Early events of spore germination and their role in *Bacillus anthracis* virulence

by

Katherine Ann Carr

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in The University of Michigan
2010

Doctoral Committee:

Associate Professor Philip C. Hanna, Chair
Professor Victor DiRita
Associate Professor Matthew Chapman
Assistant Professor Maria Sandkvist

Dedication

For my parents, the greatest cheerleaders I could ever ask for.

Acknowledgements

I would like to say thanks to all of the friends, family, and colleagues who helped me on this journey. To my friends, the Irish, Badgers, Wolverines, and everyone in between, I'd like to say thanks for the support, and the comic relief. To my home friends for supporting my science, even when they had no idea what I was doing. To my Michigan friends for being the best imaginable support system during this whole experience. To Doug, for encouraging me, and being excited for me, even when I wasn't. To the faculty and staff of the M&I department. I will truly miss the collaborative and supportive environment I've gotten to know and love these past few years. Hopefully I can bring some of that to my new assignment. To the members of the Hanna lab, past and present. The great atmosphere in our little corner lab, both scientific and social, has garnered friendships that I will have for life. To our ringleader, and fearless leader, Phil. His unending support, guidance, and honesty have turned me into a confident, independent scientist. Thanks, Phil.

And finally, to my family. Their love and support cannot be matched. Their constant enthusiasm and interest in my "baby bugs," and my "lightening bugs," even when they didn't quite understand, made the long hours much less painful.

Table of Contents

List of Figures

List of Tables

Abstract

Bacillus anthracis, the causative agent of the disease anthrax, exists in two morphological forms, the spore and the bacillus. Entry of dormant spores into the host triggers germination and outgrowth into virulent bacilli. This work focused on the spore proteins that play a role in germinant detection. *B. anthracis* encodes five distinct germinant receptor complexes, GerH, GerK, GerL, GerS, and GerX. Each complex is composed of three distinct proteins. These receptors are located in the spore inner membrane and initiate germination upon detection of combinations of L-amino acid and purine nucleoside germinants from the environment. Using mutant strains lacking one or more of these receptor complexes, I found that the germinant receptors of *B. anthracis* are essential for nutrient-mediated germination and that they have partially redundant specificities for a variety of L-amino acids. I showed also that the GerH receptor alone is sufficient to mediate a wild-type level germination response to inosine based combinations of germinants.

Germinant receptor mutants also were tested in several mouse models of infection to determine their roles in *in vivo* germination. Mutant strains exhibited different levels of virulence depending on the route of infection and the germinant receptors present in the strains. In an intratracheal infection, any one receptor was sufficient to cause disease, with the exception of GerX. In fact, a mutant expressing only GerX behaved identically to a strain lacking all five germinant receptors in any route of infection tested. The GerH

receptor was necessary and sufficient to cause full virulence when spores were inoculated subcutaneously. *In vivo* imaging suggested that spores unable to germinate at the site of inoculation cannot cause disease. Spores did not travel from the injection site, suggesting the presence of GerH specific germinants at this site of infection. GerH was also essential for wild-type levels of germination in both mammalian blood and macrophages. The addition of chemically-defined germination inhibitors to serum or macrophages suggested that these two potential *in vivo* germination sites contain both L-amino acids and inosine, explaining the need for the GerH receptor under these conditions.

In order for germination to begin, germinants must reach their receptors in the spore's inner membrane. The $gerP_{ABCDEF}$ operon has been suggested to play a role in facilitating the interaction between germinants and their receptors in spores of *Bacillus subtilis* and *Bacillus cereus.* *B. anthracis* mutants containing deletions in any of the six genes belonging to the $gerP_{ABCDEF}$ operon, or containing a deletion of the entire operon, exhibited a mild delay of germination in nutrient germinants. Chemical removal of the spore coat alleviated this defect, suggesting a role for the GerP proteins in germinant entry or spore permeability. Calcium-dipicolinic acid (Ca-DPA) must be released from the spore's core to activate cortex degrading enzymes, and can be added exogenously to stimulate a germination response. Each of the *gerP* mutants exhibited a severe defect in Ca-DPA mediated germination, suggesting a more central role for the GerP proteins in this process, than in nutrient based germination. Collectively, these data implicate all GerP proteins in the early stages of spore germination, possibly by facilitating the interaction between nutrient and non-nutrient germinants and their spore targets.

