartments, the mother cell and the forespore, in which a temporally controlled cascade of compartment specific sigma factors is responsible for proper transcriptional regulation during sporulation (Stragier and Losick 1996).

Shortly after the asymmetrical septation is formed, the early forespore specific sigma factor, σ^F, is activated which in turn activates the early mother cell-specific sigma factor, σ^E (Figure 1.2B) (Margolis, Driks et al. 1991; Harry, Pogliano et al. 1995; Webb, Decatur et al. 1995). Gene expression controlled by σ^F and σ^E leads to engulfment of the forespore by the mother cell, creating a double membrane "cell-within-a-cell" structure (Figure 1.2C) (Piggot, Moran et al. 1994). As spore development continues, proteins from σ^G dependent genes expressed in the forespore are deposited from within while proteins from σ^K dependent genes are translated in the mother cell and deposited on the developing spore from the outside (Figure 1.2D) (Stragier and Losick 1996). When spore development is complete, the mother cell is degraded and a fully developed spore is released (Figure 1.2E).

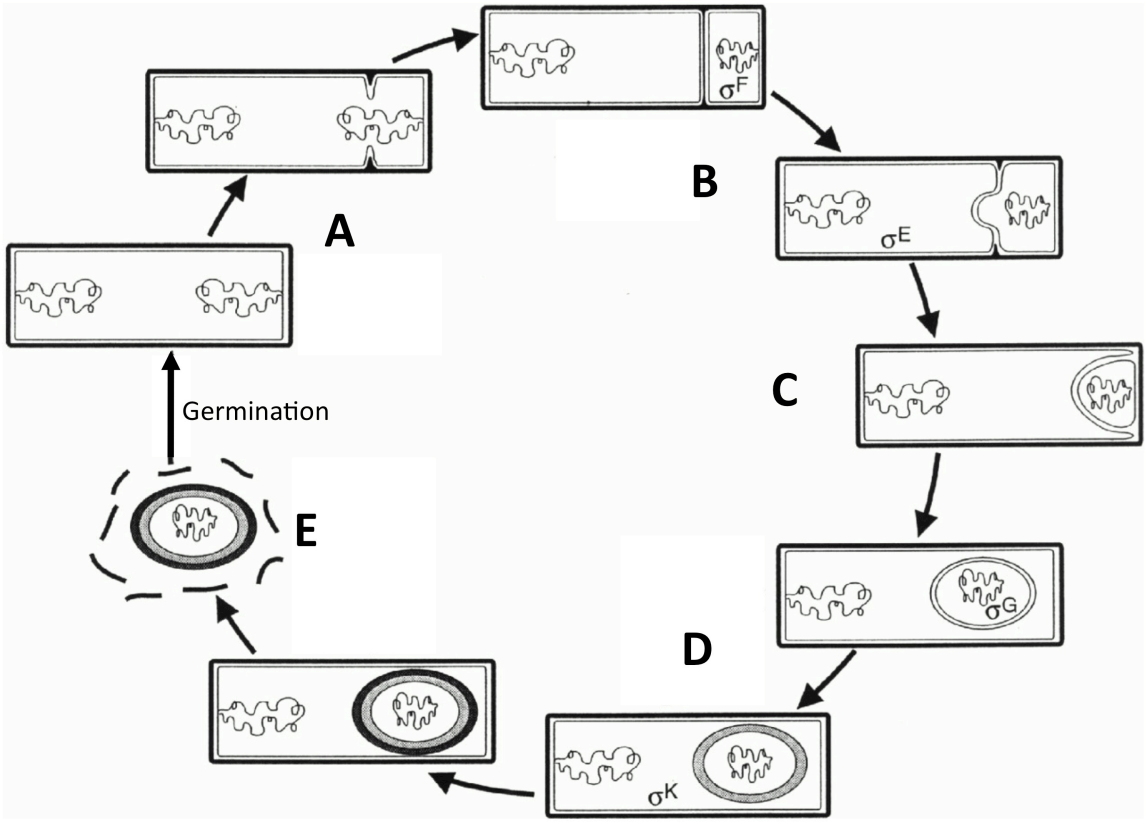

Figure 1.2: Bacillus sporulation.
(A) Sporulation begins with the formation of an asymmetric septum and subsequent asymmetric cell division. (B-C) Forespore specific gene expression is controlled by σ^F and mother cell gene expression is controlled by σ^E leading to engulfment of the forespore by the mother cell and the formation of a cell-within-a-cell structure. (D) As the spore develops, proteins are deposited from within by genes under control of a forespore specific sigma factor and from the outside by genes controlled by a mother cell specific sigma factor. (E) At the end of sporulation, the mother cell is degraded and one mature spore is released. Figure adapted from a review by Stragier and Losick (Stragier and Losick 1996).

Figure 1.3A diagrams the important structural features of a *B. anthracis* spore. At the center is the core, which houses the genetic material. The spore core exists in a highly dehydrated state (30-60% of the water content in a dividing cell). Much of the water has been replaced by a 1:1 chelate of Ca^{2+} ions and dipicolinic acid (DPA) (Setlow 2007). Dehydration of the core and the presence of DPA are critical for spore resistance to wet heat (Setlow, Atluri et al. 2006). The core is contained by the inner membrane, within which the germinant receptors are located (Sakae, Yasuda et al. 1995). *B. anthracis g*erminant receptors detect host-specific molecules (germinants) and subsequently trigger the process of germination. The process of germination will be discussed in greater detail in a later section.

Two layers of peptidoglycan, the germ cell wall and the cortex, surround the inner membrane. The germ cell wall is derived from the cell wall of the forespore and will be the basis of the cell wall in the vegetative bacillus following germination. The cortex is a much thicker layer of modified peptidoglycan (~80% of total peptidoglycan in a spore). In cortex peptidoglycan, 50% of the *N*-acetylmuramic acid residues (alternating every other residue along the polymer), are converted to a muramic-δ-lactam residues (Figure 1.3B) (Popham 2002). This modification is essential for specific degradation of the cortex by germination-specific lytic enzymes (GSLEs) during germination (Popham, Helin et al. 1996). The cortex is required for full core dehydration (Henriques and Moran 2007).

Surrounding the cortex are the spore coats and the exosporium. The composition of the spore coats and the presence or absence of the exosporium varies between spore-

forming species (Aronson and Fitz-James 1976; Giorno, Bozue et al. 2007; Henriques and Moran 2007). A combination of studies using DNA arrays and proteomic approaches have identified many of the proteins assembled into the spore coats (Kuwana, Kasahara et al. 2002; Lai, Phadke et al. 2003; Liu, Bergman et al. 2004; Giorno, Bozue et al. 2007; Henriques and Moran 2007). While the majority of coat proteins identified in *B. subtilis* are shared with members of the *B. cereus* group including *B. anthracis*, there is relatively little overlap with the coat protein profile of *Clostridium* species (Henriques and Moran 2007).

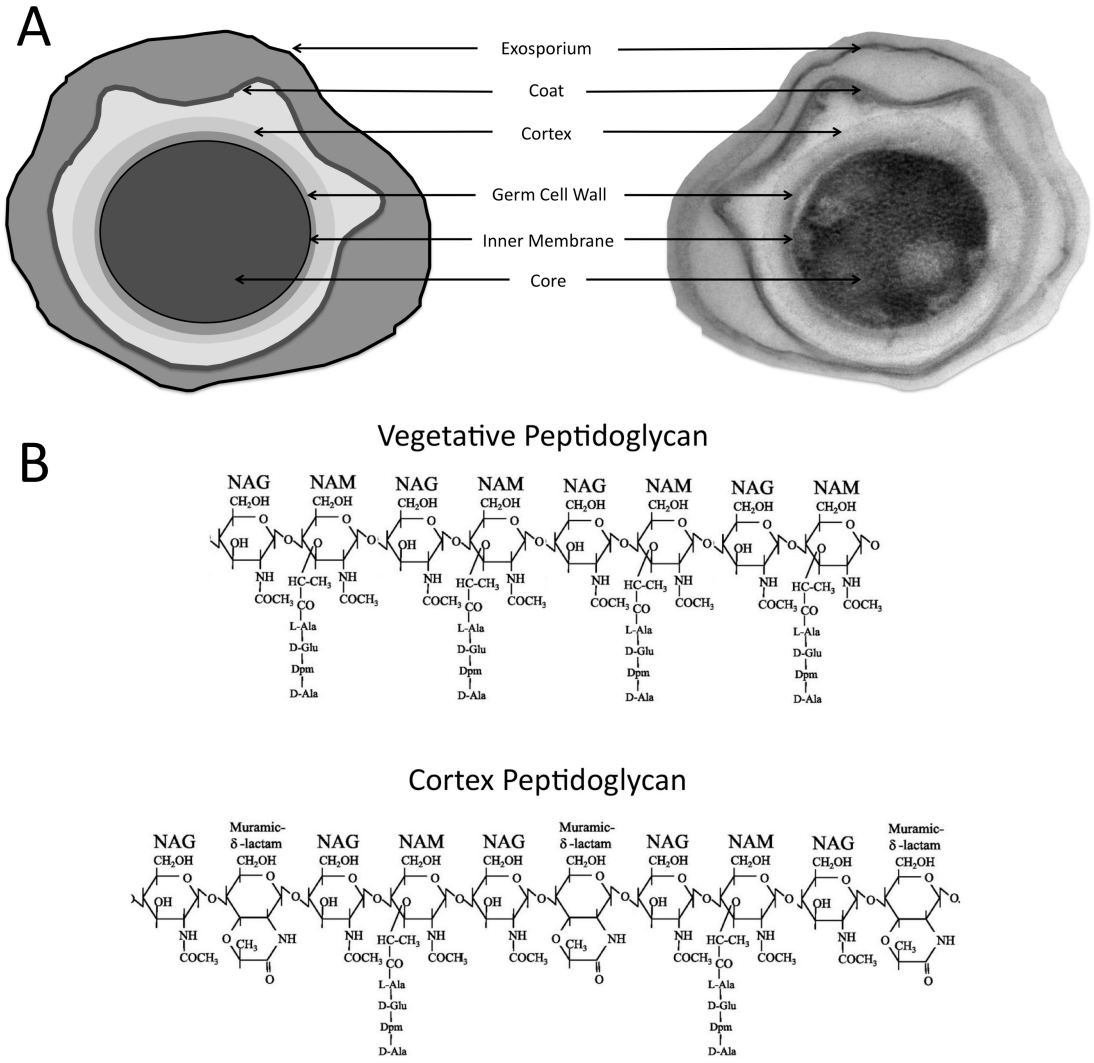

Figure 1.3: Diagram of important spore structures.
(A) Cross section of a *B. anthracis* spore and cartoon diagram of that same spore. Spores fixed in 4% paraformaldehyde and ultra-thin sectioned. The sections were examined using a Philips CM100 electron microscope at 60kV. (B) Chemical structure of cortex peptidoglycan and cell wall peptidoglycan polysaccharides. Every other NAM residue is modified to a muramic-δ-lactam residue. This modification results in reduced cross-linking in cortex peptidoglycan. NAG: *N*-acetyl glucosamine, NAM: *N*-acetyl muramic acid

Germination

Overview

Figure 1.4 diagrams the sequential steps of spore germination in *B. anthracis.* Germination is initiated by the recognition of appropriate germinants by germinant receptor complexes found on the inner membrane of the spore. Receptor-ligand interaction leads to the onset of stage-I germination by an unknown signaling pathway. At this time, it is believed that channels spanning the spore membranes, peptidoglycan and coats are opened and core rehydration begins. As water rushes into the spore core, stores of DPA and cations flow out. Partial core rehydration and release of Ca^{2+} and DPA initiate stage-II germination. In this stage, germination-specific lytic enzymes located in the cortex and coat are activated and the cortex peptidoglycan is hydrolyzed. Degradation of the cortex allows for full core rehydration. After the core is fully rehydrated, genetic and metabolic processes resume and the bacterium is able to outgrow and return to its vegetative state.

Germinant Recognition

Upon entry into the host, germination of dormant spores is dependent upon an appropriate germinant/germinant receptor interaction (Moir, Lafferty et al. 1979; Sammons, Moir et al. 1981; Zuberi, Moir et al. 1987; Corfe, Sammons et al. 1994; Ross and Abel-Santos 2010). These interactions allow for both sensitive and ongoing environmental "sensing" by the dormant spore and trigger a very rapid return to vegetative growth, even after very extended periods of dormancy, when environmental conditions are favorable. Germinant receptors localize to the spore inner membrane,

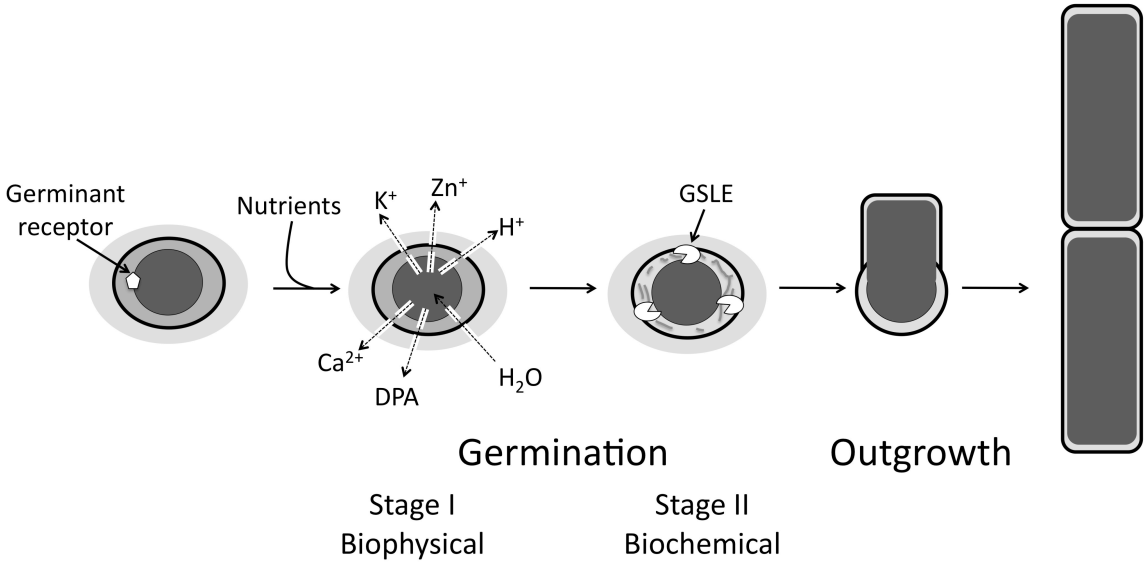

Figure 1.4: Steps of *B. anthracis* germination.
Germination begins with the recognition of nutrient germinants by germinant receptors located on the inner membrane of the spore. Ligand binding by the germinant receptors triggers the opening of channels in the spore that allow water to rush in and DPA, Ca^{2+} and other cations to flow out. Partial core rehydration and Ca^{2+}/DPA release signal the activation of GSLEs in the cortex. Cortex degradation by GSLEs allows full core rehydration and outgrowth resulting in a vegetative bacillus.

where they sense the presence of discrete germinant signals in their environment. The presence of germinants triggers an irreversible cascade of physical and biochemical changes, leading to the outgrowth of a metabolically active bacillus, with the ability to cause disease in the host (Stewart, Johnstone et al. 1981).

Germinant receptors were first identified in *B. subtilis,* which encodes three, GerA, GerB, and GerK (Sammons, Moir et al. 1981). As first shown in *B. subtilis,* the germinant receptors of all *Bacillus* species studied are encoded by tricistronic operons under the control of a σ^G promoter (Feavers, Foulkes et al. 1990; Corfe, Moir et al. 1994; Liu, Bergman et al. 2004). These receptors respond to different defined germinants, and disruption of these receptors results in the inability to respond to those germinants (Sammons, Moir et al. 1981; Corfe, Sammons et al. 1994; McCann, Robinson et al. 1996).

The *B. anthracis* chromosome encodes four known functional germinant receptor operons, *gerH, gerK, gerL,* and *gerS* (Ireland and Hanna 2002; Read, Peterson et al. 2003; Weiner, Read et al. 2003; Fisher and Hanna 2005). An additional putative receptor operon, *gerX,* is encoded on the pXO1 virulence plasmid, although its function remains to be elucidated (Read, Peterson et al. 2003). Mutational analyses indicate that at least one germinant receptor must be present to initiate disease in a murine model of anthrax infection, although there are some differences in receptor specificity depending upon route of infection (Carr, Lybarger et al. 2010). Two additional receptor-like operons, *gerA* and *gerY,* were identified on the chromosome, but are unlikely to play a role in

germination, as these open reading frames contain frame-shift mutations (Read, Peterson et al. 2003; Fisher and Hanna 2005).

The five receptors of *B. anthracis* have distinct germinant requirements, although redundancy and promiscuity exists between them. Germinants consist of a primary germinant paired with a secondary co-germinant. The primary germinant is either a purine nucleoside (typically adenosine or inosine) or L-alanine. The secondary co-germinant is one of several L-amino acids. These include L-alanine, L-serine, L-valine, L- histidine, L-methionine, L-proline, and L-tryptophan (Ireland and Hanna 2002; Weiner, Read et al. 2003; Fisher and Hanna 2005). At high concentrations, L-alanine can also germinate spores in the absence of an additional germinant. Inosine however, requires the co-stimulation provided by the L-amino acid co-germinant, although the mechanism behind this process is not yet clear (Ireland and Hanna 2002; Weiner, Read et al. 2003; Fisher and Hanna 2005; Carr, Lybarger et al. 2010).

Although it was previously thought that *B. anthracis* germination required at least two germinant receptors, evidence now shows that individual germinant receptors can respond to germinants independently (Carr, Lybarger et al. 2010). In an inhalational model of infection, the presence of any one of the chromosomally encoded receptors (GerH, GerK, GerL, or GerS) was enough to elicit a fully virulent infection. This suggests the presence of a wide range of germinants at the site of germination. In contrast, full virulence following subcutaneous inoculation requires the presence of GerH (Carr, Lybarger et al. 2010). These different receptor requirements suggest a possible explanation for the presence of so many different, yet partially overlapping, germinant specificities. These receptors may function cooperatively in the complex germinant

environment of a mammalian host, resulting in accelerated germination. In fact, recent work *in vitro* has demonstrated that different germinant receptor pathways can either synergize or block each other depending on the germinants present (Luu, Akoachere et al. 2011). Additionally, the large range of germinant specificities could provide a wider range of hosts and infectious routes available to *B. anthracis* in order to optimize its ability to germinate and cause disease.

Germination-Specific Lytic Enzymes

A critical step in the process of spore germination is the breakdown of the cortex. The cortex contributes greatly to the environmental resistance of the spore but in order for vegetative cell growth to begin it must be selectively broken down leaving the cell wall intact (Setlow 2006). This is accomplished through the action of a group of enzymes known as Germination-Specific Lytic Enzymes (GSLEs). Genes encoding GSLEs are expressed during sporulation (Popham 2002; Liu, Bergman et al. 2004). As the spore is being formed, GSLEs are packaged into the outer spore layers but remain inactive (Moriyama, Fukuoka et al. 1999). Spores in the environment may be subjected to an extended period of dormancy, possibly as long as several decades (Gest and Mandelstam 1987; Cano and Borucki 1995; Nicholson, Munakata et al. 2000). GSLEs must remain silent during this dormant period to maintain the environmental resistance of the spore. Therefore, tightly regulating the activity of these enzymes is critical for the viability of the spore, but the processes by which this is accomplished in various spore-forming bacterial species remains largely a mystery. For example, the GSLE SleB is packaged as an inactive proenzyme that is proteolytically cleaved during germination in

order to be activated in *Clostridium perfringens* as well as *Bacillus megaterium* (Foster and Johnstone 1988; Miyata, Moriyama et al. 1995; Moriyama, Fukuoka et al. 1999). However, homologs of this enzyme in *Bacillus subtilis* and *Bacillus cereus* are packaged in their active forms and do not require proteolytic processing for activation (Moriyama, Fukuoka et al. 1999). The need for this type of processing in *B. anthracis* spores remains unknown.

One common feature of all characterized GSLEs is their ability to preferentially degrade cortex peptidoglycan over cell wall peptidoglycan. This is accomplished through enzyme recognition of modifications in the sugar residues of the cortex. Bacterial cell wall peptidoglycan is comprised of repeating subunits of the saccharides *N*-acetyl glucosamine and *N*-acetyl muramic acid (Figure 1.3B)(Atrih, Zöllner et al. 1996; Popham, Helin et al. 1996; Popham 2002). These two sugars are linked in an alternating pattern to form chains. Polysaccharide chains are then cross-linked by peptide side chains found on the *N*-acetyl muramic acid residues giving the peptidoglycan its structure. In cortex peptidoglycan, 50% of the *N*-acetyl muramic acid residues are replaced by muramic δ-lactam residues (alternating every other residue) (Warth and Strominger 1969). This modification is somehow recognized by active GSLEs allowing for preferential breakdown of the cortex.

In *B. anthracis* the process of cortex degradation involves four enzymes, SleB, CwlJ1, CwlJ2, and SleL (Lambert and Popham 2008; Giebel, Carr et al. 2009; Heffron, Lambert et al. 2010). The enzymes SleB, CwlJ1 and CwlJ2 are predicted members of the group known as spore cortex lytic enzymes (SCLEs). These enzymes degrade intact

peptidoglycan. The enzyme SleL is a predicted member of the cortex fragment lytic enzyme (CFLE) family (Makino and Moriyama 2002).

SleB and CwlJ are the primary GSLEs involved in cortex degradation of *Bacillus* species. In *B. subtilis* and *B. anthracis* the presence of at least one of these genes is required for efficient spore germination indicating that these enzymes have partially redundant roles (Chirakkal, O'Rourke et al. 2002; Giebel, Carr et al. 2009; Heffron, Lambert et al. 2010). In both *B. subtilis* and *B. anthracis* the *sleB* gene is found in a bicistronic operon with the gene *ypeB*. The specific function of *ypeB* is not known; however mutant spores lacking this gene do not contain SleB (Boland, Atrih et al. 2000). This suggests that YpeB is necessary for proper expression, localization or stability of the SleB protein. *B. anthracis,* unlike the closely related *B. subtilis,* contains two homologs of *cwlJ* termed *cwlJ-1* and *cwlJ-2* (Heffron, Orsburn et al. 2009). The *B. anthracis* gene *cwlJ-1,* like the single copy of *cwlJ* found in the other species, is located in a bicistronic operon with the gene *gerQ*. Much as is the case for *ypeB* and *sleB,* the presence of *gerQ* is required for CwlJ function (Ragkousi, Eichenberger et al. 2003; Ragkousi and Setlow 2004). The unique *cwlJ2,* which is 63% identical to *cwlJ1* at the amino acid level, is not found in an operon with a *gerQ* homolog.

There are conflicting reports suggesting that SleL acts as either a glucosaminidase (Chen, Fukuoka et al. 2000; Lambert and Popham 2008) or an epimerase (Chirakkal, O'Rourke et al. 2002). *B. anthracis* mutants lacking SleL do not show defects in the speed with which they escape the cortex and initiate outgrowth suggesting that this enzyme is not required for cortex hydrolysis. However, mutants lacking SleL do retain ~65% more *N*-acetyl muramic acid than wild type spores. This finding is consistent with

the role of SleL as a CFLE that acts on partially degraded peptidoglycan (Lambert and Popham 2008). Presumably, cortex degradation, at a level sufficient for outgrowth, is carried out by other GSLEs such as SleB and CwlJ. SleL then acts on the large peptidoglycan fragments produced by these enzymes, further refining them to smaller muropeptide subunits. It is possible that this secondary processing of cortex degradation products plays a role in the interaction between *B. anthracis* spores and the host immune system during infection. A second possibility is that additional processing is involved in signaling neighboring spores in the population to begin germination. It has been shown that cell-free *B. subtilis* peptidoglycan fragments can act as a germinant for *B. anthracis* spores (Shah, Laaberki et al. 2008). However, the relative efficacy of peptidoglycan fragments collected from *sleB*, *cwlJ1* or *sleL* mutants to trigger this germination pathway has not been tested.

Summary

The successful and timely germination of spores is the essential first step in the pathogenesis of anthrax. Proper *B. anthracis* spore germination is a strictly regulated process that is programmed during sporulation and only triggered upon entry into a suitable host, possibly decades later. This process requires coordination of both germinant receptors and germination-specific lytic enzymes.

Dissertation Project

The work discussed in this dissertation examines the various mechanisms of cortex degradation during *B. anthracis* spore germination. The initial objectives of this research were to identify the specific activity of three GSLEs, SleB, CwlJ1 and CwlJ2, using *B. anthracis* strains with null mutations and to address the means by which these GSLEs remain silent in the dormant spore. Chapter 2 describes experiments designed to determine which enzymes were required and which were sufficient for spore germination in vitro as well as in a mouse model of infection. The role of SleB, CwlJ1 and CwlJ2 on germination kinetics and colony forming efficiency in vitro as well as their effects on LD_{50} and mean time to death in an animal model are described. Unexpected in vivo results led to the discovery that more than the endogenous spore germination machinery may influence spore germination inside a host. Chapter 3 details results demonstrating that host lysozyme interacts with *B. anthracis* spores within the lungs. These findings represent a new level of biological complexity in the events leading up to a *B. anthracis* infection. Initial efforts and future studies intended to identify the means of GSLE silencing in a dormant spore are described in Chapter 4. The findings produced by this dissertation shed new light on the critical step of cortex degradation during germination. A better understanding of this critical step in spore germination and subsequent disease is necessary for future development of anthrax therapeutics that target GSLE activity. Additionally they outline a previously unappreciated mechanism for spore germination in vivo that may prove to be integral to the pathogenesis of other spore-forming bacteria.

Chapter II

Germination-Specific Lytic Enzymes SleB, CwlJ1, and CwlJ2 each contribute to *Bacillus anthracis* Spore Germination and Virulence

ABSTRACT

Bacterial spore cortex is critical for spore stability and dormancy and must be hydrolyzed by germination-specific lytic enzymes (GSLEs) to allow complete germination and vegetative cell outgrowth. I created in-frame deletions of three genes that encode GSLEs that have been shown to be active in *Bacillus anthracis* germination: *sleB, cwlJ1* and *cwlJ2*. Phenotypic analysis of individual null mutations showed that the removal of any individual gene was not sufficient to disrupt spore germination in nutrient rich media. This indicates these genes have partially redundant functions. Double and triple deletions of these genes resulted in more significant defects. Although a small subset of Δ*sleB/cwlJ1* spores germinate with wild-type kinetics, the overall population has a decrease in colony-forming efficiency of three orders of magnitude below wild-type. Δ*sleB/cwlJ1/cwlJ2* spores are unable to complete germination in nutrient rich conditions *in vitro*. Both Δ*sleB/cwlJ1* and Δ*sleB/cwlJ1/cwlJ2* spores are significantly attenuated but not completely devoid of virulence in a mouse model of inhalation anthrax. Although unable to germinate in standard nutrient rich media, spores lacking SleB, CwlJ1 and CwlJ2 are able to germinate in whole blood and serum *in vitro,* which

may explain the persistent low levels of virulence observed in the mouse infections. This work contributes to an understanding of GSLE activation and function during germination. This information may provide useful therapeutic targets against the disease anthrax as well as insights into ways to induce the breakdown of the protective cortex layer facilitating easier decontamination of resistant spores.

INTRODUCTION

Bacillus anthracis, a gram-positive spore forming bacterium, is the causative agent of anthrax. The dormant spore form is the infectious particle and produces three different forms of the disease depending on route of entry into a suitable host (Dixon, Meselson et al. 1999). When spores enter through a skin lesion or are ingested, they cause cutaneous and gastrointestinal anthrax, respectively. Spores entering through the lungs cause the most severe form of the disease, inhalation anthrax, which is often fatal even with aggressive antibiotic therapy (Albrink 1961; Dixon, Meselson et al. 1999; Tournier, Quesnel-Hellmann et al. 2007). Because true pneumonias are rarely seen in victims, it is believed that inhaled spores do not germinate in the lung but are phagocytosed by alveolar macrophages and germinate intracellularly en route to the mediastinal lymph nodes leading to dissemination, septicemia, toxemia, and often death (Albrink 1961; Tournier, Quesnel-Hellmann et al. 2007). It has been shown that the bacteria are able to germinate and multiply inside macrophages both in cell culture and in the lungs of challenged animals (Ross 1955; Ross 1957; Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000).

Independent of route of infection, spore germination inside a susceptible host is essential for disease. The highly stable spore form of the bacteria can remain viable under harsh environmental conditions for many decades (Setlow 2003). However, the spore is able to return to a rapidly dividing vegetative cell upon entry into a host and recognition of specific chemical signals, or germinants, through specialized germinant receptors (Setlow 2003). The spore cortex, a thick layer of modified peptidoglycan (PG), contributes much of the spore's environmental resistance as it is necessary to maintain

dehydration of the spore core (Popham 2002). This protective barrier is broken down following the activation of germination-specific lytic enzymes (GSLEs) allowing full core rehydration and cell outgrowth (Setlow 2003). Experimentally, germination can also be triggered by non-germinant pathways such as lysozyme treatment, high pressure, exogenous Ca^{++}/dipicolinic acid treatment, and cationic surfactants (Setlow 2003). Several of these treatments likely cause spore cortex hydrolysis, triggering spore germination. This indicates the importance of cortex degradation in the spore germination process.

Bacterial cell wall PG consists of polysaccharide chains of repeating *N*-acetyl glucosamine and *N*-acetyl muramic acid, joined by β(1,4) glycosidic bonds (Popham 2002). This basic structure is modified in several ways to form spore cortex PG. In one major modification, 50% of muramic acid residues (alternating every other amino sugar) are converted to muramic-δ-lactam residues (Popham 2002). This modification is essential for specificity of GSLEs in degrading the cortex, and prevents degradation of the bacterial cell wall during cortex hydrolysis (Moir, Corfe et al. 2002).

Previous work on the role of GSLEs in *B. subtilis,* and recently in *B. anthracis,* has shown that the enzymes SleB and CwlJ serve partially redundant roles and are necessary together for full cortex hydrolysis and spore germination (Chirakkal, O'Rourke et al. 2002; Heffron, Orsburn et al. 2009). SleB is a lytic transglycosylase that when activated, by an unknown mechanism, hydrolyzes the bond between *N*-acetyl muramic acid and *N*-acetyl glucosamine (Boland, Atrih et al. 2000). In both *B. subtilis* and *B. anthracis,* the gene *sleB* is found in a bicistronic operon with *ypeB*. Although the function of YpeB is not known, the deletion of *ypeB* prevents SleB activity in spore

germination, and *sleB* and *ypeB* mutants have similar phenotypes (Boland, Atrih et al. 2000). Expression of both gene products is necessary for the presence of SleB in the cortex and inner membrane of mature spores (Boland, Atrih et al. 2000; Atrih and Foster 2001).

Although no specific enzymatic activity has been attributed to CwlJ, it is required for full germination and it shares a homologous catalytic domain with SleB (Moir 2006). In *B. subtilis* and *B. cereus, cwlJ* is found in an operon with *gerQ*. Similar to the previous finding that *ypeB* is necessary for functional SleB protein*, gerQ* is required for CwlJ activity (Ragkousi, Eichenberger et al. 2003). The *B. anthracis* genome contains two homologs of *cwlJ* (termed *cwlJ1* and *cwlJ2* (Heffron, Orsburn et al. 2009)) compared to the single copy found in *B. subtilis* and *B. cereus.* As in the related species, *cwlJ1* is found in an operon with *gerQ,* but *cwlJ2* is in a different locus and is not in an operon with a *gerQ* homolog (Heffron, Orsburn et al. 2009). It has been shown that CwlJ is localized to the spore coat and that it is necessary for spore germination by exogenous Ca^{++}/DPA treatment (Paidhungat, Ragkousi et al. 2001; Bagyan and Setlow 2002).

GSLE activation represents a critical step in the complex process of germination. The relatively small number of genes involved and the apparent essential nature of their activity make them attractive targets for new therapeutics as well as environmental decontamination compounds. The objective of this study was to test by genetic analysis the role of the GSLEs *sleB, cwlJ1*, and *cwlJ2*, in *B. anthracis* spore germination. Null mutants of these three genes were tested for effects on *in vitro* germination kinetics and colony-forming efficiency. Additionally, the virulence of these mutant strains was

examined by comparing mutant and wild-type spores in an *in vivo* mouse model of inhalational anthrax.

MATERIALS AND METHODS

Bacterial strains and antibiotics

Bacterial strains and plasmids used in this study are listed in Table 2.1. Vegetative *B. anthracis* cultures were grown in brain-heart infusion media (BHI, Difco), and *Escherichia coli* cultures were grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001). All media were supplemented with the following antibiotics and concentrations to maintain selection as described in the mutant construction section: ampicillin (100 μg/ml), erythromycin (400 μg/ml for *E. coli* and 5 μg/ml for *B. anthracis*), kanamycin (10 μg/ml), polymyxin B (60 units/ml), spectinomycin (100 μg/ml), and tetracycline (10 μg/ml).

B. anthracis spore stocks were prepared as previously described (Kim and Goepfert 1974; Dixon, Fadl et al. 2000). Briefly, vegetative cells were diluted 1:100 in modified G medium (0.2% yeast extract, 0.17 mM $CaCl_2$, 2.87 mM K_2HPO_4, 0.81 mM $MgSO_4$, 0.24 mM $MnSO_4$, 17 μM $ZnSO_4$, 20 μM $CuSO_4$, 1.8 μM $FeSO_4$, 15.5 mM $(NH_4)_2SO_4$, pH 7.2) and allowed to incubate at 37^{o}C and 300 RPM for 72 hours. Following incubation, cultures were checked microscopically to verify presence of phase-bright spores. Spores were purified as previously described and the final stock was >99% phase-bright spore particles (Liu, Bergman et al. 2004).

Table 2.1: *Bacillus anthracis* strains and plasmids used in this study.

Strain	Relevant Characteristics	Reference
Sterne $34F_2$	Wild-type ($pXO1^+$, $pXO2^-$)	(Sterne 1939)
KC61	$34F_2$, *ΔsleB*	This work
KC62	$34F_2$, *ΔypeB*	This work
KC63	$34F_2$, *ΔsleB ΔypeB*	This work
JG48	$34F_2$, *ΔcwlJ1*	This work
JG49	$34F_2$, *ΔcwlJ2*	This work
JG50	$34F_2$, *ΔsleB ΔypeB ΔcwlJ1*	This work
JG51	$34F_2$, *ΔsleB ΔypeB ΔcwlJ2*	This work
JG52	$34F_2$, *ΔcwlJ1 ΔcwlJ2*	This work
JG53	$34F_2$, *ΔsleB ΔypeB ΔcwlJ1 ΔcwlJ2*	This work

Plasmids	Relevant Characteristics	Reference
pBKJ236	Allelic exchange vector, Em^r	(Janes and Stibitz 2006)
pBKJ258	Allelic exchange vector, Kan^r	(Lee, Janes et al. 2007)
pBKJ223	Tet^r, P_{amy} I-SceI	(Janes and Stibitz 2006)
pJDG101	*ΔsleB* construct cloned in pBKJ258	This work
pJDG102	*ΔypeB* construct cloned in pBKJ258	This work
pJDG103	*ΔcwlJ1* construct cloned in pBKJ236	This work
pJDG104	*ΔcwlJ2* construct cloned in pBKJ258	This work

Mutant bacterial strains were constructed using the allelic exchange method as previously described (Janes and Stibitz 2006; Lee, Janes et al. 2007). Allelic exchange plasmids were constructed as described for the ΔGBAA1941 construct (Bergman, Anderson et al. 2006). For each gene deleted the initial 30 nucleotides were fused to the final 30 nucleotides creating an in frame deletion. In place of the deleted genetic material, three stop codons were inserted into each gene. *CwlJ1* and *cwlJ2* single mutants were constructed in a wild-type Sterne $34F_2$ background. The Δ*sleB* mutant was made by removal of the Δ*sleB/ypeB* operon in a Sterne $34F_2$ background. *YpeB* is proposed to act in either stability or targeting of *sleB*. Both proteins are required for SleB activity and there is no phenotypic difference between Δ*sleB* and Δ*ypeB* single mutants in the assays presented here (data not shown). Double and triple mutant combinations were created by deletion of additional genes in the single mutant backgrounds. All oligonucleotide sequences used in the construction and screening of mutant strains are available upon request.

Germination Assays

Spore germination was measured by loss of heat sensitivity or decrease in optical density at 600 nm. To measure loss of heat sensitivity, spore stocks were diluted to approximately $4x10^5$ spores/ml and heat activated by incubation at 65°C for 20 minutes. Germination was initiated by adding 10 μl of spores to 2.0 ml of germination medium. At each time point (2, 5, 10, 15, 20, and 30 minutes) 100 μl aliquots of this solution were removed, incubated at 65°C for 20 minutes, then 50 μl was plated onto BHI agar and incubated overnight. Colonies that formed represented spores that failed to germinate and were therefore heat resistant. As a control, 50 μl of spores at time 0 were plated

without heat treatment. The fraction of germinated spores for each time point was calculated as $(1-t_n)/t_0$ and reported as a percentage. To assay decrease in optical density, 5.0 ml of spores, OD_{600nm}=0.6, were heat activated by incubation at 65°C for 20 minutes. Germinants were then added for a starting OD_{600nm} of approximately 0.3 and the reaction was incubated at 37°C while shaking at 300 RPM. At 5 minute intervals, 1.0 ml aliquots were removed and OD_{600nm} was measured using a Genesys 10UV spectrophotometer (Spectronic Unicam, Rochester, NY).

The germination media used in this study were BHI (Difco), whole bovine blood (HemoStat Laboratories), and bovine serum. Bovine serum was collected by pelleting whole blood at 4,000 RPM in an eppendorf 5810 R swinging bucket centrifuge. Following centrifugation, serum was filtered through a 0.2 μm syringe filter (Nalgene) and stored at 4°C. Heat-treated serum was incubated at 65°C for 30 minutes.

In this study, colony-forming efficiency is defined as the number of spore particles that represent one cfu. An improved Neubauer haemocytometer was used to assay spore particles per ml for each stock assayed. The same spore stock was germinated in one of the germination media listed above for 10 minutes at 37°C. Germination reactions were then titered on BHI plates to measure cfu/ml.

Murine Challenges

Intratracheal infections of DBA/2J mice (Jackson Laboratories) were done as previously described (Heffernan, Thomason et al. 2007). Groups of eight mice were infected with doses ranging between $1.5x10^3$ and $1.5x10^7$ spores per mouse of mutant or wild-type strains. Mice were monitored for a period of 14 days. *B. anthracis* bacteria

was recovered from blood, spleen, heart, and lungs of randomly selected individuals that died following infection. The strain identity was verified by diagnostic PCR.

RESULTS

Effects of GSLE mutations on *in vitro* spore germination in BHI. Germination appears to be a well-conserved process among spore forming bacteria including *B. subtilis* and *B. cereus* (Moriyama, Kudoh et al. 1996; Ishikawa, Yamane et al. 1998; Atrih and Foster 2001). Independently, and using different assays than those presented in this study, Heffron *et al.*. (Heffron, Orsburn et al. 2009) have shown that single deletion mutations of the GSLEs *sleB, cwlJ1* and *cwlJ2* in *B. anthracis,* cause minor deficiencies in *in vitro* germination kinetics when compared to wild-type spores, and that the double mutant Δ*sleB/cwlJ1* has severe germination deficiencies. I had simultaneously constructed defined in-frame deletion mutations in each of the three GSLEs individually and in double and triple combinations to better determine the contributions of these three genes to spore germination *in vitro* and to determine if these genes play a role in virulence in an *in vivo* mouse model of inhalation anthrax.

No strains constructed exhibited a gross defect in sporulation or vegetative growth rates (data not shown). These strains were germinated *in vitro* using BHI as a germinant and germination was monitored as loss of heat resistance (Fisher and Hanna 2005). All single mutants reached ~100% germination in roughly the same time period, 2-5 min (Figure 2.1A). Small variations in germination rates were observed from 0-5 min including slight delays by Δ*cwlJ1* and Δ*cwlJ2* at the 5 min time point and a temporary increase in the germination kinetics of Δ*sleB* at the 2 min time point. Measuring spore germination by loss of heat resistance is limited in that it can only score viable spores that are ultimately able to outgrow. Therefore I also measured germination by monitoring the drop in optical density at 600 nm, following the addition of germinants. A decrease of

60% of the initial optical density is considered 100% germination (Nicholson and Setlow 1990). The Δ*sleB* and Δ*cwlJ2* mutants did not reach 100% germination (Figure 2.1B). The Δ*cwlJ1* mutant did reach 100% germination but was slightly delayed compared to wild-type (Figure 2.1B). These results indicate that loss of any one GSLE only caused minor deficiencies in the germination kinetics compared to wild-type.

Germination of double mutants in BHI also showed small fluctuations in germination kinetics when measured by loss of heat resistance (Figure 2.1C). Spores of the strain Δ*sleB/cwlJ2* germinated with near wild-type kinetics. Spores of the strain Δ*cwlJ1/cwlJ2* had a delay in germination at the 5 minute time point when compared to wild-type spores. Despite this delay, mutant spores still reached nearly 100% germination by the end of the 30 min time course. Similarly, I found only minor germination defects in Δ*sleB/cwlJ1* spores compared to wild-type. As seen in the Δ*sleB* single mutant, spores of this double mutant were accelerated after 2 minutes but otherwise germinated in BHI in a manner similar to wild-type germination kinetics (Figure 2.1C). However, as seen by Heffron *et al.* (Heffron, Orsburn et al. 2009), I found severe defects in Δ*sleB/cwlJ1* and additionally in Δ*sleB/cwlJ1/cwlJ2* spore germination when measured by drop in optical density (Figure 2.1D). Although the drop in optical density assay is better able to detect early events in spore germination, small subsets of spores will be lost in the background of the population. I believe that this explains the different results for Δ*sleB/cwlJ1* spore germination when measured by the two different methods. Although the Δ*sleB/cwlJ1* spore population as a whole has major germination defects and cannot outgrow, the small number of spores that do outgrow germinate with wild-type kinetics (Figure 2.1C-D).

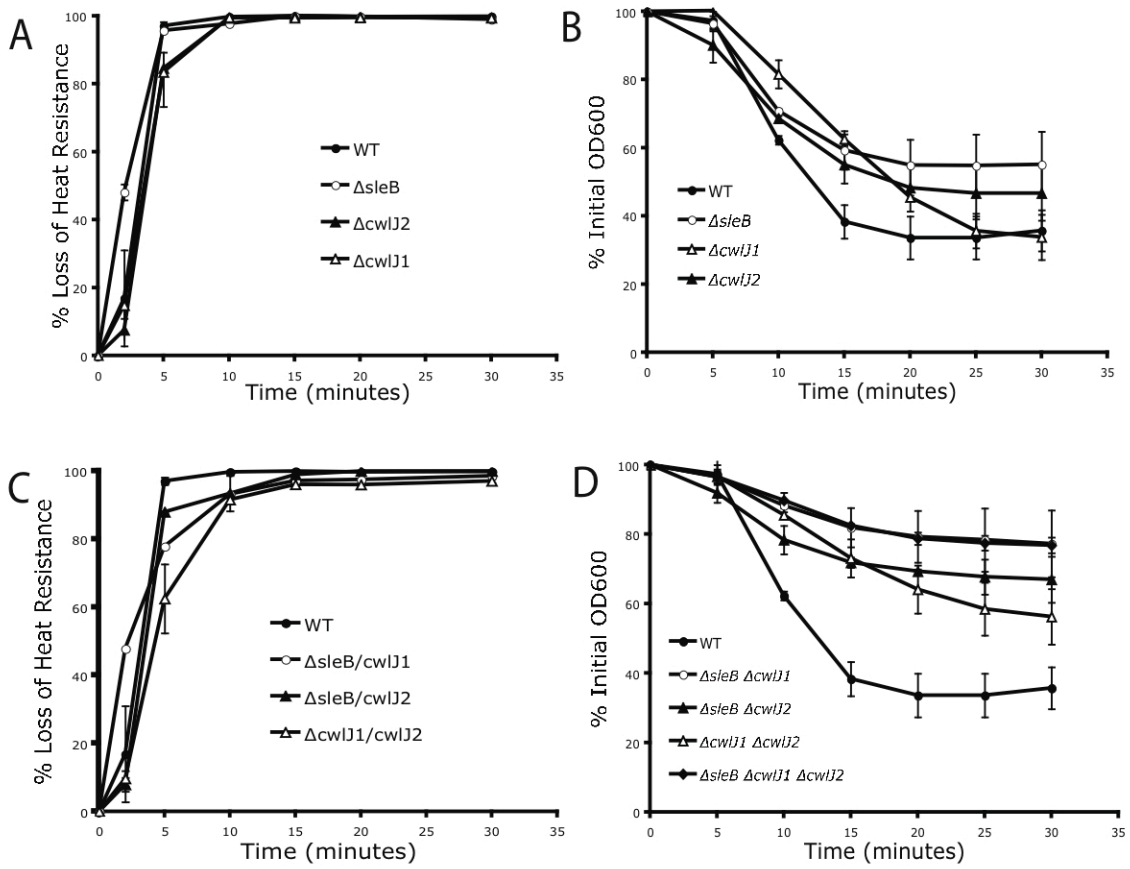

Figure 2.1: In vitro germination of spores in BHI.
Loss of heat resistance (A and C) and drop in OD_{600nm} (B and D) were measured as markers of spore germination. A 60% decrease in OD_{600nm} is approximately 100% germination. Spores were incubated in BHI at 37°C for 0-30 min. Germination kinetics of the triple mutant strain Δ*sleB/cwlJ1/cwlJ2* could not be assayed by loss of heat resistance because this strain was unable to germinate in response to BHI. All panels represent mean values from 2-3 independent experiments, error bars represent ±standard error.