Together these experiments give us a better understanding of the signals required to stimulate germination in a host. These findings may lead us to additional therapeutic candidates, and give us a greater appreciation for the earliest events of disease initiation.

Chapter I

Introduction

Bacillus anthracis is a Gram positive spore forming bacterium, and the causative agent of the disease anthrax. This disease has received much attention during recent years when spores were released either intentionally, as was the case in the attacks after September 11, 2001, or unintentionally, like in the Sverdlovsk incident in 1979 (Meselson *et al.*, 1994, Jernigan *et al.*, 2001). Although it has gained negative notoriety in the past few decades, *B. anthracis* has provided invaluable insight into the microbial world. In fact, Koch's postulates were developed using *B. anthracis*, and these criteria are still used as benchmarks to determine infectious disease etiology (Koch, 1876). Additionally, Pasteur's early vaccine studies were conducted using heat killed bacilli to confer immunity to sheep challenged with *B. anthracis* spores (Pasteur, 1881, Turnbull, 1991, Scorpio *et al.*, 2006). These contributions to bacteriology have made *B. anthracis* an important organism both historically and currently as a public health threat.

Bacteriology

The *B. anthracis* genome consists of 5,508 predicted open reading frames (ORFs) on the chromosome. The bacterium also carries two virulence plasmids, the 185 kilobase pair (kbp) pXO1 encoding 143 predicted ORFs, and the 95 kbp pXO2, encoding 85

predicted ORFs (Okinaka *et al.*, 1999, Pannucci *et al.*, 2002, Read *et al.*, 2003). These plasmids carry the virulence genes encoding for toxins and capsule that are responsible for the hallmark pathogenesis of anthrax (Green *et al.*, 1985, Uchida *et al.*, 1985).

B. anthracis has a number of notable family members, both pathogenic and non-pathogenic. Taxonomically, *B. anthracis* is a member of the *Bacillus cereus* group, with indistinguishable 16S rRNA and a very high level of sequence homology between the two species (Ash *et al.*, 1991, Read *et al.*, 2003, Rasko *et al.*, 2004). The *B. cereus* group also consists of additional genetically similar strains including *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis,* and *Bacillus thuringiensis* (Arnesen *et al.*, 2008). It has been argued that *B. cereus, B. anthracis,* and *B. thuringiensis* should be classified as one species based on genetic sequence identity (Helgason *et al.*, 2000). For example, the only known difference between *B. cereus* and *B. thuringiensis* is the presence of a group of genes encoding insecticidal toxins in *B. thuringiensis* (Wilcks *et al.*, 1998). These three strains are still listed as separate, however, in part due to the distinctive pathogenesis and host tropisms of these species. *B. cereus* is best known as a food-borne pathogen, transmitted through a wide variety of contaminated food products (Andersson *et al.*, 1995). *B. thuringiensis* has been characterized as an insect pathogen and several of its toxins have been optimized for pesticidal purposes (Hofte and Whiteley, 1989). In contrast, *B. anthracis* is historically considered a pathogen of grazing herbivores, with humans being accidental hosts (Turnbull *et al.*, 1999). The disease caused by *B. anthracis* will be described in much more detail below. Most virulence genes for these three *Bacillus* species are encoded on plasmids, however, so correct taxonomic classification is still under debate. Genetic

similarities have led to concerns that horizontal gene transfer may have implications in public health issues in the future, with strains having the potential of gaining new virulence genes (Gonzalez *et al.*, 1982, Wilcks *et al.*, 1998).

Natural reservoirs

B. anthracis is endemic in some areas of the world, particularly in regions with large populations of grazing herbivores including arid regions in Africa and Asia (OIE, 1997, Turnbull *et al.*, 1999). Anthrax is primarily a disease of herbivores, although most mammals have at least some level of susceptibility. Animals contract the disease by feeding in contaminated fields, where they either inhale or ingest infectious spores. Depending upon route of infection lethality can be very high. If a host succumbs to the disease, vegetative bacilli are released back into the environment, where they sporulate and lie dormant until contact with the next mammalian host (Turnbull *et al.*, 1999). Until the advent of animal vaccines, anthrax had the potential to cause significant losses in livestock populations in the event of an outbreak (Sterne, 1939). Humans are accidental hosts of *B. anthracis*, with natural forms of the disease occurring after contact with infected animals or animal products (Turnbull *et al.*, 1999).