Germination kinetics of the triple mutant strain Δ*sleB/cwlJ1/cwlJ2* could not be assayed by loss of heat resistance because this strain was unable to germinate in response to BHI. However, this mutant had the identical germination defect of Δ*sleB/cwlJ1* spores when measured by drop in optical density (Figure 2.1D). To better characterize the nature of this dramatic defect and to test if similar defects were present in the double mutant strains, colony-forming efficiency was measured for each strain. I tested colony-forming efficiency by visually quantifying the number of spores in a sample using a haemocytometer and then inducing germination of those same spores with a variety of germinants. By comparing the difference between the concentration of a spore stock (spores/ml) when counted on a haemocytometer and the cfu/ml of the same stock when titered on BHI plates I determined the colony-forming efficiency of all double and triple GSLE mutants in nutrient rich media (Figure 2.2). The colony-forming efficiency of Δ*sleB/cwlJ1/cwlJ2* was found to be 8.2×10^7 spore particles per cfu, a decrease of seven orders of magnitude compared to wild-type spores. Consistent with a recent report (Heffron, Orsburn et al. 2009), the strain Δ*sleB/cwlJ1* also exhibited a significant decrease in colony-forming efficiency of approximately three orders of magnitude when compared to wild-type. All other double mutants and wild-type strains yielded an approximate colony-forming efficiency of 1 (Figure 2.2). These data show that although these GSLEs do not play a major role in germination kinetics, they are necessary for spore outgrowth. Furthermore, it appears that all three genes have partially redundant functions as any one is sufficient for at least some level of colony-forming ability. In order of increasing colony-forming efficiency, the triple mutant Δ*sleB/cwlJ1/cwlJ2* had

the lowest colony forming efficiency followed by Δ*sleB/cwlJ1*. The remaining strains Δ*sleB/cwlJ2* and Δ*cwlJ1/cwlJ2* had the highest colony-forming efficiency at levels comparable to wild-type. This indicates that contributions from *sleB* and *cwlJ1* have greater influence on colony-forming efficiency than *cwlJ2*.

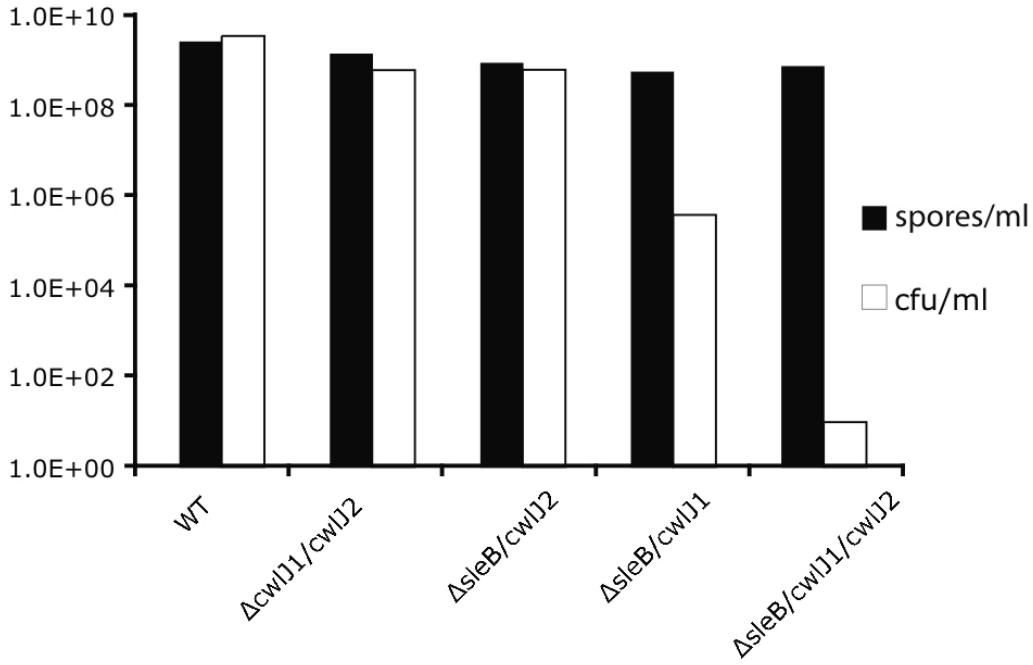

Figure 2.2: Colony-forming efficiency of GSLE mutants.
Colony-forming efficiency was examined by visually assaying the number of spores/ml (black bars) of a given stock as enumerated on an improved Neubauer haemocytometer and comparing that to cfu/ml (white bars) of that same stock when plated on BHI. Data shown above is a representative sample of typical results from three independent experiments.

Virulence of GSLE mutant strains in mice. Although the *B. anthracis* Sterne strain is attenuated in many mammalian species, certain strains of inbred mice including DBA/2J are susceptible to the bacteria making them an excellent model system for virulence studies (Welkos, Trotter et al. 1989; Harvill, Lee et al. 2005). I used intratracheal injection of spores into the lungs as a model for inhalational anthrax (Fisher, Shetron-Rama et al. 2006). Virulence in DBA/2J mice when challenged with all double and triple mutants described above was compared to wild-type bacteria. DBA/2J mice infected with wild-type spores had a median time to death of two days at a dose of $1.5x10^6$ spores/mouse and an LD_{50} of $1.5x10^4$ spores/mouse (Table 2.2).

The Δ*cwlJ1/cwlJ2* double mutant, which had a slight delay in germination kinetics *in vitro* (Figure 2.1C-D), showed no attenuation in virulence compared to wild-type at a dose of $1.5x10^6$ spores/mouse (100x the wild-type LD_{50}) (Figure 2.3A), and no significant increase in median time to death (Table 2.2). The Δ*sleB/cwlJ2* double mutant strain, which showed the least amount of variation from wild-type germination kinetics in BHI, was slightly attenuated (Figure 2.3B) and had a slightly longer delay in median time to death (Table 2.2). Although these values were not significantly different from wild-type ($p=0.058$) they did trend away from wild-type spores. As expected from the *in vitro* colony-forming efficiency, the strain Δ*sleB/cwlJ1* exhibited a highly significant ($p=0.0001$) attenuation compared to wild-type spores (Figure 2.3C). Median time to death of this strain was increased from 2 days with wild-type spores to 5.5 days, and even at a 10 fold higher dose ($1.5X10^7$ spores/mouse) a delay in median time to death was observed (2 days wild-type to 3.5 days mutant) compared to the lower dose of wild-type bacteria (Table 2.2).

Table 2.2: DBA/2J Mouse Virulence

Median Time to Death (days)

Strain	$1.5x10^6$ spores	$1.5x10^7$ spores	LD_{50}** (spores/mouse)
34F2	2	ND*	1.5×10^4
Δ*sleB/cwlJ1*	5.5	3.5	1.2×10^6
Δ*sleB/cwlJ2*	3.5	ND*	ND*
Δ*cwlJ1/cwlJ2*	3	ND*	ND*
Δ*sleB/cwlJ1/cwlJ2*	Undefined***	4	8.3×10^6

* ND: not determined

** LD_{50} calculations were done by Reed and Muench method (Reed and Muench 1938) and were based on survival data from 4 groups of mice (n=8) at 4 different doses for each of the strains assayed.

*** Because only 2 out of 8 mice in the group died during the course of the experiment, the dose was not lethal enough to determine median time to death.

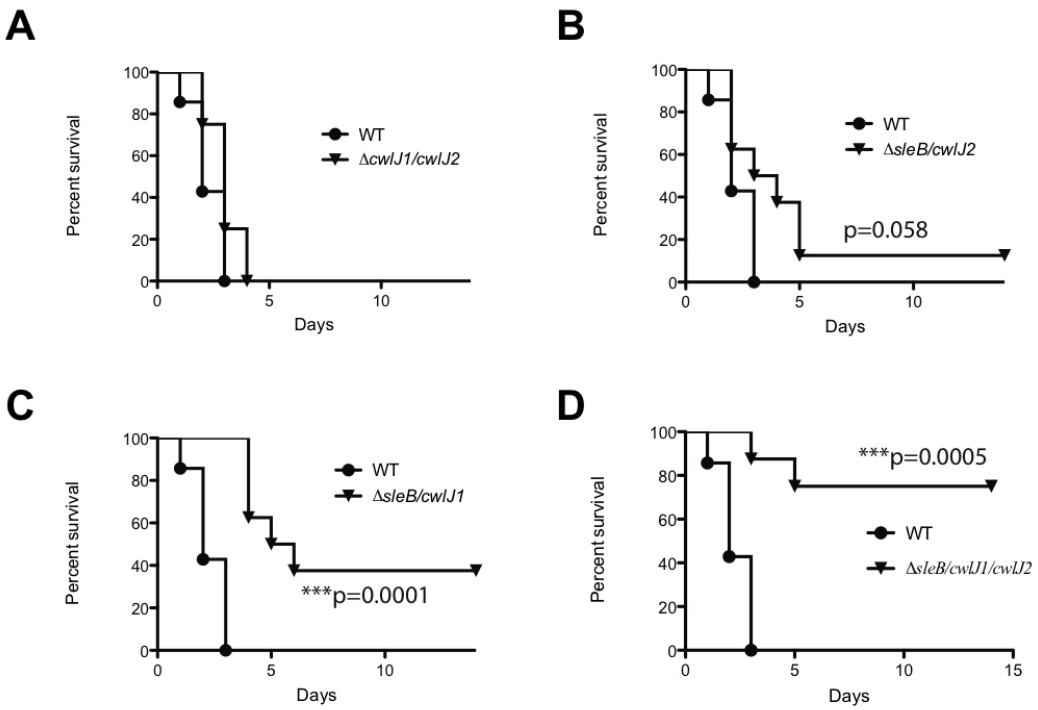

Figure 2.3: Survival curves of GSLE mutants in mice.
DBA/2J mice (n=8 mice per group) were challenged by intratracheal infection with WT (●) and mutant (▼) spores at a dose of $1.5x10^6$ spores/mouse. The mice were monitored for 14 days at which point all surviving mice were euthanized. Survival curves for Δ*sleB/cwlJ1* (C) and Δ*sleB/cwlJ1/cwlJ2* (D) were found to be significantly different from wild-type p=0.0001 and p=0.0005 respectively, by log-rank (Mantel-Cox) test. Although not meeting the significance threshold, the Δ*sleB/cwlJ2* (B) survival curve did show a trend away from wild-type (p=0.058).

Next I tested the virulence of the Δ*sleB/cwlJ1/cwlJ2* triple mutant in our mouse model. The triple mutant was significantly attenuated (Figure 2.3D, p=0.0005), but it did retain some measure of virulence, killing 25% of mice challenged in 4 days. This was somewhat surprising because the triple mutant was functionally unable to germinate in rich media. On BHI plates, only one spore per 82 million could germinate. As each mouse was given a dose of only 1.5 million spores, I did not expect to see any germination or subsequent mortality. Necropsies were performed on a subset of animals that succumbed to the infection and mutant bacteria were isolated on BHI plates, confirming that mutant spores were able to geminate inside mice and multiply to near wild-type levels (data not shown). Median time to death for this strain could not be determined at the same dose as the above listed strains ($1.5x10^6$ spores/mouse) because it was not virulent enough to kill at least half the mice challenged within the time course of the experiment (Table 2.2). At a 10-fold higher dose, $1.5x10^7$ spores/mouse, the median time to death was 4 days. This was delayed compared to the lower dose of wild-type spores (2 days) but was approximately the same as the equally high dose of double mutant Δ*sleB/cwlJ1* spores (3.5 days) (Table 2.2). Although both are significantly attenuated compared to wild-type, the highest doses of Δ*sleB/cwlJ1* and Δ*sleB/cwlJ1/cwlJ2* tested, $1.5x10^7$ spores/mouse and $8.4x10^7$ spores/mouse respectively, did not produce significantly different survival curves and had the same median time to death, 3.5 days (data not shown). This suggested that despite increasing numbers of mutant spores in the challenge dose, only a certain subset were able to germinate and cause disease.

To further define the virulence effects of the attenuated mutants, LD_{50} values were determined for wild-type, Δ*sleB/cwlJ1,* and Δ*sleB/cwlJ1/cwlJ2*. Groups of mice (n=8) were infected by intratracheal injection with 4 different doses of spores suspended in water. The parental Sterne strain $34F_2$ (wild-type) had an LD_{50} of $1.5x10^4$ spores. LD_{50} values of Δ*sleB/cwlJ1* and Δ*sleB/cwlJ1/cwlJ2* were found to be, $1.2x10^6$ spores and $8.3x10^6$ spores respectively, or 80 fold and 550 fold higher than wild-type respectively (Table 2.2). Taken together, these data show that lack of germination on nutrient rich media *in vitro,* does not indicate a block of germination *in vivo*, and that another mechanism of spore germination may be active in the host.

Effects of GSLE mutations on *in vitro* germination in blood. Although it was expected that the colony-forming deficient mutant Δ*sleB/cwlJ1* would be at least somewhat attenuated in mice, it did not seem likely that the extreme colony-forming deficient mutant Δ*sleB/cwlJ1/cwlJ2* would retain any ability to cause disease. Based solely on colony-forming units on BHI, even the highest challenge dose of Δ*sleB/cwlJ1/cwlJ2* would be effectively 1 germinating spore/mouse, 10,000-fold lower than the wild-type LD_{50}. These data lead to the hypothesis that a host factor(s) was able to cause germination at levels higher than those calculated from using BHI as a germinant and perhaps circumvent the requirement for GSLE activities.

To test if such factors were in blood, colony-forming efficiency assays were repeated with Δ*sleB/cwlJ1/cwlJ2* spores using defibrinated bovine whole blood as the germination medium. Spores were first incubated for 10 minutes in defibrinated bovine blood to initiate germination. Then the spores were plated on BHI and allowed to outgrow at 37°C. The colony-forming efficiency of Δ*sleB/cwlJ1/cwlJ2* increased from $8.2x10^7$ spores per cfu when germinated in BHI to $9.2x10^3$ spores per cfu when germinated in blood. This represents an increase of almost 4 orders of magnitude over colony-forming efficiency in BHI (Figure 2.4). Triple mutant colony-forming efficiency in blood was about 3 orders of magnitude below that of wild-type spores germinated in blood (data not shown).

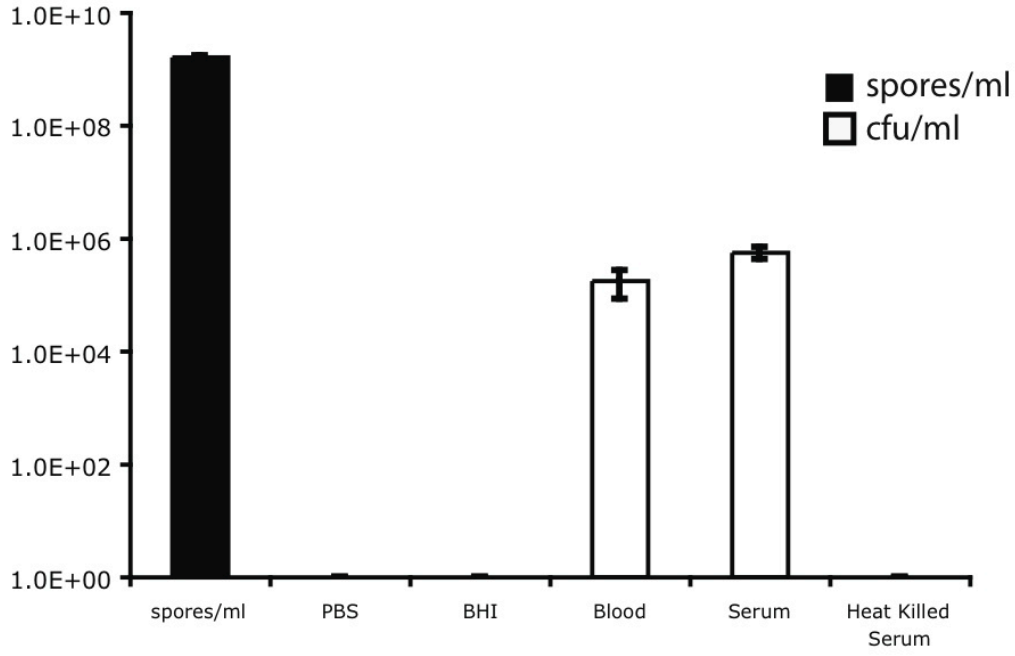

Figure 2.4: In vitro germination of ΔsleB/cwlJ1/cwlJ2 spores in blood.
Colony-forming efficiency of Δ*sleB/cwlJ1/cwlJ2* spores in blood and serum was examined. Spore dilutions were quantified visually with an improved Neubauer haemocytometer (spores/ml) (black bar) and compared to cfu/ml (white bars) when spores were allowed to germinate in PBS (pH=7.5), brain heart infusion (BHI), whole bovine blood, bovine serum, or heat killed bovine serum for 10 min before being plated on BHI plates and incubated at 37^{o}C overnight.

Next I wanted to further examine the source of the host germination factor by removing the contribution of whole cells in the blood. To do this, I used filtered bovine serum and heat-treated filtered bovine serum as germination media for triple mutant spores. Mutant spores germinated in serum at a rate comparable to whole blood, $2.9x10^3$ spores per cfu in bovine serum compared to $9.2x10^3$ spores per cfu in whole blood (Figure 2.4). This indicates that serum contains all the necessary host components contained in whole blood to cause mutant spore germination and that whole cells in blood do not play a direct role. Bovine serum heat-treated at 65^oC for 30 minutes lost the ability to cause germination in Δ*sleB/cwlJ1/cwlJ2* spores (Figure 2.4). This suggests that the host factor causing mutant-spore germination is heat labile and may be an enzyme in serum.

DISCUSSION

GSLEs are expressed during *B. anthracis* sporulation and are active during germination (Bergman, Anderson et al. 2006; Heffron, Orsburn et al. 2009). Deletion of the genes that encode the enzymes SleB, CwlJ1, and CwlJ2, was sufficient to significantly disrupt spore germination and, in the case of the triple mutant, even lead to complete loss of germination in rich medium *in vitro*. However, these germination-deficient spores retained some virulence in our mouse model, suggesting an alternate mechanism of spore germination.

Individual deletions of the genes *sleB, cwlJ1* and *cwlJ2* did not cause major differences in germination kinetics or colony forming efficiency (Figure 2.1). These mutants were not tested for virulence in mice. The double mutants Δ*sleB/cwlJ2* and Δ*cwlJ1/cwlJ2* also only produced minor effects on germination kinetics and had no effect on colony-forming efficiency (Figs. 2.1-2). However, the double mutant Δ*sleB/cwlJ1* was decreased by three orders of magnitude in colony-forming efficiency on BHI (Figure 2). Despite this defect, the small percentage of the spore population that was able to outgrow, germinated with kinetics very similar to wild-type spores (Figure 2.1C). I believe the assay measuring a drop in optical density was not sensitive enough to detect the germination kinetics of the subpopulation of spores that managed to germinate. Because only outgrowing spores can be detected in the assay measuring loss of heat resistance, I was able to see that in fact the population defect was not uniform and that while the bulk of the spores are defective, a small number germinate like wild type. The triple mutant Δ*sleB/cwlJ1/cwlJ2* had a decrease of seven orders of magnitude in colony-

forming efficiency in BHI compared to wild-type (Figure 2.2). Because of this severe defect in germination, the germination kinetics of outgrowing spores could not be measured; however, the population exhibited the same severe defect as Δ*sleB/cwlJ1* spores when assayed by drop in optical density (Figure 2.1D).

B. subtilis and other related species of spore-forming bacteria contain one copy of the gene *cwlJ*, while the *B. anthracis* genome contains a second copy referred to as *cwlJ2* (Chirakkal, O'Rourke et al. 2002; Heffron, Orsburn et al. 2009; Setlow, Peng et al. 2009). The single copy of *cwlJ* in other species, and the gene *cwlJ1* in *B. anthracis* are found in an operon with *gerQ,* a gene that has been shown to be required for proper targeting of *cwlJ* in the developing forespore (Ragkousi, Eichenberger et al. 2003). This conserved gene structure implies the relative importance of *cwlJ1* over *cwlJ2*. Mutations of *cwlJ2* show no major defect in colony-forming efficiency (Figure 2.2), suggesting that CwlJ2 is a less functional or redundant protein. Although CwlJ2 does not appear to be the primary protein involved, it is active during germination and can compensate in the absence of CwlJ1. The double mutant Δ*cwlJ1/cwlJ2* has a greater germination defect than either of the single mutations (Figure 1). Also, the triple mutant Δ*sleB/cwlJ1/cwlJ2,* has a 100,000-fold decrease in colony forming ability compared to the double mutant Δ*sleB/cwlJ1* in BHI (Figure 2.2). These experiments show that SleB or CwlJ1 are sufficient for near wild-type levels of spore germination. CwlJ2 is sufficient for low-level germination, which can be observed when this protein is compensating for the absence of SleB and CwlJ1.

GSLEs play a critical role in the germination of all spore-forming bacteria, and it has long been thought that targeting the activity of these enzymes could provide useful

therapies. The complete lack of *in vitro* germination of Δ*sleB/cwlJ1/cwlJ2* supports this theory, but the continued virulence in a mouse model indicates that there are alternate mechanisms of germination that can result in disease. I calculated the LD_{50} of wild-type Sterne $34F_2$ to be $1.5x10^4$ spores/mouse in the DBA/2J mouse model. Mutant strains Δ*sleB/cwlJ1* and Δ*sleB/cwlJ1/cwlJ2* were attenuated and had increased LD_{50s} of 80-fold and 550-fold that of wild-type respectively (Table 2.2). Despite an increase in LD_{50} and a large decrease in colony-forming efficiency for Δ*sleB/cwlJ1/cwlJ2* compared to Δ*sleB/cwlJ1*, at no dose tested was there a significant difference in murine survival following infection with these two mutant strains (Figure 2.3 and data not shown). This suggests that an alternate method of germination may involve limiting host factors *in vivo*. It remains possible that these factors are host-specific germinants that activate unknown GSLEs. However, it is much more likely that a host factor is able to complement missing GSLE functions. These potential factors are unknown, and are currently under investigation. They may include host enzymes with peptidoglycan hydrolyzing activity such as lysozyme. The ability of Δ*sleB/cwlJ1/cwlJ2* spores to germinate in bovine serum but not heat-treated bovine serum suggests the role of a host enzyme in germination and supports a host factor complementation mechanism (Figure 4). Interestingly, the triple mutant colony-forming efficiency in bovine serum is about 3 orders of magnitude below that of wild-type (Figure 2.4) which is consistent with the 550-fold decrease in LD_{50} between the triple mutant and the wild-type strain (Table 2.2).

It is generally believed that inhalation anthrax is the result of spore germination within alveolar macrophages (Ross 1957; Guidi-Rontani, Weber-Levy et al. 1999). Inhaled spores remain dormant in the lungs, possibly for many weeks, until they are

engulfed by lung macrophages and trafficked to the nearest lymph node. *B. anthracis* spores engulfed by macrophages are not killed, but undergo germination and replication within the cell until it is ruptured (Dixon, Fadl et al. 2000). It would be interesting to know if the alternate germination mechanism provided by components of the serum was also active in alveolar macrophages.

SleB, CwlJ1, and to a lesser extent CwlJ2 enzymes play important roles in the successful germination and outgrowth of *B. anthracis* spores. Removal of all three enzymes is sufficient to block *in vitro* germination under standard laboratory conditions. Continued virulence of these strains despite their lack of *in vitro* germination sheds new light on secondary, host-directed mechanisms of germination *in vivo.* Although it is clear that many benefits in disease therapies and spore decontamination can come from targeting germination specific lytic enzymes, it will be important to understand and control these secondary factors to fully combat disease.

Chapter III

Host lysozyme contributes to *Bacillus anthracis* spore germination in vivo.

Abstract

Germination-specific lytic enzymes (GSLEs) are required for typical *Bacillus anthracis* spore germination. Here I investigate the ability of lysozyme to complement GSLE activity in a *B. anthracis* mutant lacking *sleB, cwlJ1* and *cwlJ2* (Δ*sleB/cwlJ1/cwlJ2*). Germination of mutant spores was measured in vitro using nutrient rich medium (BHI), murine serum, and a solution of purified hen egg white lysozyme. Δ*sleB/cwlJ1/cwlJ2* spore germination was readily detected in serum and in purified lysozyme but not in heat-treated serum. This germination phenotype correlated with the presence of lysozyme activity in each sample. Bone marrow-derived macrophages from wild-type mice were able to induce germination of Δ*sleB/cwlJ1/cwlJ2* spores while macrophages derived from lysozyme M-deficient mice could not. Finally, Lysozyme $M^{-/-}$ mice were protected against infection with Δ*sleB/cwlJ1/cwlJ2* spores compared to wild-type mice. Spore germination is a critical step in the disease anthrax. Therefore GSLEs are attractive therapeutic targets. This work increases our understanding of the interaction between *B. anthracis* spores and the host, specifically as it relates to cortex degradation and GSLE function during germination.

Introduction

During an inhalational anthrax infection, *Bacillus anthracis* spores are taken up into the alveolar spaces of the host lungs (Dixon, Meselson et al. 1999). Here they are engulfed by macrophages or dendritic cells and the process of spore germination, a critical first step in disease initiation, begins (Ross 1957; Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000; Cleret, Quesnel-Hellmann et al. 2007). The proteins required for germination, including those involved in host recognition, breaking down spore structures, and transitioning to vegetative cell growth, are prepackaged into endospores during sporulation (Kroos and Yu 2000; Liu, Bergman et al. 2004). Once these proteins are activated, the germination process is irreversible (Foster and Johnstone 1988). While dormant, germinant receptor proteins located in the inner membrane of the spore passively sense the spore's external environment (Moir, Corfe et al. 2002). In *B. anthracis* spores, these receptors are activated by specific combinations of purine nucleosides, typically inosine, and L-amino acids (Carr, Lybarger et al. 2010). Activation of germinant receptors initiates stage I germination, during which channels are opened in the spore allowing partial core rehydration. As water flows into the spore, cations and dipicolinic acid flow out (Moir, Corfe et al. 2002). These events trigger stage II germination which involves the activation of germination-specific lytic enzymes (GSLEs) (Setlow 2003). GSLEs hydrolyze peptidoglycan containing muramic-δ-lactam, a modified muramic acid residue found only in the spore cortex (Atrih, Zöllner et al. 1996; Popham, Helin et al. 1996). This modification allows for specific degradation of the cortex, leaving the germ cell wall peptidoglycan intact. GSLEs must degrade the

spore cortex to allow full rehydration of the core and the transition to a vegetative bacillus (Setlow, Melly et al. 2001). Since cortex breakdown and full core rehydration are required for normal enzymatic function, GSLE activation is a critical checkpoint during germination (Setlow, Melly et al. 2001).

B. anthracis germination involves the action of four GSLEs (Heffron, Orsburn et al. 2009). Two of these enzymes, SleB and CwlJ1, are individually sufficient to complete germination (Giebel, Carr et al. 2009; Heffron, Lambert et al. 2010). The two remaining enzymes, CwlJ2 and SleL, serve secondary roles in cortex degradation and cortex fragment modification (Lambert and Popham 2008; Giebel, Carr et al. 2009). When the genes *sleB, cwlJ1* and *cwlJ2* are disrupted (Δ*sleB/cwlJ1/cwlJ2*), nutrient-induced spore germination as outlined above is abolished (Giebel, Carr et al. 2009). When these mutant spores come into contact with traditional *B. anthracis* germinants, such as in the rich bacterial broth BHI, stage I germination events proceed normally (Giebel, Carr et al. 2009). However, in stage II germination, cortex degradation is blocked and the cells cannot complete core rehydration, which is necessary for transition to vegetative growth (Setlow, Melly et al. 2001). I have previously shown that when Δ*sleB/cwlJ1/cwlJ2* spores are plated on BHI, only 1 in 80 million spores successfully germinate and subsequently multiply, creating a colony-forming unit (cfu) (Giebel, Carr et al. 2009).

Since germination is essential to initiate disease, these in vitro findings would predict that Δ*sleB/cwlJ1/cwlJ2* spores would be incapable of infecting and killing mice. However, DBA/2J mice are more susceptible to infection by this mutant than would be predicted by this in vitro germination rate. The LD_{50} for DBA/2J mice infected with wild-type ($34F_2$ Sterne strain) *B. anthracis* spores intratracheally is 15,000 spores/mouse,

which based on the 1:1 colony-forming efficiency in wild-type *B. anthracis* represents 15,000 cfu/mouse (Giebel, Carr et al. 2009). However, when DBA/2J mice were infected with 1.5 million Δ*sleB/cwlJ1/cwlJ2* spores, predicted to represent less than 1 cfu based on their severely decreased colony-forming rate, 25% of mice succumb to the infection. This suggests that spores within a mouse complete germination more efficiently than predicted, either by means of a previously unidentified GSLE or using a non-nutrient pathway.

B. anthracis spores are known to germinate in several nutrient-independent pathways including by exogenous lysozyme treatment (Setlow 2003). Like native GSLEs, lysozyme hydrolyzes the β-1,4 glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid (Ibrahim, Aoki et al. 2002). Lysozyme treatment can induce germination in mutants that affect GSLE activity, and would likely be encountered by spores in vivo making it a likely candidate for increased Δ*sleB/cwlJ1/cwlJ2* germination and virulence (Cross, Mangelsdorf et al. 1988; Sekiguchi, Akeo et al. 1995).

Mutant spores of the related species, *Bacillus subtilis*, that lack the gene *cwlD,* fail to incorporate the muramic-δ-lactam modification in their cortex peptidoglycan (Popham, Helin et al. 1996). Since this modification is required for GSLE recognition and activity, a *cwlD* mutant effectively prevents the action of all GSLEs. In this case, loss of *cwlD* completely blocked colony formation much like the defect observed for Δ*sleB/cwlJ1/cwlJ2* spores. When *cwlD* spores were treated with lysozyme, a small percentage was able to germinate and grow, overcoming the loss of GSLE activity (Sekiguchi, Akeo et al. 1995).

Lysozyme is expressed in a variety of tissues within a broad range of animals, including mice (Fleming 1922; Cross, Mangelsdorf et al. 1988). Unlike humans, which express only one lysozyme gene, mice have evolved two genes that encode for this protein, lysozyme M and lysozyme P (Hammer, Schilling et al. 1987). Lysozyme M is the most closely related to human lysozyme and is found in the majority of mouse tissues. In contrast, lysozyme P is predominantly expressed in the small intestine and may have evolved to function in digestion (Hammer, Schilling et al. 1987; Hammer and Wilson 1987; Cross, Mangelsdorf et al. 1988). Lysozyme M is the more relevant form of this protein to *B. anthracis* infections because it is abundant in the lungs, macrophages, and in serum, environments that spores encounter early in infection. This protein has also been shown to function in host defense against bacterial infections (Cross, Mangelsdorf et al. 1988; Akinbi, Epaud et al. 2000; Markart, Korfhagen et al. 2004). During a laboratory challenge, Δ*sleB/cwlJ1/cwlJ2* spores injected into the lungs would immediately encounter lysozyme M secreted onto the airway surface by lung epithelial cells. Spores would also be exposed to high concentrations of lysozyme M after being phagocytosed by alveolar macrophages, as is predicted at the onset of a typical anthrax infection. Because it has been shown that lysozyme can induce spore germination in a nutrient independent manner and that spores inoculated intratracheally will repeatedly encounter host lysozyme, I set out to test the hypothesis that lysozyme-induced germination leads to increased virulence in Δ*sleB/cwlJ1/cwlJ2* infection. In this study, I test the ability of murine serum and purified lysozyme to induce Δ*sleB/cwlJ1/cwlJ2* spore germination in vitro, as well as the role of lysozyme M during experimental infections.

I would like to thank Jill Fritz and Dr. Henry Akinbi for providing lysozyme deficient mice for this work. Also for their expert advice in revising this text.

Materials and Methods

Bacterial culture conditions and spore preparation

B. anthracis strain 34F_2:Δ*sleB/cwlJ1/cwlJ2* (Giebel, Carr et al. 2009) was cultured in brain heart infusion medium (BHI) (Difco) and colony-forming units were titered on BHI plus 1.5% agarose plates. Spore stocks were prepared as previously described (Kim and Goepfert 1974; Dixon, Fadl et al. 2000). Briefly, actively dividing vegetative cells, taken from a log-phase culture, were diluted 1:100 in modified G medium (0.2% yeast extract, 0.17 mM $CaCl_2$, 2.87 mM K_2HPO_4, 0.81 mM $MgSO_4$, 0.24 mM $MnSO_4$, 17 μM $ZnCl_2$, 20 μM $CuSO_4$, 1.8 μM $FeCl_3$, 15.5 mM $(NH_4)_2SO_4$, pH 7.2) and incubated at 37^{o}C and 300 RPM for 72 hours. Following incubation, cultures were observed microscopically to verify the presence of phase-bright spores. Spores were purified by washing three times in sterile deionized H_2O and passage through a 3.1 micron filter (National Scientific Company, part #F2500-20). Following filtration, spore suspensions were heat treated at 65 ^{o}C for 20 minutes to kill any remaining vegetative bacilli.

Germination assays and lysozyme activity assays

Colony-forming efficiency is defined as the number of spore particles that represent one cfu. An improved Neubauer haemocytometer was used to assay spore particles per milliliter. $2x10^6$ spore particles were incubated in each germinant for 10 minutes at 37 ^{o}C. The entire reaction was plated on BHI to determine cfu/ml.

Germinants that were assayed included 10 μg/ml HEWL (Sigma), mouse serum (MP Biomedicals), and BHI. Lysozyme activity was measured using the EnzChek Lysozyme Assay Kit (Molecular Probes) according to the manufacturer's protocols and recommendations.

Mouse strains and virulence assays

Lysozyme M deficient mice were generated by inserting the enhanced green fluorescent protein gene in exon 1 of the lysozyme M gene resulting in a null mutation. The mice are indistinguishable from WT mice. Characterization of the lysozyme $M^{-/-}$ has been previously reported (Faust, Varas et al. 2000; Markart, Faust et al. 2004). The mice were backcrossed to FVB/N through 10 generations. For all experiments, age- and sex-matched WT FVB/N were used as controls.

Intratracheal infections of wild-type and lysozyme $M^{-/-}$ mice were conducted as previously described (Heffernan, Thomason et al. 2007). Groups of eight female mice age 6-8 weeks were infected with $1.5x10^7$ spores/mouse. Mice were monitored for up to 14 days following infection.

Spore germination in bone marrow-derived macrophages (BMM)

BMM were cultured and harvested as previously described (Swanson 1989). Briefly, bone marrow from the hind leg bones of two mice (4 femurs, 4 tibia), was pooled for wild-type or lysozyme $M^{-/-}$ genotypes. Cells were differentiated in DMEM with 20% FBS and 30% L cell-conditioned medium. Cultures were allowed to differentiate for one week. Additional medium containing 30% L-cell conditioned medium was added at day-3.

For spore germination assays, $4x10^5$ BMM/well were seeded into 12-well tissue culture treated plates (Corning) and incubated overnight at 37°C and 5% CO_2. Cells were washed three times in 1.0 ml of HBSS (pre-warmed to 37°C). Spores were diluted to $4x10^7$ spores/ml in MEM + L-glutamine and 1.0 ml was added to each well. Plates were centrifuged at 200x*g* for 30 seconds to settle spores onto macrophages. Spores and macrophages were incubated at 37°C and 5% CO_2 for one hour. Non-internalized spores were removed by washing macrophages with 1.0 ml of HBSS. BMM were incubated for 20 minutes at room temperature in 500 μl of MEM + L-glutamine + 20 μg/ml of gentamicin to kill any extracellular spores that may have germinated. The gentamicin-containing medium was removed by washing cells three times in 1.0 ml of HBSS. Cells were scraped and lysed in 200 μl of MEM + 1% saponin. Cell lysate was plated on BHI and cultured overnight at 37°C to measure the number of cfu.

Results

Spores lacking GSLEs germinate in murine serum and purified lysozyme in vitro. A Δ*sleB/cwlJ1/cwlJ2* mutant strain of *B. anthracis* is incapable of germinating in response to nutrients in vitro (Giebel, Carr et al. 2009). Although these spores are highly attenuated in a mouse model of inhalation anthrax, they maintain an unexpected level of virulence (Giebel, Carr et al. 2009). This suggests that the Δ*sleB/cwlJ1/cwlJ2* germination rate must be higher in vivo than it is in nutrient rich medium in vitro.

Colony-forming efficiency is increased when Δ*sleB/cwlJ1/cwlJ2* spores are incubated in murine serum compared to BHI (Figure 3.1A). Serum is an easily obtainable bodily fluid, and although not likely encountered during inhalational anthrax, it is likely to come into contact with spores in a cutaneous infection. Colony-forming efficiency was determined by comparing the number of spores/ml, quantified visually using a haemocytometer, to the numbers of cfu/ml when incubated in various germinants. When Δ*sleB/cwlJ1/cwlJ2* spores were incubated in BHI prior to plating, colony-forming efficiency was below our limit of detection, as expected (Figure 3.1A). When Δ*sleB/cwlJ1/cwlJ2* spores were incubated in murine serum prior to plating on BHI, colony-forming efficiency was increased to 5.0×10^4 spores/cfu (Figure 3.1A). When serum was heat-treated at 65^{o}C for 20 minutes, the ability to induce Δ*sleB/cwlJ1/cwlJ2* spore germination (Figure 3.1A) was abolished. These findings show that murine serum is capable of inducing Δ*sleB/cwlJ1/cwlJ2* spore germination, and are in agreement with earlier published results using bovine serum (Giebel, Carr et al. 2009).

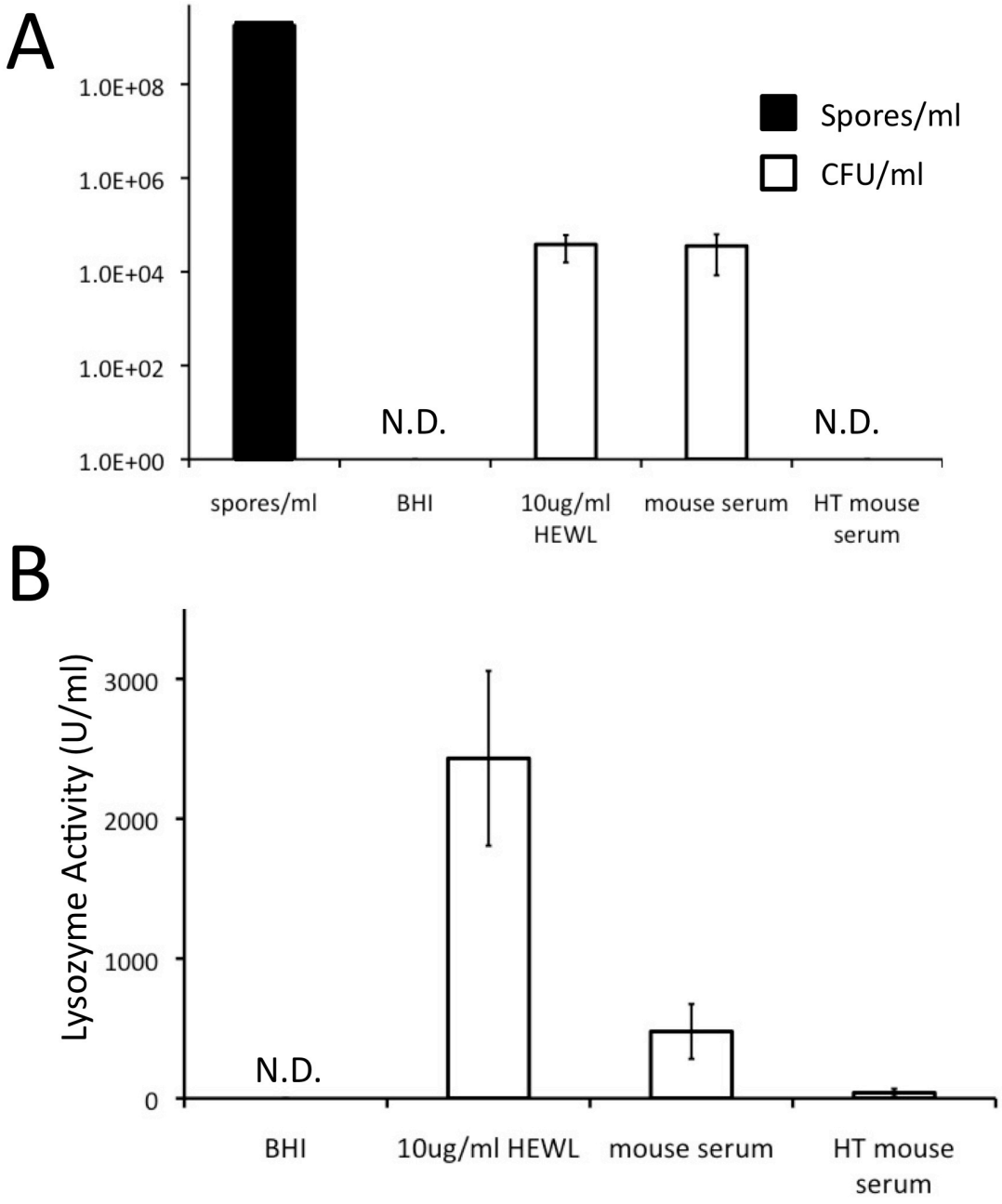

Figure 3.1: Colony-forming efficiency and corresponding lysozyme activity.
Colony forming efficiency (A) was determined by visually assaying the number of spore particles/ml (black bar) using an improved Neubauer haemocytometer compared to the number cfu/ml (white bars) when tittered on BHI. Lysozyme activity (B) was measured by release of fluorescently labeled substrate compared to a standard curve generated using purified lysozyme. Data shown represent the mean value of three replicate experiments; error bars represent ±standard error. N.D.= none detected

The ability of serum to induce mutant spore germination is heat-sensitive; therefore I hypothesized that an enzyme might be involved. As previously discussed, lysozyme was a prime candidate to compensate for the loss of native GSLE activity; therefore I tested the ability of purified lysozyme to induce Δ*sleB/cwlJ1/cwlJ2* spore germination (Figure 3.1A). When Δ*sleB/cwlJ1/cwlJ2* spores were preincubated in a solution of purified hen egg-white lysozyme (10μg/ml) (HEWL), colony-forming efficiency was increased to $4.7x10^4$ spores/cfu. This indicated that lysozyme alone was sufficient to induce germination of Δ*sleB/cwlJ1/cwlJ2* spores.

I next measured lysozyme activity in murine serum and heat-treated murine serum to assess the correlation between the loss of the Δ*sleB/cwlJ1/cwlJ2* germination phenotype and the loss of lysozyme activity (Figure 3.1B). The average lysozyme activity in murine serum was found to be 480 ± 110 U/ml. Following heat treatment, the lysozyme activity in mouse serum was reduced to 40 ± 17 U/ml. These data show a correlation between lysozyme activity and Δ*sleB/cwlJ1/cwlJ2* spore germination.

Lysozyme M enhances the virulence of a GSLE mutant in mice.

To test whether increased colony-forming efficiency of Δ*sleB/cwlJ1/cwlJ2* spores in vitro is responsible for increased virulence in mice, I infected lysozyme M deficient mice with mutant spores. These mice were in the FVB/NJ parental background rather than the DBA/2J background that was previously used to test the virulence of Δ*sleB/cwlJ1/cwlJ2* spores.