Anthrax

Disease occurs when a host encounters dormant spores, which are the infectious form of the bacteria (Kang *et al.*, 2005). Anthrax can present in three major ways in humans, depending on route of infection. Cutaneous anthrax is the most common form of the disease, with an estimated 2000 cases reported each year accounting for about 95%

of naturally occurring infections (Dixon *et al.*, 1999, Friedlander, 1999). This form is typically seen in farmers and other workers that handle the hides or carcasses of infected livestock. Spores enter the body via cuts or abrasions in the skin of the head, neck, and extremities, where they then germinate. The characteristic black skin eschars that form as a result of infection are typically painless and self-limiting, although antibiotic therapy is recommended to avoid the possibility of a systemic infection (Smego *et al.*, 1998). The black color of these eschars is responsible for the name anthrax after "anthros," the Greek work for coal (Dixon *et al.*, 1999).

Gastrointestinal anthrax occurs following ingestion of spores, typically through the consumption of undercooked meat (Sirisanthana *et al.*, 1984). Infection occurs after the introduction of spores in the upper or lower gastrointestinal (GI) tract. Infection of the upper GI tract in humans results in oral-pharyngeal anthrax, causing ulceration that leads to regional lymphadenopathy, edema, and sepsis (Sirisanthana *et al.*, 1984, Sirisanthana *et al.*, 1988). Spores inoculated into the lower GI tract causes intestinal lesions in the terminal ileum or cecum. This infection initially presents with nausea and vomiting, eventually leading to bloody diarrhea, acute abdomen, and/or sepsis (Abramova *et al.*, 1993). If bacilli reach the bloodstream, prognosis is poor, as they can replicate to greater than 10^8 bacteria/mL of blood. The host is eventually overwhelmed by toxin production, leading to death (Turnbull *et al.*, 1999). Due to the difficulty of diagnosis of gastrointestinal anthrax, mortality rates can be high (Dixon *et al.*, 1999).

The most severe form of anthrax occurs when dormant spores are inhaled and enter the alveolar spaces of the airway, aided by the small size of spore particles (Druett *et al.*, 1953). Historically, workers that handled the wool and other hairs of infected

livestock were susceptible, resulting in what was commonly referred to as 'woolsorters' disease (Metcalfe, 2004). Upon entry into alveolar spaces, spores are engulfed by alveolar phagocytes, and carried toward the regional lymph nodes in the mediastinum (Chakrabarty *et al.*, 2006). During transport, spores germinate and rapidly multiply, killing the phagocytes in which they were carried. This results in a release of vegetative bacilli in the lymph nodes, causing hemorrhagic mediastinitis (Albrink, 1961). The bacteria eventually enter the bloodstream causing systemic infection, hemorrhaging of the lymphoid organs, and massive deregulation of vascular homeostasis resulting in death (Dixon *et al.*, 1999). This form of the disease is often fatal if left untreated, as evidenced by the United States anthrax attacks of 2001. Victims of these attacks had a mortality rate of 40%, even with rapid diagnosis and aggressive antibiotic therapy (Jernigan *et al.*, 2001). The incidence of naturally occurring inhalation anthrax cases is low, with less than one case reported annually in the United States in the past two decades, and only 11 total cases from 1955 to 1994 (Pile *et al.*, 1998). Exact numbers globally are unknown, as anthrax is not a reportable disease in some areas of the world, including some African countries (WHO, 1994, Pile *et al.*, 1998).

A newly emerging form of the disease, referred to as "injectional" anthrax, results from direct injection of spores into the bloodstream. Thus far, the reported cases of injectional anthrax have been from intravenous drug users injecting contaminated heroin. Spores injected directly into the bloodstream rapidly germinate and multiply, resulting in rapid death in about a third of reported cases. At the time of this publication, 39 confirmed cases were reported in Europe, with 12 total deaths. Contamination of heroin

with spores likely occurred during the preparation or cutting of the drug in its nation of origin, where *B. anthracis* spores are endemic (Booth *et al.*, 2010).