It has been shown that different inbred mouse strains have varying degrees of susceptibility to *B. anthracis* infection primarily based on the presence of the hemolytic complement deficient allele Hc^0 (Welkos, Keener et al. 1986; Welkos and Friedlander 1988). Mouse strains containing the Hc^0 allele are much more susceptible to $34F_2$ Sterne strain spores than those with wild-type alleles. Because both DBA/2J and FVB/NJ mice contain the Hc^0 allele, I predicted that FVB/NJ and Lysozyme $M^{-/-}$ mice would be similarly susceptible to $34F_2$ infection as DBA/2J mice were. To verify this, FVB/NJ and lysozyme $M^{-/-}$ mice were infected with a single dose of wild-type spores (Figure 3.2A). Mice were infected intratracheally with $1.5x10^7$ spores/mouse to simulate inhalational anthrax infection. FVB/NJ mice were highly susceptible to this dose with 100% of the mice succumbing to the disease by day-3 post infection (Figure 3.2A). This lethality rate is similar to previously published virulence studies using DBA/2J mice (Giebel, Carr et al. 2009; Carlson, Dixon et al. 2010; Carr, Lybarger et al. 2010). At this dosage, lysozyme $M^{-/-}$ mice appear slightly more susceptible to $34F_2$ spores ($p=0.06$) (Figure 3.2A). All Lysozyme $M^{-/-}$ mice challenged with wild-type spores succumb to the infection after only one day. This shows that FVB/NJ and lysozyme $M^{-/-}$ mice do not

have a generally increased resistance to *B. anthracis* infection and lysozyme $M^{-/-}$ might even be more susceptible than their parental background. This suggests that lysozyme M may be involved in innate immunity during anthrax infection. Although this is an intriguing observation, the experiments necessary to confirm this hypothesis were beyond the scope of this study.

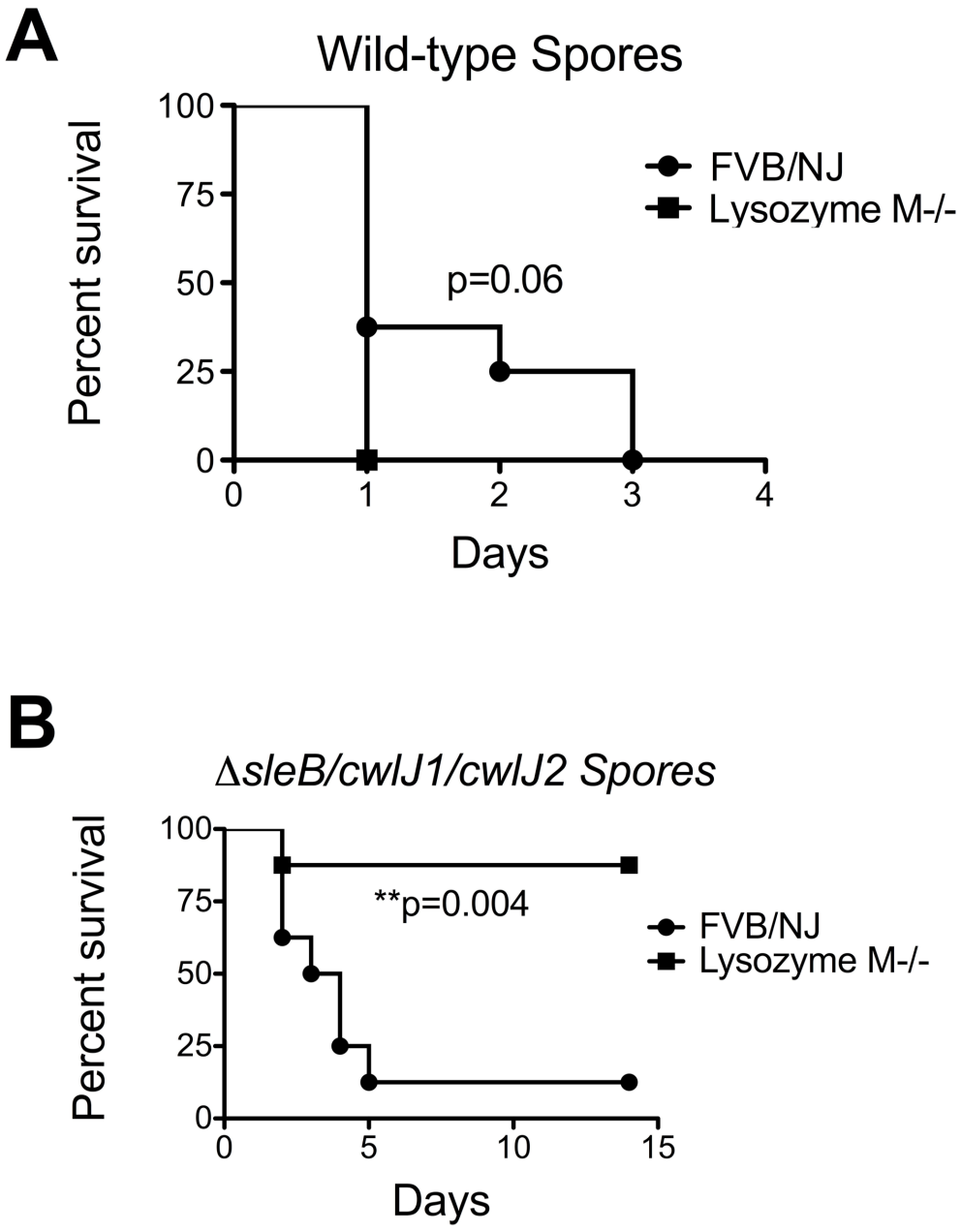

Figure 3.2: Survival curves of FVB/NJ and Lysozyme M-/- mice challenged with wild-type and ΔsleB/cwlJ1/cwlJ2 spores.
FVB/NJ (●) and lysozyme $M^{-/-}$ (■) mice (n=8 per group) were challenged intratracheally with $1.5x10^7$ $34F_2$ spores/mouse (A) or $1.5x10^7$ Δ*sleB/cwlJ1/cwlJ2* spores/mouse. Mice were monitored for a period of 14 days post infection at which point any surviving mice were euthanized. Lysozyme $M^{-/-}$ mice were found to be significantly protected from infection by Δ*sleB/cwlJ1/cwlJ2* spores compared to FVB/NJ mice (p=0.004), by log-rank (Mantel-Cox) test.

FVB/NJ and Lysozyme $M^{-/-}$ mice were next challenged with Δ*sleB/cwlJ1/cwlJ2* spores to test the role of lysozyme M in Δ*sleB/cwlJ1/cwlJ2* virulence. Again mice were challenged intratracheally with $1.5x10^7$ spores/mouse. At this dose, 90% of wild-type mice succumb to the infection after 5 days (Figure 3.2B). However, lysozyme $M^{-/-}$ mice infected with the same high dose of Δ*sleB/cwlJ1/cwlJ2* spores did not develop disease (Figure 3.2B). Only one mouse succumbed to the infection during the course of the experiment (*p=0.004)*. These results show that deficiency of lysozyme M was associated with enhanced resistance to infection by Δ*sleB/cwlJ1/cwlJ2* spores, likely due to decreased spore germination in vivo.

Spore germination in bone marrow-derived macrophages and serum from knockout mice

In inhalation anthrax, spore germination takes place in association with alveolar phagocytes following engulfment (Ross 1957). To mimic this essential step in disease in vitro, I harvested bone marrow cells from the femurs of wild-type and lysozyme $M^{-/-}$ transgenic mice and cultured them in macrophage colony-stimulating factor to obtain bone marrow-derived macrophages (BMM). $4x10^5$ BMM were infected at an MOI of 100 and were treated with extracellular gentamicin following the challenge to ensure that only intracellular germination was being measured. When BMM from wild-type mice were infected with Δ*sleB/cwlJ1/cwlJ2* spores, an average of 99 ± 16 spores germinated following internalization (Figure 3.3). However, no germination could be detected from Δ*sleB/cwlJ1/cwlJ2* spores internalized by lysozyme $M^{-/-}$ BMM (Figure 3.3). These data indicate that lysozyme M is required for Δ*sleB/cwlJ1/cwlJ2* spore germination within macrophages.

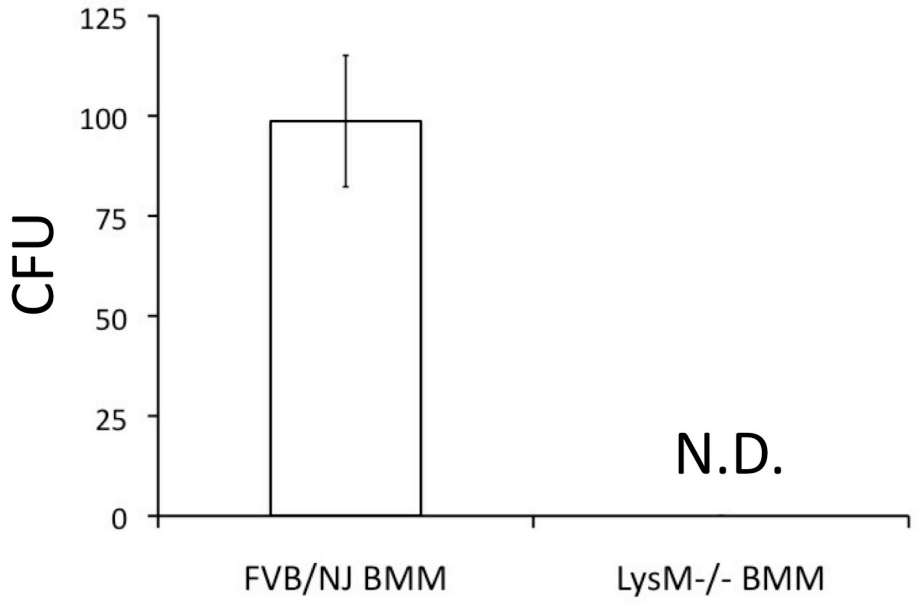

Figure 3.3: ΔsleB/cwlJ1/cwlJ2 spore germination within bone marrow-derived macrophages.
BMM were harvested from the hind leg bones of wild-type (FVB/NJ) and lysozyme $M^{-/-}$ mice. Cells were differentiated in culture containing macrophage colony-stimulating factor. Following incubation with spores, BMM were treated with gentamicin to kill any remaining extracellular spores that may have germinated. This insured only internalized spores were measured. Data shown represent the mean value of three replicate experiments; error bar represents ±standard error. N.D.= none detected

In addition to primary macrophages, filtered serum was also collected from lysozyme $M^{-/-}$ mice. Colony-forming efficiency assays with Δ*sleB/cwlJ1/cwlJ2* spores were preformed using wild-type serum as well as mutant mouse serum as germinants. Colony-forming efficiency of spores incubated in lysozyme $M^{-/-}$ serum remained at wild-type serum levels (Figure 3.4A). However, when lysozyme activity in this germinant was measured it was found to be approximately equal to that of wild-type serum (Figure 3.4B). Presumably, lysozyme P expression was upregulated in the absence of lysozyme M in serum. To overcome this I collected filtered serum from a newly developed lysozyme $MP^{-/-}$ mouse strain. This strain is completely devoid of lysozyme expression as verified by a lysozyme activity assay (Figure 3.4B). Mutant spore germination could not be detected following incubation in this lysozyme negative serum (Figure 3.4A). This shows that lysozyme alone is responsible for mutant spore germination in murine serum.

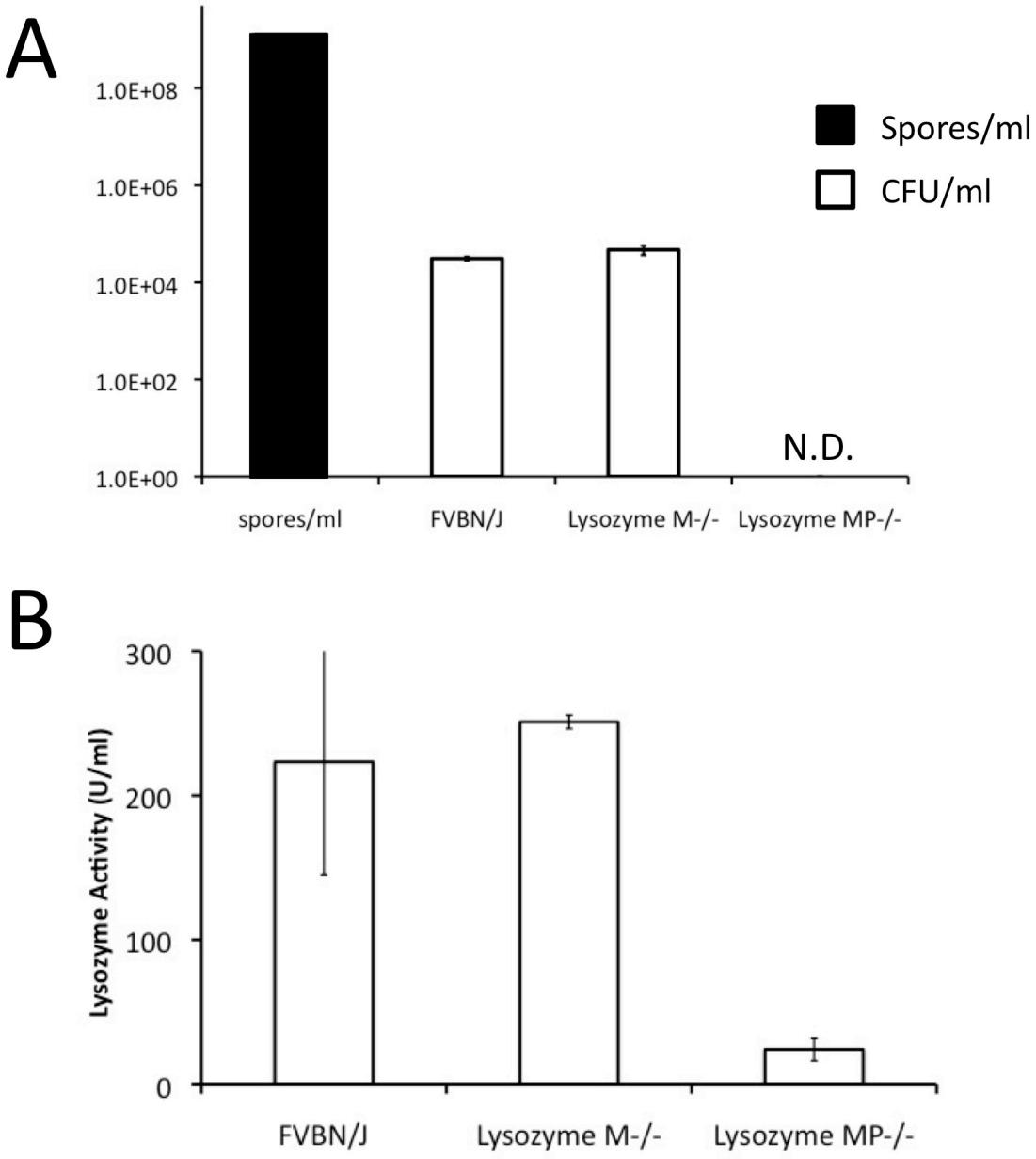

Figure 3.4: Colony forming efficiency of Δ*sleB/cwlJ1/cwlJ2* spores in lysozyme deficient serum.
Colony forming efficiency (A) was determined by visually assaying the number of spore particles/ml (black bar) using an improved Neubauer haemocytometer compared to the number cfu/ml (white bars) when tittered on BHI. Lysozyme activity (B) was measured by release of fluorescently labeled substrate compared to a standard curve generated using purified lysozyme. Data shown represent the mean value of two replicate experiments; error bars represent ±standard error.

Discussion

I have shown previously that mutant *B. anthracis* spores lacking *sleB, cwlJ1* and *cwlJ2* are completely blocked from in vitro germination in response to nutrients, but that these spores have higher than expected virulence in a mouse model of inhalation anthrax (Giebel, Carr et al. 2009). Those results suggested that Δ*sleB/cwlJ1/cwlJ2* germination rates in vivo were significantly increased. The increased germination rate could be a result of an in vivo specific germinant, but this would require the presence of an unknown GSLE that can be activated by the in vivo specific germinant. A much more likely explanation is that germination is being triggered in a non-nutrient dependent manner that bypasses the need for GSLEs.

Besides nutrient-based germination, spores have been shown to germinate in response to a variety of non-nutrient stimuli including a 1:1 chelate of Ca^{2++} and dipicolinic acid (Paidhungat, Ragkousi et al. 2001), cationic surfactants (Rode and Foster 1961; Setlow, Cowan et al. 2003), high physical pressure (Gould, Sale et al. 1969; Gould and Sale 1970) and lysozyme (Sekiguchi, Akeo et al. 1995; Popham, Helin et al. 1996). Exogenous calcium ions and dipicolinic acid induce germination by activating CwlJ (Paidhungat, Ragkousi et al. 2001). Both *cwlJ1* and *cwlJ2* are lacking in the Δ*sleB/cwlJ1/cwlJ2* mutant, so this germination pathway cannot account for its increased virulence. High pressure would not be exerted on spores in vivo, and as such, would not contribute to the increased virulence.

The most likely pathway for increased Δ*sleB/cwlJ1/cwlJ2* spore germination in vivo is cortex degradation by lysozyme. Lysozyme degrades peptidoglycan by hydrolyzing the β-1,4 glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid (Ibrahim, Aoki et al. 2002). Presumably, this enzymatic activity leads to spore germination by sufficiently disrupting the cortex allowing core rehydration and outgrowth without the need for GSLEs. Also, there is a high likelihood that *B. anthracis* spores contact a high concentration of lysozyme in the airways of the host. It is estimated that the concentration of lysozyme in airway surface liquid is >100 μg/ml (Travis, Conway et al. 1999). In addition to being located in several bodily fluids, such as saliva, tears and serum, lysozyme is abundant in alveolar macrophages and lung epithelial cells (Fleming 1922; Gordon, Todd et al. 1974; Cross, Mangelsdorf et al. 1988). Very early in an inhalational anthrax infection, spores are thought to encounter both of these cell types (Ross 1957).

The role of lysozyme in Δ*sleB/cwlJ1/cwlJ2* spore germination was further supported by in vitro germination assays. Incubating Δ*sleB/cwlJ1/cwlJ2* spores in BHI results in an extremely low colony-forming efficiency, below the limit of detection ($2x10^6$ spores/cfu) in our assay (Figure 3.1A). When mutant spores were incubated in a solution of HEWL (10μg/ml), their colony-forming efficiency was increased at least 40-fold to $4.7x10^4$ spores/cfu (Figure 3.1A). This demonstrates that lysozyme alone is sufficient to increase germination rates of Δ*sleB/cwlJ1/cwlJ2* spores. To test whether lysozyme could induce spore germination in a more natural environment, I began by exposing Δ*sleB/cwlJ1/cwlJ2* spores to murine serum in vitro. Although spores will not likely encounter serum-associated factors during inhalation anthrax, they would be

exposed to them during a cutaneous infection. Similar to incubation in purified lysozyme, the colony-forming efficiency of Δ*sleB/cwlJ1/cwlJ2* spores incubated in murine serum was increased at least 40-fold over that of spores incubated in BHI (Figure 3.1A). When serum was heat-treated, lysozyme activity was abrogated and its ability to induce Δ*sleB/cwlJ1/cwlJ2* spore germination was abolished (Figure 3.1A and 3.1B).

Although this showed that lysozyme activity was correlated with the ability to cause germination, I did not observe a linear relationship. Colony-forming efficiency of Δ*sleB/cwlJ1/cwlJ2* spores incubated in serum or a solution of HEWL (10μg/ml) was approximately the same ($5.0x10^4$ spores/ml and $4.7x10^4$ spores/ml respectively). However, the lysozyme activity of purified lysozyme was found to be approximately five times higher than that of murine serum (Figure 3.1B). This suggests that there is a lysozyme activity threshold and that increased activity above this threshold will not lead to increased spore germination. One possible explanation for these findings is that only a small percentage of spores allow lysozyme access to their cortex, presumably due to rare defects in the outer layers of the spore. If true, this observation suggests a new function for these protective layers in the environment. It is possible that the coat and exosporium have evolved to help maintain spore dormancy. Without these layers, the spore would be susceptible to lysozyme and other peptidoglycan hydrolyzing enzymes that could cause premature germination. The coats may shield the spore from these foreign enzymes allowing the spore's natural nutrient sensing pathway to dictate the most favorable time to initiate germination.

GSLEs play a critical role in *B. anthracis* virulence and their function is required for nutrient based spore germination (Giebel, Carr et al. 2009). However, as

demonstrated by the Δ*sleB/cwlJ1/cwlJ2* mutant, even if these critical enzymes could be completely inhibited some spores may still germinate leading to an active infection. When wild-type mice were challenged with $1.5x10^7$ Δ*sleB/cwlJ1/cwlJ2* spores, 90% succumb to the infection (Figure 3.2B). However, when lysozyme M-deficient mice were challenged with the same dose, only one mouse succumbed to the infection (Figure 3.2B). This shows that host lysozyme is capable of germinating spores that would be otherwise unable to exit dormancy. Based on our current understanding of anthrax pathogenicity, it is likely that the host lysozyme responsible for germinating these spores was encountered within alveolar macrophages and in the airway surface lining fluid. This theory is supported by in vitro data using BMM from wild-type and lysozyme $M^{-/-}$ mice. A small percentage of Δ*sleB/cwlJ1/cwlJ2* spores internalized by wild-type BMM were germinated (Figure 3.3). However, no germination could be measured when spores were incubated in the presence of BMM from lysozyme $M^{-/-}$ mice. This shows that the presence of lysozyme within a host macrophage, the most likely environment for a spore following inhalation, is sufficient to induce germination.

Although lysozyme is able to enhance/induce *B. anthracis* spore germination both in vitro and in vivo, it is unlikely that this pathway significantly contributes to disease in a natural infection. Only a very small number of spores are susceptible to germination by lysozyme. Therefore, lysozyme-induced germination events would certainly be overwhelmed by the number of spores that undergo germination in response to typical nutrient germinants encountered during infection. Rather, these data collected using Δ*sleB/cwlJ1/cwlJ2* spores as a tool to observe rare germination events, provides insight and questions into the use of spores as a dormant vaccine vector, the feasibility of

blocking GSLE activity to prevent disease, and the role of outer spore layers in preserving dormancy.

Chapter IV

Discussion and Future Directions

The focus of this research was to further our understanding of cortex hydrolysis in *B. anthracis*. Breakdown of the cortex is a critical transition step in germination and work by many groups over the past several decades has contributed greatly to our understanding of conserved hydrolytic enzymes that are needed to accomplish this. Chapter 2 outlines a traditional cortex hydrolysis pathway of *B. anthracis.* As in other spore forming bacteria, *B. anthracis* employs the coordinated effort of several GSLEs that are packaged into the spore during sporulation and are only activated at a precise time when cortex hydrolysis is required. Unexpected germination of mutants developed in Chapter 2 lead to the realization of an entirely separate mechanism for cortex hydrolysis. This new mechanism, host-lysozyme induced germination, is characterized in Chapter 3. Below I will discuss how this new pathway impacts current beliefs in the spore germination field.

Host-lysozyme induced germination among other spore forming bacteria

The role of host-lysozyme induced germination, identified in Chapter 2 and further characterized in Chapter 3, has recently been applied to *Clostridium perfringens* spore germination research (Paredes-Sabja and Sarker 2011). Indeed, published work

from Chapter 2 was cited as the impetus for this study. *C. perfringens* is a Gram-positive spore forming bacteria that can cause a wide spectrum of disease (Amimoto, Noro et al. 2007; Sayeed, Li et al. 2007; Sayeed, Li et al. 2010). Following spore germination inside a host, toxins secreted by *C. perfringens* vegetative cells can cause gas gangrene, antibiotic-associated diarrhea, and a variety of other diseases. As is the case for anthrax, spore germination inside the host is a critical step in disease initiation. However, relatively little is known about *C. perfringens* spore germination in vivo. A recent study by Paredes-Sabja and Sarker has suggested that host-lysozyme induced germination may be an important step in the early stages of *C. perfringens* pathogenesis (Paredes-Sabja and Sarker 2011).

Three disease causing clinical isolates of *C. perfringens* showed rates of germination varying from 20% to 100% when incubated in the rich medium BHI (Paredes-Sabja and Sarker 2011). However, all of these strains reached nearly 100% germination when they were incubated in sheep blood, sheep serum, or human serum. Blood or serum-induced germination was not diminished in mutants lacking germinant receptors. This showed that serum-induced germination of *C. perfringens* spores is not dependent on nutrients and does not require traditional spore germination machinery. As is the case in *B. anthracis* germination, *C. perfringens* spores lacking GSLEs do not germinate when incubated in BHI. However, approximately 10% of GSLE deficient *C. perfringens* spores do germinate when incubated in serum or blood. This phenotype is similar to serum based germination assays with Δ*sleB/cwlJ1/cwlJ2 B. anthracis* spores (Figure 2.4 and 3.1). Size exclusion filtration and Western-blotting strongly implicated lysozyme as the serum component that was responsible for non-nutrient *C. perfringens*

germination (Paredes-Sabja and Sarker 2011). The role of host lysozyme-induced germination on *C. perfringens* pathogenesis was not determined. However, based on my findings in *B. anthracis,* I would predict that GSLE deficient *C. perfringens* spores would be attenuated in lysozyme deficient mice compared to wild-type mice.

It appears that both *B. anthracis* and *C. perfringens* spores are able to germinate in response to host lysozyme. However, because of the much greater number of spores that can germinate in this way, host-lysozyme induced germination may be more biologically relevant in the case of *C. perfringens* infection. As discussed in Chapter 2, only ~0.01% of GSLE deficient *B. anthracis* spores germinate in serum. In analogous *C. perfringens* mutants, ~10% of spores are able to germinate when incubated in serum, an increase of 1000-fold over the *B. anthracis* response. This suggests that compared to the minimal role of host lysozyme-induced germination in a *B. anthracis* infection, a much higher percentage of *C. perfringens* spores may germinate through this alternate route in vivo. *C. perfringens* pathogenesis studies must be conducted to determine if host-lysozyme induced germination does in fact play a critical role.

A fundamental difference in spore coat composition and structure is a likely explanation for the varying rates of lysozyme-induced spore germination between these two species. Removal of spore coats has shown that this structure provides protection from exogenous lytic enzymes such as lysozyme (Popham, Helin et al. 1996; Paredes-Sabja and Sarker 2011). In *B. subtilis,* the spore coat prevents degradation of spores phagocytosed by the protozoan *Tetrahymena thermophila* (Klobutcher, Ragkousi et al. 2006). The protein composition of spore coats in *Bacillus* species is quite conserved; however, very few of these proteins are found in the coats of *Clostridium* species

(Henriques and Moran 2007). This difference in protein composition and likely coat structure could lead to varying accessibility of the cortex to exogenous lysozyme and therefore altered lysozyme-induced germination rates.

Another possible explanation for variable sensitivity to host lysozyme between these two species is the exosporium. The presence and morphology of this spore structure is highly variable across species but its function is very poorly understood (Hodgkiss, Ordal et al. 1967; Rode 1971; Driks 2007). The molecular makeup of the *B. anthracis* exosporium has been partially defined but little is known about the chemical makeup of *C. perfringens* exosporium (Steichen, Chen et al. 2003; Liu, Bergman et al. 2004). Structural differences between the two species can be seen by electron microscopy but both appear to have a crystalline like basal layer with small pores that may allow the passage of small molecule germinants but probably do not allow large molecules such as lysozyme to pass through (Hoeniger, Stuart et al. 1968; Ball, Taylor et al. 2008). Future studies using *B. anthracis* and *C. perfringens* spores with and without their exosporium are required to determine if the structural differences in this layer result in varying sensitivity to host-lysozyme.

The chemical structure of cortex peptidoglycan has been studied in several spore-forming bacteria including, *B. anthracis* and *C. perfringens* (Popham 2002; Dowd, Orsburn et al. 2008). Only minor differences have been found between the two species making cortex sensitivity to lysozyme treatment an unlikely explanation for differential germination rates. Regardless of the mechanism behind variable rates in host-lysozyme induced germination, it remains to be seen whether or not this difference represents an evolutionary strategy of either species to optimize virulence.

Vaccinology implications of lysozyme induced germination

Many attempts have been made to use *Bacillus* spores as vaccine vector platforms expressing recombinant antigen on their surface (Duc le, Hong et al. 2003; Oggioni, Ciabattini et al. 2003; Ferreira, Ferreira et al. 2005). This approach offers potentially important advantages in vaccine development. Spores are extremely resistant to environmental stresses. They are able to withstand desiccation, extreme heat, and radiation (Setlow 2007). The stability of the spore allows for an extremely long shelf life of the potential vaccine as well as transportation without regard to maintaining a cold chain. Another potential advantage of bacterial spore vaccine vectors is an enhanced immune response due to cross presentation of spore surface proteins with the antigen of interest (Duc le, Hong et al. 2003).

An obvious drawback to the use of live bacteria as a vaccine vector is a potential for pathogenesis. For this reason *B. subtilis* is typically chosen because although it may pose a concern for severely immunocompromised patients, it is generally believed to be non-pathogenic in humans and animals (Sonenshein, Hoch et al. 1993). However, some studies suggest that *B. anthracis* may produce better local and systemic immune responses due to its increased antigenicity (Ferreira, Ferreira et al. 2005). Live *B. anthracis* spores of the attenuated Sterne strain have been used as vectors for veterinary vaccines to protect animals from *B. anthracis* infection, *L. monocytogenes* infection and *C. perfringens* toxins (Brossier, Mock et al. 1999). Although *B. anthracis* Sterne strain has been used since the 1930's as an anthrax vaccine for animals, and to a lesser extent humans, the remote possibility of infection and side effects such as fever and edema

caused by anthrax toxin prevent more widespread applications. Attempts have been made to further inhibit toxin activity, through point mutations, without affecting immunogenicity (Pezard, Berche et al. 1991; Pezard, Weber et al. 1995). An attractive alternative to fully detoxifying *B. anthracis* is blocking germination rendering the spores inert and incapable of causing disease.

As attractive as this is however, this approach would be complicated by host lysozyme-induced germination. As outlined in Chapter 3, even if spore germination machinery is completely disabled, some fraction of the population may still germinate. This alternate germination pathway must be considered in any future attempts to create vaccines based on *B. anthracis* spores.

Future Directions: The link between GSLE localization and silencing

A next step in understanding cortex hydrolysis in vivo is determining the silencing and activation mechanism of GSLEs. The astonishing ability of GSLEs to remain silent for hundreds of years while in close proximity to their substrate and then be activated within a matter of minutes is very poorly understood. Some information regarding the activation of CwlJ is known (Paidhungat, Ragkousi et al. 2001; Ragkousi, Eichenberger et al. 2003); however, nothing is known about the activation of SleB or the way in which germinant receptors transmit the signal to begin germination to these down stream enzymes. One possible mechanism for GSLE silencing is sequestration during packaging. Spore assembly during sporulation is a tightly regulated process. Mother cell and forespore specific sigma factors ensure that most proteins packaged into the mature spore, including GSLEs, are expressed at a precise time point and from a precise location. It is possible that GSLE localization dictated during sporulation is the mechanism by which these enzymes remain silent.

Specific localization of GSLEs has been demonstrated by immunoelectron microscopy. SleB in *B. subtilis* and *B. cereus,* and its homologue SleC in *C. perfringens,* are expressed in the forespore and localize to the outer layer of the cortex, just inside the spore coat (Miyata, Kozuka et al. 1997; Moriyama, Fukuoka et al. 1999). The specific localization of SleB in *B. anthracis* spores has not yet been determined. More importantly, nothing is known about the functionality of this enzyme if this localization pattern is disrupted.

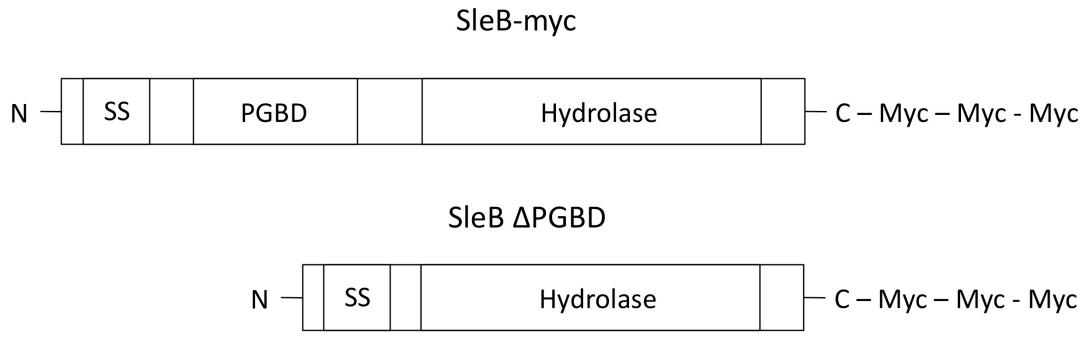

Figure 4.1: Conserved domain structure of full length and truncated SleB
SS: signal sequence. PGBD: peptidoglycan binding domain

SleB is a 253-amino-acid protein with a predicted signal sequence at its amino-terminus and two conserved domains, a hydrolytic domain and a peptidoglycan-binding domain (PGBD) (Figure 4.1). The hydrolytic domain is a member of the pfam07486 hydrolases and is homologous to the hydrolytic domain in CwlJ1 and CwlJ2. The PGBD is conserved among SleB homologues in *B. subtilis* and *B. cereus,* as well as a variety of other peptidoglycan hydrolyzing enzymes (Miyata, Moriyama et al. 1995; Moriyama, Hattori et al. 1996; Masayama, Fukuoka et al. 2006).

In vitro studies showed that the hydrolytic domain was active in the absence of the PGBD but that SleB activity was increased 10-fold when the PGBD is present (Heffron, Sherry et al. 2011). Full length SleB was able to bind purified peptidoglycan with nearly 100% efficiency. The hydrolytic domain alone was also able to bind peptidoglycan (approximately 50% efficiency). Interestingly, while full length SleB showed no preferential binding to cortex peptidoglycan over cell wall peptidoglycan, the hydrolytic domain alone preferentially bound to purified cortex (Heffron, Sherry et al. 2011). This suggests that the hydrolytic domain might dictate proper substrate binding during cortex hydrolysis. Based on these in vitro studies, I developed the following hypothesis for SleB silencing and activation. SleB is bound by its PGBD to the outermost region of the cortex where it does not have access to its substrate. When the germination process is initiated, SleB is released and cortex hydrolysis begins.

To date I do not have conclusive evidence to support or reject this hypothesis, but I have begun preliminary experiments to visualize the localization of wild-type SleB as well as a truncated SleB protein that lacks a peptidoglycan-binding domain. I have made

sleB constructs that fuse a carboxy-terminal myc tag onto full length SleB (SleB-myc) and truncated SleB that lacks a PGBD (SleBΔPGBD) (Figure 4.1). These proteins were expressed in a GSLE null strain of *B. anthracis* (*ΔsleB/cwlJ1/cwlJ2)*. Using these strains expressing tagged proteins, I have begun to track wild-type and truncated SleB localization by immunoelectron microscopy. Preliminary results show possible full-length SleB localization to the outer layer of the cortex, similar to what has been observed in *B. subtilis* and *B. cereus* (Figure 4.2A). However, localization of SleBΔPGBD has not been detected as of yet (Figure 4.2B). Experimental conditions need to be refined to increase the detection of full length as well as truncated protein.

In addition to testing the role of the PGBD in SleB localization in the dormant spore, this system could be used to track any movement of SleB throughout germination. Once the experimental conditions have been optimized, spores expressing myc-tagged full length SleB could be fixed at various time points following incubation in germinants. This experiment would show if SleB localization changes upon initiation of germination as predicted by my hypothesis. Future work to refine these results can identify the cellular localization of SleB throughout this process and whether or not proper localization requires the PGBD.

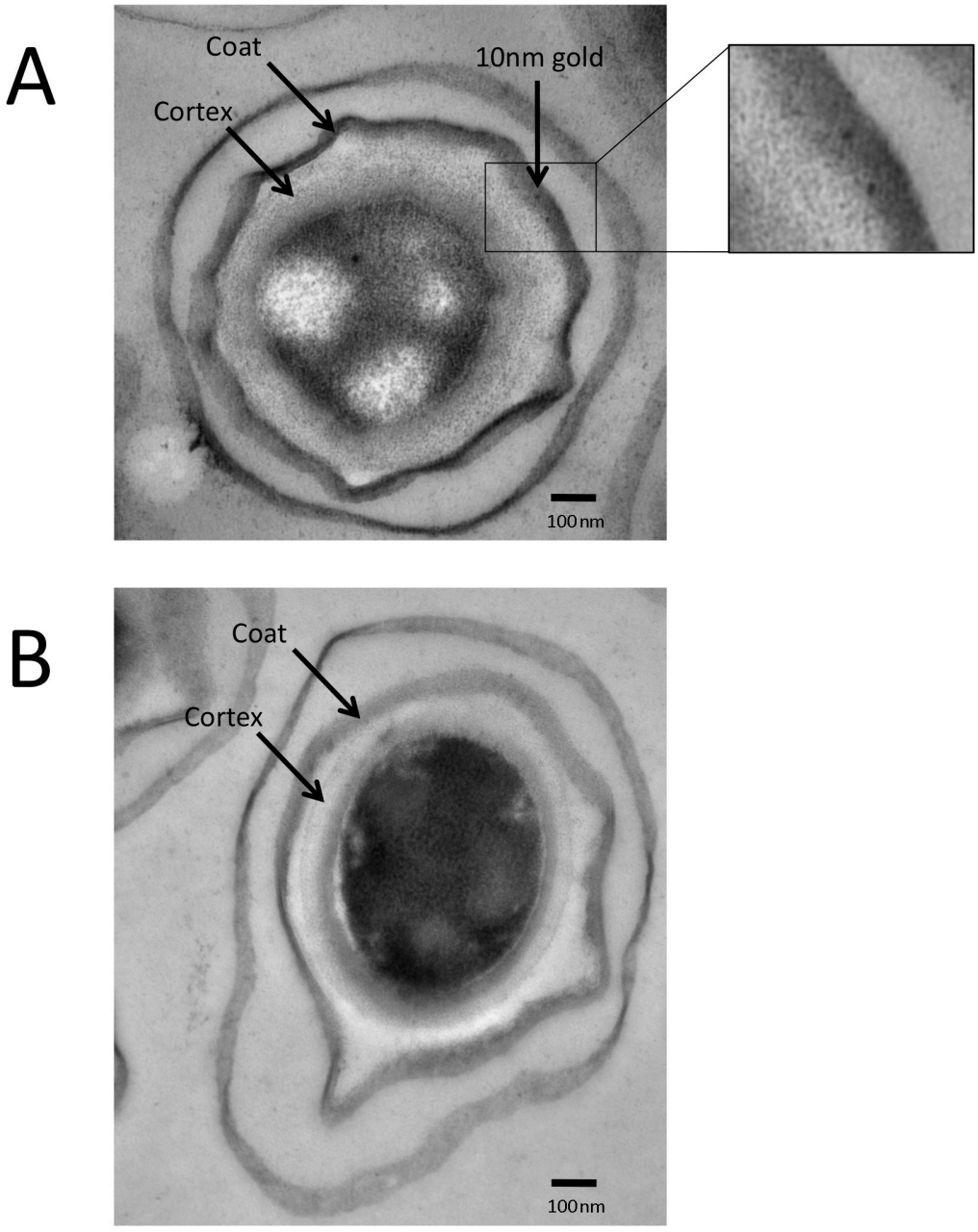

Figure 4.2: Immunoelectron microscopic localization of full length and truncated SleB.

Spores expressing myc-tagged SleB (A) and myc-tagged SleBΔPGBD (B) were fixed in 4% paraformaldehyde and ultra-thin sections were taken. Samples were stained with C-myc mouse monoclonal antibody [9E10] and goat anti-mouse IgG secondary antibody conjugated to 10nm gold particles. The sections were examined using a Philips CM100 electron microscope at 60kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Conclusion

The entire germination process, and especially cortex degradation, represents a fascinating example of enzymatic regulation. The bacterial spore is nature's loaded gun. Everything it needs to fire is incorporated during sporulation and yet it is able to remain silent for centuries, if not millennia, until the trigger is pulled by the proper germinant and the spore's germination potential is released with incredible speed.

The studies presented in this thesis outline a conventional role for *B. anthracis* GSLEs that has been seen in the germination of many other spore-forming species. Much more interestingly, this work goes on to describe a new mechanism by which spores exit dormancy in vivo. The existence of this alternate germination pathway that relies on host-lysozyme sheds new light on potential functions of spore coats and exosporium. It is unclear whether host-induced lysozyme germination is an evolutionarily designed mechanism for in vivo germination or is a detrimental process that should be avoided until the spore germinates via its endogenous machinery following recognition of traditional nutrient germinants. It is very possible that the positive or negative implications of this pathway are species specific and that different spore forming pathogens have optimized their outer protective layers, to enhance or inhibit this event. Future studies on the vast array of spore coats and spore appendages, as well as the role of host-lysozyme germination in the pathogenesis of other spore forming bacteria will further our understanding of host pathogen interactions in these diseases.

Appendix I

Germination by Calcium and Dipicolinic Acid

This appendix includes data describing the activation of CwlJ1 and CwlJ2 by exogenous Ca^{2+} and dipicolinic acid.

BACKGROUND

Spore germination is an irreversible chain of events that is preprogrammed during sporulation (Setlow 2003). At that time, all of the proteins required to complete the transition back to a vegetative cell are packaged into the mature spore (Liu, Bergman et al. 2004). However, during a spore's extended dormant phase, these proteins must remain silent to maintain the spore's integrity in the environment. As outlined in Chapter 1 (Figure 1.4), germination events are separated into early events (stage I germination) and late events (stage II) germination. Recognition of germinants by receptors in the inner membrane is the signal that sets these pre-programmed events into action; however, the mechanism by which that signal is conveyed to GSLEs, which are activated in stage II germination, is unclear (Setlow, Melly et al. 2001).

To date, the only evidence of a possible mechanism for this signaling is the activation of CwlJ by a 1:1 chelate of exogenous Ca^{2+} and DPA (Paidhungat, Ragkousi et al. 2001; Ragkousi, Eichenberger et al. 2003). It was first shown by Riemann and Ordal in 1961 that a 1:1 ratio of Ca^{2+} and DPA would induce spore germination in *C. perfringens, B. subtilis, B. cereus*, and several other *Bacillus* species (Riemann and Ordal

1961). This method of spore germination was not dependent on the presence of germinant receptors and therefore believed to act downstream in the germination cascade (Paidhungat and Setlow 2000). In *B. subtilis,* CwlJ is required for Ca^{2+}/DPA germination (Paidhungat, Ragkousi et al. 2001; Ragkousi, Eichenberger et al. 2003). It was suggested that this method of CwlJ activation may occur in natural germination when ions and DPA are released from the spore core following stage-I germination (Paidhungat, Ragkousi et al. 2001). Unlike *B. subtilis, B. anthracis* encodes two CwlJ homologues, CwlJ1 and CwlJ2. The effect of Ca^{2+}/DPA on activation of these enzymes or their relative contributions to germination in response to Ca^{2+}/DPA has not been defined. Here I will present data from germination assays of wild-type and GSLE deficient spores incubated in Ca^{2+} and DPA to define the response of SleB, CwlJ1 and CwlJ2 to this non-nutrient germination pathway.

Materials and Methods

Bacterial strains

Bacterial strains and plasmids used in this study are listed in Table A1.1. Vegetative *B. anthracis* cultures were grown in brain-heart infusion media (BHI, Difco) and *Escherichia coli* cultures were grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001). *B. anthracis* spore stocks were prepared as previously described (Kim and Goepfert 1974; Dixon, Fadl et al. 2000). Mutant bacterial strains were constructed using the allelic exchange method as previously described (Janes and Stibitz 2006; Lee, Janes et al. 2007).

Germination Assays

Spore germination was measured by loss of heat sensitivity. Spore stocks were diluted to approximately $4x10^5$ spores/ml and heat activated by incubation at 65^oC for 20 minutes. Germination was initiated by adding 10 μl of spores to 2.0 ml of 60mM $CaCl_2$ and DPA pH=8.5. At each time point (2, 5, 10, 15, 20, and 30 minutes) 100 μl aliquots of this solution were removed, incubated at 65^oC for 20 minutes, then 50 μl was plated onto BHI agar and incubated overnight. Colonies that formed represented spores that failed to germinate and were therefore heat resistant. As a control, 50 μl of spores at time 0 were plated without heat treatment. The fraction of germinated spores for each time point was calculated as $(1-t_n)/t_0$ and reported as a percentage.

Table A1.1: Bacterial strains and plasmids

Strain	Relevant Characteristics	Reference
Sterne $34F_2$	Wild-type ($pXO1^+$, $pXO2^-$)	(Sterne 1939)
KC63	$34F_2$, *ΔsleB ΔypeB*	This work
JG48	$34F_2$, *ΔcwlJ1*	This work
JG49	$34F_2$, *ΔcwlJ2*	This work
JG50	$34F_2$, *ΔsleB ΔypeB ΔcwlJ1*	This work
JG51	$34F_2$, *ΔsleB ΔypeB ΔcwlJ2*	This work
JG52	$34F_2$, *ΔcwlJ1 ΔcwlJ2*	This work
JG54	*E. coli,* BL21 pJDG105	This work

Plasmids	Relevant Characteristics	Reference
pBKJ236	Allelic exchange vector, Em^r	(Janes and Stibitz 2006)
pBKJ258	Allelic exchange vector, Kan^r	(Lee, Janes et al. 2007)
pBKJ223	Tet^r, P_{amy} I-SceI	(Janes and Stibitz 2006)
pJDG101	*ΔsleB* construct cloned in pBKJ258	This work
pJDG102	*ΔypeB* construct cloned in pBKJ258	This work
pJDG103	*ΔcwlJ1* construct cloned in pBKJ236	This work
pJDG104	*ΔcwlJ2* construct cloned in pBKJ258	This work
pJDG105	pET300/NT-DEST:*cwlJ1*	This work

Western Blot and Antibody Production

Recombinant CwlJ was expressed under an IPTG-inducible promoter with an N-terminal 6xHistidine fusion in BL21 *E. coli* cells. Protein purification was preformed using Talon metal affinity resin. Purified recombinant protein was sent to Rockland Immunochemicals Inc. (Gilbertsville, PA) for rabbit immunization and serum collection.

CwlJ1 protein expression in *B. anthracis* spores was measured by SDS-PAGE and Western blotting with a rabbit polyclonal antibody. Prior to separation on a gel, spores were mechanically disrupted using a bead beater and 0.1mm glass beads.

Results and Discussion

Loss of heat resistance was used as a marker for germination of wild-type and mutant *B. anthracis* strains incubated in 60mM $CaCl_2$ and DPA (Figure A1.1). Wild-type Sterne $34F_2$ spores reach approximately 100% germination within the first 15 minutes (Figure A1.1A). Previous studies have shown that spore germination in response to Ca^{2+} and DPA requires the enzyme CwlJ (Paidhungat, Ragkousi et al. 2001; Ragkousi, Eichenberger et al. 2003). *B. anthracis* mutants lacking *cwlJ1, cwlJ2*, or both (*ΔcwlJ1/cwlJ2*) were tested for germination rates in 60mM $CaCl_2$ and DPA to assay whether one or both of these genes were required for this type of germination. Individual gene deletions *ΔcwlJ1* and *ΔcwlJ2* showed intermediate defects in response to Ca^{2+}/DPA (Figure A1.1A). Spores lacking CwlJ2 were delayed in the start of germination and only reached 74±12% germination in 30 minutes compared to 95±6% for wild-type. Spores lacking CwlJ1 were even more defective in response to Ca^{2+}/DPA with only 26±11% of spores being able to germinate within 30 minutes. When spores lacking both CwlJ1 and CwlJ2 were incubated in Ca^{2+} and DPA, germination was completely abolished. These results suggest that although both CwlJ1 and CwlJ2 contribute to Ca^{2+}/DPA germination, CwlJ1 activity is more important.

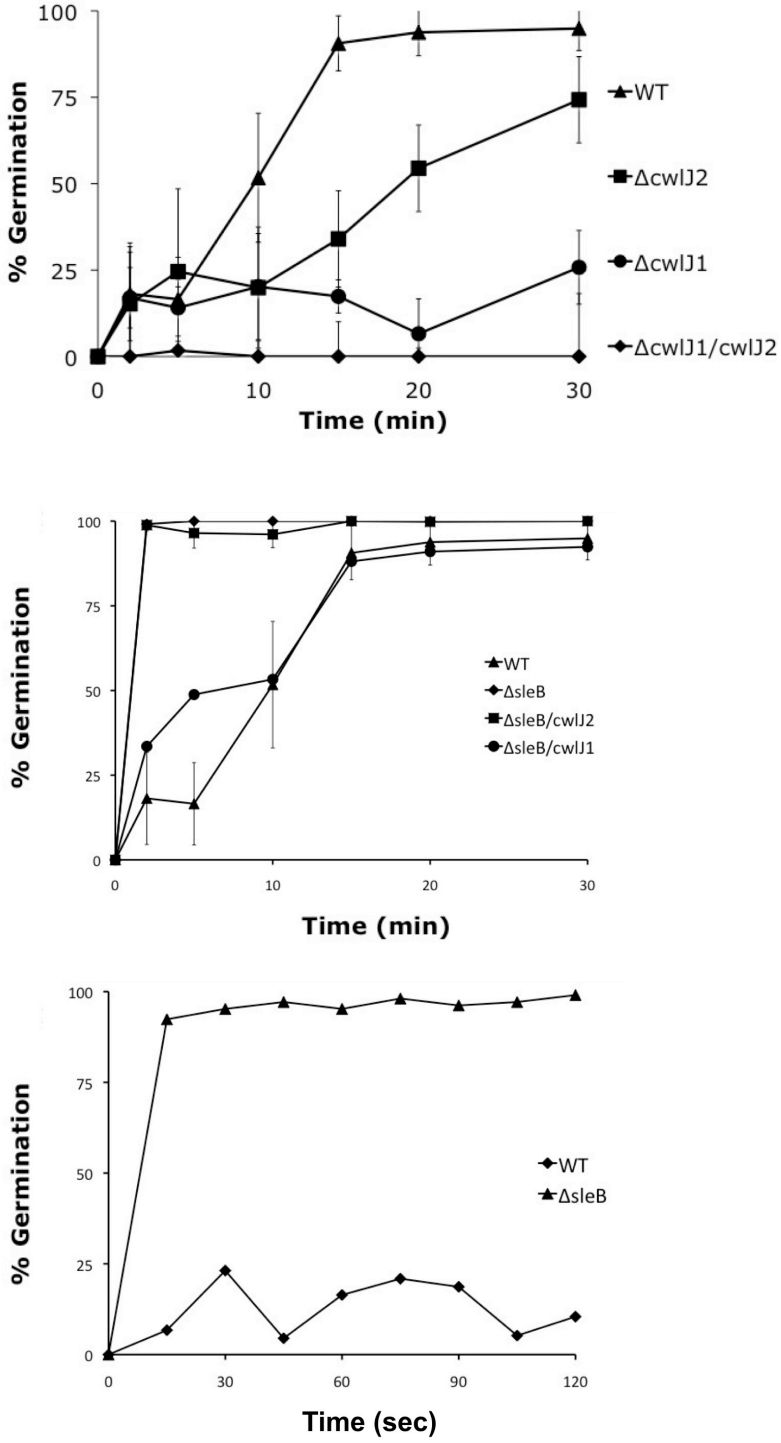

Figure A1.1: *B. anthracis* spore germination in 60mM Ca^{2+} and DPA.
Spores were incubated in 60mM $CaCl_2$ and DPA pH8.5 at 37°C for 0-30 min (A and B) or 0-120 sec (C). Percent germination was monitored by loss of heat resistance. Panels A and B represent mean values from at least 3 independent experiments, error bars represent ±standard error.

In addition to CwlJ mutants, spores lacking SleB were assayed to test whether exogenous Ca^{2+} and DPA affected SleB activity. Spores lacking SleB (*ΔsleB)* reached 100% germination within the first two minutes, the first time point measured (Figure A1.1B). This hypergermination phenotype was a very surprising result, as the SleB homolog in *B. subtilis* has no reported effect on Ca^{2+}/DPA germination. It is also counterintuitive that the loss of a GSLE (SleB is highly active in all other germinants tested) could lead to an increase in germination rate. To measure how quickly *ΔsleB* spores lost their heat resistance, the germination assay was repeated, this time taking 15-second time points for 0-2 minutes (Figure A1.1C). At the earliest time point, 15 seconds, *ΔsleB* spores had already reached 92% germination. These data indicate that *ΔsleB* spores lose their heat resistance almost immediately upon exposure to exogenous Ca^{2+} and DPA. As shown in Chapter 2 this phenotype is specific to Ca^{2+}/DPA germination and is not seen with nutrient based germinants (Figure 2.1).

Double mutants *ΔsleB/cwlJ1* and *ΔsleB/cwlJ2* and the triple mutant *ΔsleB/cwlJ1/cwlJ2* were also tested for the ability to germinate in exogenous Ca^{2+} and DPA (Figure A1.1B and data not shown). Spores lacking SleB and CwlJ2 exhibited a hypergermination phenotype very similar to *ΔsleB*. Spores lacking SleB and CwlJ1 did not have a hypergermination phenotype, however the germination rate was significantly increased, approximately to that of wild-type spores. Spores lacking all GSLEs (*ΔsleB/cwlJ1/cwlJ2*) did not germinate when incubated in Ca^{2+} and DPA (data not shown). These data suggest that loss of SleB induces a hypergermination phenotype, but that this response requires CwlJ1.

I hypothesized that loss of *sleB* affected the expression level of *cwlJ1,* causing hypergermination as a result of increased CwlJ1 protein levels. I tested this by Western blot using a polyclonal antibody against CwlJ1. No obvious change in CwlJ1 protein level could be detected between wild-type, *ΔsleB,* and *ΔsleB/cwlJ2* (Figure A2.1).

It is possible that lack of SleB affects CwlJ1 activity, localization, or substrate binding affinity. Enzymatic activity assays using purified enzymes in vitro could identify possible regulatory roles of SleB on CwlJ1. Similarly, SleB may bind to CwlJ1 in the spore and limit its ability to respond to Ca^{2+} and DPA. Direct binding studies would be difficult because the harsh conditions required to break down the spore would likely disrupt protein-protein interactions between these enzymes. It may be possible to visualize potential co-localization of SleB and CwlJ1 by immunoelectron microscopy with two different secondary antibodies conjugated to different sized gold particles. Further studies are required to define the mechanism of this surprising phenotype.

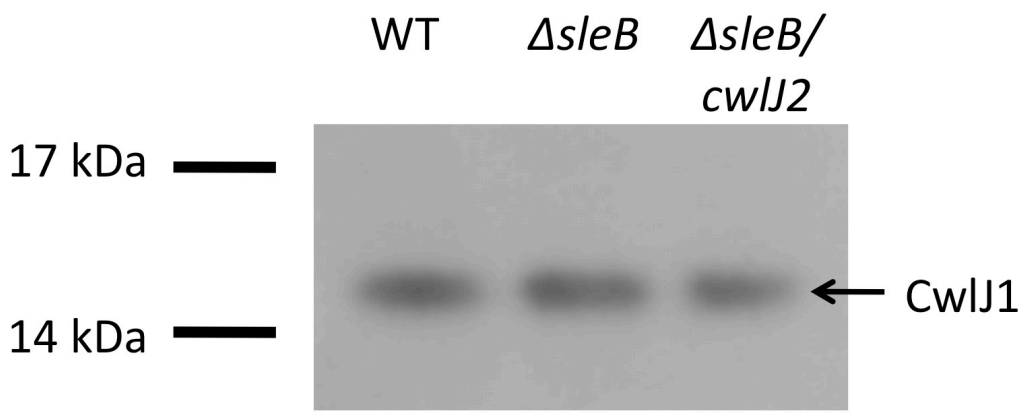

Figure A1.2: CwlJ1 expression in *ΔsleB* spores.
Total protein extract from wild-type (WT), *ΔsleB,* and *ΔsleB/cwlJ2* spores was separated by SDS-PAGE. CwlJ1 protein was detected by western blot with an anti-CwlJ1 polyclonal antibody.

References

Akinbi, H. T., R. Epaud, et al. (2000). Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. J Immunol. **165:** 5760-5766.

Albrink, W. S. (1961). "Pathogenesis of inhalation anthrax." Bacteriol Rev **25**: 268-273.

Amimoto, K., T. Noro, et al. (2007). "A novel toxin homologous to large clostridial cytotoxins found in culture supernatant of *Clostridium perfringens* type C." Microbiology **153**(Pt 4): 1198-1206.

Aronson, A. I. and P. Fitz-James (1976). "Structure and morphogenesis of the bacterial spore coat." Bacteriol Rev **40**(2): 360-402.

Ascenzi, P., P. Visca, et al. (2002). "Anthrax toxin: a tripartite lethal combination." FEBS Lett **531**(3): 384-388.

Atrih, A. and S. J. Foster (2001). "In vivo roles of the germination-specific lytic enzymes of *Bacillus subtilis* 168." Microbiology **147**(Pt 11): 2925-2932.

Atrih, A., P. Zöllner, et al. (1996). Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. Journal of Bacteriology. **178:** 6173-6183.

Bagyan, I. and P. Setlow (2002). "Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*." J Bacteriol **184**(4): 1219-1224.

Ball, D. A., R. Taylor, et al. (2008). "Structure of the exosporium and sublayers of spores of the *Bacillus cereus* family revealed by electron crystallography." Mol Microbiol **68**(4): 947-958.

Basu, S., T. J. Kang, et al. (2007). "Role of *Bacillus anthracis* spore structures in macrophage cytokine responses." Infect Immun **75**(5): 2351-2358.

Beauregard, K. E., R. J. Collier, et al. (2000). "Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization." Cell Microbiol **2**(3): 251-258.

Bergman, N. H., E. C. Anderson, et al. (2006). "Transcriptional profiling of the *Bacillus anthracis* life cycle in vitro and an implied model for regulation of spore formation." J Bacteriol **188**(17): 6092-6100.

Boland, F. M., A. Atrih, et al. (2000). "Complete spore-cortex hydrolysis during germination of *Bacillus subtilis* 168 requires SleB and YpeB." Microbiology **146 (Pt 1)**: 57-64.

Brossier, F., M. Mock, et al. (1999). "Antigen delivery by attenuated *Bacillus anthracis*: new prospects in veterinary vaccines." J Appl Microbiol **87**(2): 298-302.

Candela, T. and A. Fouet (2005). "Bacillus anthracis CapD, belonging to the gamma-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan." Mol Microbiol **57**(3): 717-726.

Candela, T., M. Mock, et al. (2005). "CapE, a 47-amino-acid peptide, is necessary for *Bacillus anthracis* polyglutamate capsule synthesis." J Bacteriol **187**(22): 7765-7772.

Cano, R. J. and M. K. Borucki (1995). "Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber." Science **268**(5213): 1060-1064.

Carlson, P. E., Jr., S. D. Dixon, et al. (2010). "Genetic analysis of petrobactin transport in *Bacillus anthracis*." Mol Microbiol.

Carr, K. A., S. R. Lybarger, et al. (2010). "The role of *Bacillus anthracis* germinant receptors in germination and virulence." Mol Microbiol **75**(2): 365-375.

Chang, L. and M. Karin (2001). "Mammalian MAP kinase signalling cascades." Nature **410**(6824): 37-40.

Chen, Y., S. Fukuoka, et al. (2000). "A novel spore peptidoglycan hydrolase of *Bacillus cereus*: biochemical characterization and nucleotide sequence of the corresponding gene, sleL." J Bacteriol **182**(6): 1499-1506.

Chirakkal, H., M. O'Rourke, et al. (2002). "Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination." Microbiology **148**(Pt 8): 2383-2392.

Cleret, A., A. Quesnel-Hellmann, et al. (2007). "Lung dendritic cells rapidly mediate anthrax spore entry through the pulmonary route." J Immunol **178**(12): 7994-8001.

Corfe, B. M., A. Moir, et al. (1994). "Analysis of the expression and regulation of the gerB spore germination operon of *Bacillus subtilis* 168." Microbiology **140 (Pt 11)**: 3079-3083.

Corfe, B. M., R. L. Sammons, et al. (1994). "The gerB region of the *Bacillus subtilis* 168 chromosome encodes a homologue of the gerA spore germination operon." Microbiology **140 (Pt 3)**: 471-478.

Cote, C. K., N. Van Rooijen, et al. (2006). "Roles of macrophages and neutrophils in the early host response to *Bacillus anthracis* spores in a mouse model of infection." Infect Immun **74**(1): 469-480.

Cross, M., I. Mangelsdorf, et al. (1988). "Mouse lysozyme M gene: isolation, characterization, and expression studies." Proc Natl Acad Sci U S A **85**(17): 6232-6236.

Dixon, T., M. Meselson, et al. (1999). Anthrax. N Engl J Med. **341:** 815-826.

Dixon, T. C., A. A. Fadl, et al. (2000). "Early *Bacillus anthracis*-macrophage interactions: intracellular survival survival and escape." Cell Microbiol **2**(6): 453-463.

Dowd, M. M., B. Orsburn, et al. (2008). "Cortex peptidoglycan lytic activity in germinating *Bacillus anthracis* spores." J Bacteriol **190**(13): 4541-4548.

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

Driks, A. (2007). "Surface appendages of bacterial spores." Mol Microbiol **63**(3): 623-625.

Druett, H. A., D. W. Henderson, et al. (1953). "Studies on respiratory infection. I. The influence of particle size on respiratory infection with anthrax spores." J Hyg (Lond) **51**(3): 359-371.

Drysdale, M., S. Heninger, et al. (2005). "Capsule synthesis by *Bacillus anthracis* is required for dissemination in murine inhalation anthrax." EMBO J **24**(1): 221-227.

Duc le, H., H. A. Hong, et al. (2003). "Bacterial spores as vaccine vehicles." Infect Immun **71**(5): 2810-2818.

Duesbery, N. S., C. P. Webb, et al. (1998). "Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor." Science **280**(5364): 734-737.

Faust, N., F. Varas, et al. (2000). "Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages." Blood **96**(2): 719-726.

Feavers, I. M., J. Foulkes, et al. (1990). "The regulation of transcription of the gerA spore germination operon of *Bacillus subtilis*." Mol Microbiol **4**(2): 275-282.

Ferreira, L. C., R. C. Ferreira, et al. (2005). "Bacillus subtilis as a tool for vaccine development: from antigen factories to delivery vectors." An Acad Bras Cienc **77**(1): 113-124.

Fisher, N. and P. Hanna (2005). "Characterization of *Bacillus anthracis* germinant receptors in vitro." J Bacteriol **187**(23): 8055-8062.

Fisher, N., L. Shetron-Rama, et al. (2006). "The dltABCD operon of *Bacillus anthracis* sterne is required for virulence and resistance to peptide, enzymatic, and cellular mediators of innate immunity." J Bacteriol **188**(4): 1301-1309.

Fleming, A. (1922). "On a remarkable bacteriolytic element found in tissues and secretions." Proceedings of the Royal Society of London Series B-Containing Papers of a Biological Character **93**(653): 306-317.

Foster, S. J. and K. Johnstone (1988). "Germination-specific cortex-lytic enzyme is activated during triggering of *Bacillus megaterium* KM spore germination." Mol Microbiol **2**(6): 727-733.

Friedlander, A. (1999). Clinical aspects, diagnosis and treatment of anthrax. J Appl Microbiol. **87:** 303.

Friedlander, A. M. (1999). Anthrax. Vaccines. S. Plotkin and W. Orenstein. Philadelphia, WB Saunders Company**:** 629-637.

Friedlander, A. M., R. Bhatnagar, et al. (1993). "Characterization of macrophage sensitivity and resistance to anthrax lethal toxin." Infect Immun **61**(1): 245-252.

Fritze, D. (2004). "Taxonomy of the genus bacillus and related genera: the aerobic endospore-forming bacteria." Phytopathology **94**(11): 1245-1248.

Gest, H. and J. Mandelstam (1987). Longevity of microorganisms in natural environments. Microbiol Sci. **4:** 69-71.

Giebel, J. D., K. A. Carr, et al. (2009). "The germination-specific lytic enzymes SleB, CwlJ1, and CwlJ2 each contribute to *Bacillus anthracis* spore germination and virulence." J Bacteriol **191**(18): 5569-5576.

Giorno, R., J. Bozue, et al. (2007). "Morphogenesis of the *Bacillus anthracis* spore." J Bacteriol **189**(3): 691-705.

Glomski, I. J., J. P. Corre, et al. (2007). "Noncapsulated toxinogenic *Bacillus anthracis* presents a specific growth and dissemination pattern in naive and protective antigen-immune mice." Infect Immun **75**(10): 4754-4761.

Glomski, I. J., F. Dumetz, et al. (2008). "Inhaled non-capsulated *Bacillus anthracis* in A/J mice: nasopharynx and alveolar space as dual portals of entry, delayed dissemination, and specific organ targeting." Microbes Infect **10**(12-13): 1398-1404.

Gordon, S., J. Todd, et al. (1974). "In vitro synthesis and secretion of lysozyme by mononuclear phagocytes." J Exp Med **139**(5): 1228-1248.

Gould, G. W., A. J. Sale, et al. (1969). "Germination of Bacterial Spores by High Hydrostatic Pressures." Journal of General Microbiology **57**: R28-&.

Gould, G. W. and A. J. H. Sale (1970). "Initiation of Germination of Bacterial Spores by Hydrostatic Pressure." Journal of General Microbiology **60**: 335-&.

Green, B. D., L. Battisti, et al. (1985). "Demonstration of a capsule plasmid in *Bacillus anthracis*." Infect Immun **49**(2): 291-297.

Guidi-Rontani, C., M. Weber-Levy, et al. (1999). "Germination of *Bacillus anthracis* spores within alveolar macrophages." Mol Microbiol **31**(1): 9-17.

Gut, I. M., B. Tamilselvam, et al. (2011). *Bacillus anthracis* spore interactions with mammalian cells: Relationship between germination state and the outcome of in vitro infections. BMC microbiology. **11:** 46.

Hammer, M. F., J. W. Schilling, et al. (1987). "Recruitment of lysozyme as a major enzyme in the mouse gut: duplication, divergence, and regulatory evolution." J Mol Evol **24**(3): 272-279.

Hammer, M. F. and A. C. Wilson (1987). "Regulatory and structural genes for lysozymes of mice." Genetics **115**(3): 521-533.

Hammond, P. M. and G. B. Carter (2002). From biological warfare to healthcare: Porton Down 1940-2000. Basingstoke, UK, Palgrave.

Hanna, P. C., D. Acosta, et al. (1993). "On the role of macrophages in anthrax." Proc Natl Acad Sci U S A **90**(21): 10198-10201.

Hanna, P. C., S. Kochi, et al. (1992). "Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells." Mol Biol Cell **3**(11): 1269-1277.

Hanna, P. C., B. A. Kruskal, et al. (1994). "Role of macrophage oxidative burst in the action of anthrax lethal toxin." Mol Med **1**(1): 7-18.

Harry, E. J., K. Pogliano, et al. (1995). "Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*." J Bacteriol **177**(12): 3386-3393.

Harvill, E. T., G. Lee, et al. (2005). "Complement depletion renders C57BL/6 mice sensitive to the *Bacillus anthracis* Sterne strain." Infect Immun **73**(7): 4420-4422.

Heffernan, B. J., B. Thomason, et al. (2007). "*Bacillus anthracis* anthrolysin O and three phospholipases C are functionally redundant in a murine model of inhalation anthrax." FEMS Microbiol Lett **271**(1): 98-105.

Heffron, J. D., E. A. Lambert, et al. (2010). Contributions of four cortex lytic enzymes to germination of *Bacillus anthracis* spores. Journal of Bacteriology. **192:** 763-770.

Heffron, J. D., B. Orsburn, et al. (2009). Roles of germination-specific lytic enzymes CwlJ and SleB in *Bacillus anthracis*. Journal of Bacteriology. **191:** 2237-2247.

Heffron, J. D., N. Sherry, et al. (2011). In vitro studies of peptidoglycan binding and hydrolysis by the *Bacillus anthracis* germination-specific lytic enzyme SleB. Journal of Bacteriology. **193:** 125-131.

Heine, H. S., J. Bassett, et al. (2007). Determination of antibiotic efficacy against *Bacillus anthracis* in a mouse aerosol challenge model. Antimicrobial Agents and Chemotherapy. **51:** 1373-1379.

Helgason, E., D. A. Caugant, et al. (2000). "Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections." J Clin Microbiol **38**(4): 1615-1622.

Helgason, E., O. A. Okstad, et al. (2000). "*Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*--one species on the basis of genetic evidence." Appl Environ Microbiol **66**(6): 2627-2630.

Henderson, D. W., S. Peacock, et al. (1956). "Observations on the prophylaxis of experimental pulmonary anthrax in the monkey." J Hyg (Lond) **54**(1): 28-36.

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

Hodgkiss, W., Z. J. Ordal, et al. (1967). "The morphology and ultrastructure of the spore and exosporium of some *Clostridium* species." Journal of General Microbiology **47**(2): 213-225.

Hoeniger, J. F., P. F. Stuart, et al. (1968). "Cytology of spore formation in *Clostridium perfringens*." J Bacteriol **96**(5): 1818-1834.

Hoffmaster, A. R., J. Ravel, et al. (2004). "Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax." Proc Natl Acad Sci U S A **101**(22): 8449-8454.

Hofte, H. and H. R. Whiteley (1989). "Insecticidal crystal proteins of *Bacillus thuringiensis*." Microbiol Rev **53**(2): 242-255.

Hu, H., J. Emerson, et al. (2007). "Factors involved in the germination and inactivation of *Bacillus anthracis* spores in murine primary macrophages." FEMS Microbiol Lett **272**(2): 245-250.

Ibrahim, H. R., T. Aoki, et al. (2002). "Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules." Curr Pharm Des **8**(9): 671-693.

Ireland, J. and P. Hanna (2002). Macrophage-enhanced germination of *Bacillus anthracis* endospores requires gerS. Infect Immun. **70:** 5870-5872.

Ireland, J. A. and P. C. Hanna (2002). "Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* DeltaSterne endospores: gerS mediates responses to aromatic ring structures." J Bacteriol **184**(5): 1296-1303.

Ishikawa, S., K. Yamane, et al. (1998). "Regulation and characterization of a newly deduced cell wall hydrolase gene (cwlJ) which affects germination of *Bacillus subtilis* spores." J Bacteriol **180**(6): 1375-1380.

Ivanova, N., A. Sorokin, et al. (2003). "Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*." Nature **423**(6935): 87-91.

Janes, B. K. and S. Stibitz (2006). "Routine markerless gene replacement in *Bacillus anthracis*." Infect Immun **74**(3): 1949-1953.

Jensen, G. B., B. M. Hansen, et al. (2003). "The hidden lifestyles of *Bacillus cereus* and relatives." Environ Microbiol **5**(8): 631-640.

Jernigan, D. B., P. L. Raghunathan, et al. (2002). Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings. Emerging Infect Dis. **8:** 1019-1028.

Jernigan, J. A., D. S. Stephens, et al. (2001). Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. Emerging Infect Dis. **7:** 933-944.

Kang, T. J., M. J. Fenton, et al. (2005). "Murine macrophages kill the vegetative form of *Bacillus anthracis*." Infect Immun **73**(11): 7495-7501.

Kim, H. U. and J. M. Goepfert (1974). "A sporulation medium for *Bacillus anthracis*." J Appl Bacteriol **37**(2): 265-267.

Klobutcher, L. A., K. Ragkousi, et al. (2006). "The *Bacillus subtilis* spore coat provides "eat resistance" during phagocytic predation by the protozoan Tetrahymena thermophila." Proc Natl Acad Sci U S A **103**(1): 165-170.

Koch, R. (1877). "The aetiology of anthrax based on the ontogeny of the anthrax bacillus." Beitrage zur Biologie der Phlanzen **2**: 277-282.

Koch, R. (1881). On the etiology of anthrax. Essays of Robert Koch. New York, Greenwood Press**:** 57-81.

Kroos, L. and Y. T. Yu (2000). "Regulation of sigma factor activity during *Bacillus subtilis* development." Curr Opin Microbiol **3**(6): 553-560.

Kuwana, R., Y. Kasahara, et al. (2002). "Proteomics characterization of novel spore proteins of *Bacillus subtilis*." Microbiology **148**(Pt 12): 3971-3982.

Lai, E. M., N. D. Phadke, et al. (2003). "Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*." J Bacteriol **185**(4): 1443-1454.

Lambert, E. A. and D. L. Popham (2008). "The *Bacillus anthracis* SleL (YaaH) protein is an N-acetylglucosaminidase involved in spore cortex depolymerization." J Bacteriol **190**(23): 7601-7607.

Lee, J. Y., B. K. Janes, et al. (2007). "Biosynthetic analysis of the petrobactin siderophore pathway from *Bacillus anthracis*." J Bacteriol **189**(5): 1698-1710.

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**(10): 3162-3166.

Liu, H., N. H. Bergman, et al. (2004). "Formation and composition of the *Bacillus anthracis* endospore." J Bacteriol **186**(1): 164-178.

Loving, C. L., M. Kennett, et al. (2007). Murine aerosol challenge model of anthrax. Infect Immun. **75:** 2689-2698.

Luu, H., M. Akoachere, et al. (2011). "Cooperativity and Interference of Germination Pathways in *Bacillus anthracis* Spores." J Bacteriol **193**(16): 4192-4198.

Makino, S. and R. Moriyama (2002). "Hydrolysis of cortex peptidoglycan during bacterial spore germination." Med Sci Monit **8**(6): RA119-127.

Makino, S., C. Sasakawa, et al. (1988). "Cloning and CO2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*." Mol Microbiol **2**(3): 371-376.

Makino, S., I. Uchida, et al. (1989). "Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in *Bacillus anthracis*." J Bacteriol **171**(2): 722-730.

Margolis, P., A. Driks, et al. (1991). "Establishment of cell type by compartmentalized activation of a transcription factor." Science **254**(5031): 562-565.

Markart, P., N. Faust, et al. (2004). Comparison of the microbicidal and muramidase activities of mouse lysozyme M and P. Biochem J. **380:** 385-392.

Markart, P., T. R. Korfhagen, et al. (2004). Mouse lysozyme M is important in pulmonary host defense against *Klebsiella pneumoniae* infection. American Journal of Respiratory and Critical Care Medicine. **169:** 454-458.

Masayama, A., H. Fukuoka, et al. (2006). "Subcellular localization of a germiantion-specific cortex-lytic enzyme, SleB, of Bacilli during sporulation." Genes Genet Syst **81**(3): 163-169.

McCann, K. P., C. Robinson, et al. (1996). "Alanine germination receptors of *Bacillus subtilis*." Lett Appl Microbiol **23**(5): 290-294.

Meselson, M., J. Guillemin, et al. (1994). The Sverdlovsk anthrax outbreak of 1979. Science. **266:** 1202-1208.

Miyata, S., S. Kozuka, et al. (1997). "Localization of germination-specific spore-lytic enzymes in *Clostridium perfringens* S40 spores detected by immunoelectron microscopy." FEMS Microbiol Lett **152**(2): 243-247.

Miyata, S., R. Moriyama, et al. (1995). "A gene (sleC) encoding a spore-cortex-lytic enzyme from *Clostridium perfringens* S40 spores; cloning, sequence analysis and molecular characterization." Microbiology **141 (Pt 10)**: 2643-2650.

Mock, M. and A. Fouet (2001). "Anthrax." Annu Rev Microbiol **55**: 647-671.

Mogridge, J., M. Mourez, et al. (2001). "Involvement of domain 3 in oligomerization by the protective antigen moiety of anthrax toxin." J Bacteriol **183**(6): 2111-2116.

Moir, A. (2006). "How do spores germinate?" J Appl Microbiol **101**(3): 526-530.

Moir, A., B. M. Corfe, et al. (2002). "Spore germination." Cell Mol Life Sci **59**(3): 403-409.

Moir, A., E. Lafferty, et al. (1979). "Genetics analysis of spore germination mutants of *Bacillus subtilis* 168: the correlation of phenotype with map location." Journal of General Microbiology **111**(1): 165-180.

Moriyama, R., H. Fukuoka, et al. (1999). "Expression of a germination-specific amidase, SleB, of Bacilli in the forespore compartment of sporulating cells and its localization on the exterior side of the cortex in dormant spores." J Bacteriol **181**(8): 2373-2378.

Moriyama, R., A. Hattori, et al. (1996). "A gene (sleB) encoding a spore cortex-lytic enzyme from *Bacillus subtilis* and response of the enzyme to L-alanine-mediated germination." J Bacteriol **178**(20): 6059-6063.

Moriyama, R., S. Kudoh, et al. (1996). "A germination-specific spore cortex-lytic enzyme from *Bacillus cereus* spores: cloning and sequencing of the gene and molecular characterization of the enzyme." J Bacteriol **178**(17): 5330-5332.

Navacharoen, N., T. Sirisanthana, et al. (1985). "Oropharyngeal anthrax." J Laryngol Otol **99**(12): 1293-1295.

Nicholson, W. L., N. Munakata, et al. (2000). "Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments." Microbiol Mol Biol Rev **64**(3): 548-572.

Nicholson, W. L. and P. Setlow (1990). Sporulation, Germination and Outgrowth. Molecular Biological Methods for Bacillus. C. R. Harwood and S. M. Cutting. Chichester, John Wiley & Sons Ltd**:** 391-450.

O'Brien, J., A. Friedlander, et al. (1985). "Effects of anthrax toxin components on human neutrophils." Infect Immun **47**(1): 306-310.

Oggioni, M. R., A. Ciabattini, et al. (2003). "*Bacillus* spores for vaccine delivery." Vaccine **21 Suppl 2**: S96-101.

Okinaka, R. T., K. Cloud, et al. (1999). "Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes." J Bacteriol **181**(20): 6509-6515.

Paidhungat, M., K. Ragkousi, et al. (2001). "Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca(2+)-dipicolinate." J Bacteriol **183**(16): 4886-4893.

Paidhungat, M. and P. Setlow (2000). "Role of ger proteins in nutrient and nonnutrient triggering of spore germination in *Bacillus subtilis*." J Bacteriol **182**(9): 2513-2519.

Pannucci, J., R. T. Okinaka, et al. (2002). "DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria." BMC Genomics **3**(1): 34.

Paredes-Sabja, D. and M. R. Sarker (2011). "Host serum factor triggers germination of *Clostridium perfringens* spores lacking the cortex hydrolysis machinery." J Med Microbiol.

Petosa, C., R. J. Collier, et al. (1997). "Crystal structure of the anthrax toxin protective antigen." Nature **385**(6619): 833-838.

Pezard, C., P. Berche, et al. (1991). "Contribution of individual toxin components to virulence of *Bacillus anthracis*." Infect Immun **59**(10): 3472-3477.

Pezard, C., M. Weber, et al. (1995). "Protective immunity induced by *Bacillus anthracis* toxin-deficient strains." Infect Immun **63**(4): 1369-1372.

Pickering, A. K., M. Osorio, et al. (2004). Cytokine response to infection with *Bacillus anthracis* spores. Infect Immun. **72:** 6382-6389.

Piggot, P., C. Moran, et al. (1994). Morphogenesis and gene expression during sporulation. Regulation of Bacterial Differentiation. Washington, DC, American Society of Microbiology**:** 113-137.

Popham, D. L. (2002). "Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox." Cell Mol Life Sci **59**(3): 426-433.

Popham, D. L., J. Helin, et al. (1996). Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. Journal of Bacteriology. **178:** 6451-6458.

Popham, D. L., J. Helin, et al. (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**(26): 15405-15410.

Quinn, C. and P. Turnbull (1998). Anthrax. Topley and Wilson's Microbiology and Microbial Infections. L. Collier, A. Balows, M. Sussman and W. Hausler. London, Arnold. **3:** 799-818.

Ragkousi, K., P. Eichenberger, et al. (2003). "Identification of a new gene essential for germination of *Bacillus subtilis* spores with Ca2+-dipicolinate." J Bacteriol **185**(7): 2315-2329.

Ragkousi, K. and P. Setlow (2004). "Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis* spores." J Bacteriol **186**(17): 5567-5575.

Rasko, D. A., M. R. Altherr, et al. (2005). "Genomics of the *Bacillus cereus* group of organisms." FEMS Microbiol Rev **29**(2): 303-329.

Rasko, D. A., M. J. Rosovitz, et al. (2007). "Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the B. cereus-group plasmids, including *Bacillus anthracis* pXO1." J Bacteriol **189**(1): 52-64.

Read, T. D., S. N. Peterson, et al. (2003). "The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria." Nature **423**(6935): 81-86.

Reed, L. J. and H. Muench (1938). "A simple method of estimating fifty per cent endpoints." American Journal of Hygiene(27): 493-497.
Ribot, W. J., R. G. Panchal, et al. (2006). "Anthrax lethal toxin impairs innate immune functions of alveolar macrophages and facilitates *Bacillus anthracis* survival." Infect Immun **74**(9): 5029-5034.
Riemann, H. and Z. J. Ordal (1961). "Germination of bacterial endospores with calcium and dipicolinic acid." Science **133**: 1703-1704.
Rode, L. J. (1971). "Bacterial spore appendages." CRC Crit Rev Microbiol **1**(1): 1-27.
Rode, L. J. and J. W. Foster (1961). "Germination of bacterial spores with alkyl primary amines." J Bacteriol **81**: 768-779.
Ross, C. and E. Abel-Santos (2010). "The Ger receptor family from sporulating bacteria." Curr Issues Mol Biol **12**(3): 147-158.
Ross, J. M. (1955). "On the histopathology of experimental anthrax in the guinea-pig." Br J Exp Pathol **36**(3): 336-339.
Ross, J. M. (1957). "The pathogenesis of anthrax following the administration of spores by the respiratory route." J Pathol Bacteriol **73**: 485-494.
Sakae, Y., Y. Yasuda, et al. (1995). "Immunoelectron microscopic localization of one of the spore germination proteins, GerAB, in Bacillus subtilis spores." J Bacteriol **177**(21): 6294-6296.
Sambrook, J. and D. W. Russell (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.
Sammons, R. L., A. Moir, et al. (1981). "Isolation and Properties of Spore Germination Mutants of *Bacillus subtilis* 168 Deficient in the Initiation of Germination." Microbiology **124**(2): 229-241.
Sanz, P., L. D. Teel, et al. (2008). "Detection of *Bacillus anthracis* spore germination in vivo by bioluminescence imaging." Infect Immun **76**(3): 1036-1047.
Sayeed, S., J. Li, et al. (2007). "Virulence plasmid diversity in *Clostridium perfringens* type D isolates." Infect Immun **75**(5): 2391-2398.
Sayeed, S., J. Li, et al. (2010). "Characterization of virulence plasmid diversity among *Clostridium perfringens* type B isolates." Infect Immun **78**(1): 495-504.
Sekiguchi, J., K. Akeo, et al. (1995). "Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, cwlD, which affects germination in *Bacillus subtilis*." J Bacteriol **177**(19): 5582-5589.
Setlow, B., S. Atluri, et al. (2006). "Role of dipicolinic acid in resistance and stability of spores of *Bacillus subtilis* with or without DNA-protective alpha/beta-type small acid-soluble proteins." J Bacteriol **188**(11): 3740-3747.
Setlow, B., A. E. Cowan, et al. (2003). "Germination of spores of *Bacillus subtilis* with dodecylamine." J Appl Microbiol **95**(3): 637-648.
Setlow, B., E. Melly, et al. (2001). "Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination." J Bacteriol **183**(16): 4894-4899.
Setlow, B., L. Peng, et al. (2009). "Characterization of the germination of *Bacillus megaterium* spores lacking enzymes that degrade the spore cortex." J Appl Microbiol **107**: 318-328.
Setlow, P. (2003). "Spore germination." Curr Opin Microbiol **6**(6): 550-556.
Setlow, P. (2006). "Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals." J Appl Microbiol **101**(3): 514-525.

Setlow, P. (2007). "I will survive: DNA protection in bacterial spores." Trends Microbiol **15**(4): 172-180.

Shah, I. M., M.-H. Laaberki, et al. (2008). A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell. **135:** 486-496.

Shetron-Rama, L. M., A. C. Herring-Palmer, et al. (2010). "Transport of *Bacillus anthracis* from the lungs to the draining lymph nodes is a rapid process facilitated by CD11c+ cells." Microb Pathog **49**(1-2): 38-46.

Sirisanthana, T. and A. E. Brown (2002). "Anthrax of the gastrointestinal tract." Emerg Infect Dis **8**(7): 649-651.

Sirisanthana, T., N. Navachareon, et al. (1984). "Outbreak of oral-oropharyngeal anthrax: an unusual manifestation of human infection with *Bacillus anthracis*." Am J Trop Med Hyg **33**(1): 144-150.

Sirisanthana, T., K. E. Nelson, et al. (1988). Serological studies of patients with cutaneous and oral-oropharyngeal anthrax from northern Thailand. Am J Trop Med Hyg. **39:** 575-581.

Smego, R. A., Jr., B. Gebrian, et al. (1998). "Cutaneous manifestations of anthrax in rural Haiti." Clin Infect Dis **26**(1): 97-102.

Sonenshein, A. L., J. A. Hoch, et al. (1993). *Bacillus subtilis* and other gram positive bacteria: biochemistry, physiology, and molecular genetics. *Bacillus subtilis* and other Gram-positive bacteria. A. L. Sonenshein, J. A. Hoch and R. Losick. Washington, D.C., American Society of Microbiology**:** 917-937.

Steichen, C., P. Chen, et al. (2003). "Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium." J Bacteriol **185**(6): 1903-1910.

Sterne, M. (1939). "The immunization of laboratory animals against anthrax." J. Vet. Sci. Anim. Ind. **13**: 313-317.

Stewart, G. S., K. Johnstone, et al. (1981). "Commitment of bacterial spores to germinate. A measure of the trigger reaction." Biochem J **198**(1): 101-106.

Stragier, P. and R. Losick (1996). "Molecular genetics of sporulation in *Bacillus subtilis*." Annu Rev Genet **30**: 297-241.

Swanson, J. A. (1989). "Phorbol esters stimulate macropinocytosis and solute flow through macrophages." J Cell Sci **94 (Pt 1)**: 135-142.

Tournier, J. N., A. Quesnel-Hellmann, et al. (2007). "Contribution of toxins to the pathogenesis of inhalational anthrax." Cell Microbiol **9**(3): 555-565.

Travis, S. M., B. A. Conway, et al. (1999). "Activity of abundant antimicrobials of the human airway." Am J Respir Cell Mol Biol **20**(5): 872-879.

Turnbull, P. (1996). "Stubborn contamination with anthrax spores." Environmental health series **106**: 171.

Uchida, I., T. Sekizaki, et al. (1985). "Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid." Journal of General Microbiology **131**(2): 363-367.

Vitale, G., R. Pellizzari, et al. (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**(3): 706-711.

Warth, A. D. and J. L. Strominger (1969). Structure of the peptidoglycan of bacterial spores: occurrence of the lactam of muramic acid. Proc Natl Acad Sci USA. **64:** 528-535.

Watts, C. J., B. L. Hahn, et al. (2008). "Progressive and destructive hair follicle infections in a murine cutaneous anthrax model." Microb Pathog **44**(5): 363-369.

Watts, C. J., B. L. Hahn, et al. (2009). "Resistance of athymic nude mice to experimental cutaneous *Bacillus anthracis* infection." J Infect Dis **199**(5): 673-679.

Webb, C. D., A. Decatur, et al. (1995). "Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*." J Bacteriol **177**(20): 5906-5911.

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

Weiner, M., T. Read, et al. (2003). Identification and characterization of the gerH operon of *Bacillus anthracis* endospores: a differential role for purine nucleosides in germination. J Bacteriol. **185:** 1462-1464.

Welkos, S. L. and A. M. Friedlander (1988). "Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*." Microb Pathog **4**(1): 53-69.

Welkos, S. L., T. J. Keener, et al. (1986). "Differences in susceptibility of inbred mice to *Bacillus anthracis*." Infect Immun **51**(3): 795-800.

Welkos, S. L., R. W. Trotter, et al. (1989). "Resistance to the Sterne strain of *B. anthracis*: phagocytic cell responses of resistant and susceptible mice." Microb Pathog **7**(1): 15-35.

Wheelis, M. (1999). Biological sabotage in World War I. Biological and Toxin Weapons: Research, Developments and Use from the Middle Ages to 1945. E. Geissler and J. E. van Courtland Moon. Oxford, Oxford University Press**:** 35-62.

WHO (2008). Anthrax in humans and animals.

Zuberi, A. R., A. Moir, et al. (1987). "The nucleotide sequence and gene organization of the gerA spore germination operon of *Bacillus subtilis* 168." Gene **51**(1): 1-11.