Virulence factors

The major virulence factors of *B. anthracis* are encoded on the pXO1 and pXO2 virulence plasmids. Both plasmids are essential for full virulence in most mammals (Sterne, 1939, Welkos *et al.*, 1986). pXO1 is the larger of the two plasmids, encoding 143 predicted ORFs, including the components of two secreted exotoxins (Friedlander, 1986, Hammond and Hanna, 1998, Okinaka *et al.*, 1999, Read *et al.*, 2003). These exotoxins are both are AB toxins, containing an enzymatically active (A) polypeptide linked to a binding (B) polypeptide that binds to cell-surface receptors and facilitates entry of the complex into the cell. The two toxins of *B. anthracis* are formed by three components, two A moieties and a B moiety: lethal factor (LF), edema factor (EF), and protective antigen (PA), respectively (Smith *et al.*, 1955, Stanley and Smith, 1963, Leppla, 1982). Toxin expression is regulated by temperature and CO_2, and requires the transcription factor AtxA, whose own expression is controlled by temperature (Bartkus and Leppla, 1989, Uchida *et al.*, 1993, Sirard *et al.*, 1994, Dai and Koehler, 1997). PA is co-transcribed with its transcriptional repressor PagR (Hoffmaster and Koehler, 1999).

Protective antigen (PA) serves as the "B" moiety for both lethal factor and edema factor, competitively binding either protein. These binding events result in the formation of two AB toxins, lethal toxin and edema toxin, respectively (Leppla, 1982, Elliott *et al.*, 2000, Pimental *et al.*, 2004). The toxin subunits, PA, EF, and LF, are independently secreted from the cell, and then assembled at the surface of mammalian cells into their

respective toxin complexes (Smith *et al.*, 1955, Singh *et al.*, 1991, Klimpel *et al.*, 1992, Milne *et al.*, 1994). Binding and assembly requires the presence of a receptor on the host cell. Thus far, PA has been shown to bind to the integrin-like inserted domains on two host receptors, TEM8/ANTXR1 and CMG2/ANTXR2, which are expressed in a variety of tissues (Bradley *et al.*, 2001, Scobie *et al.*, 2003). After host cell binding, PA is cleaved and the carboxy-terminal fragment forms a heptamer allowing binding of either lethal factor or edema factor. These toxin complexes are then translocated across the membrane and trafficked to an acidic compartment (Gordon *et al.*, 1988, Singh *et al.*, 1991, Klimpel *et al.*, 1992, Beauregard *et al.*, 2000, Collier and Young, 2003). The acidic environment causes a conformational change in the PA heptamer, converting it from a pre-pore to a pore, allowing its insertion into the membrane and the formation of an ion-conductive channel (Blaustein *et al.*, 1989, Koehler and Collier, 1991, Milne and Collier, 1993). Acidification also leads to the unfolding of EF and LF, which allows for their translocation through the pore into the cytoplasm, where they can refold and exert their enzymatic effects on the cell (Krantz *et al.*, 2004).

The lethal factor component of lethal toxin is a zinc metalloprotease that cleaves mitogen-activated protein kinase kinase and stimulates macrophages to release TNF-α and IL-1β, which contribute to rapid death in systemic anthrax infections (Hanna *et al.*, 1992, Duesbery *et al.*, 1998, Hammond and Hanna, 1998). Lethal factor has been shown to induce apoptosis in RAW 264.7 macrophages, providing a defense against the innate immune response and allowing escape of the bacilli (Friedlander, 1986, Kim *et al.*, 2003). In contrast, a more recent report shows that human alveolar macrophages are actually resistant to killing by lethal factor, suggesting that an additional cell type may provide the

vehicle for trafficking of spores to the lymph nodes (Wu *et al.*, 2009). Indeed, dendritic cells also facilitate germination of spores and may likely play a role in trafficking in the host (Brittingham *et al.*, 2005, Cleret *et al.*, 2007).

Edema factor (EF) is a calmodulin-dependent adenylate cyclase. After transport into the cell via PA, EF is activated by host calmodulin and a flux of exogenous calcium into the cell (Kumar *et al.*, 2002). Activated EF catalyzes the synthesis of cyclic AMP (cAMP) in a variety of immune cells (Leppla, 1982, Kumar *et al.*, 2002). The increase of cAMP causes a disruption in water homeostasis, resulting in the massive edema associated with anthrax infections (Leppla, 1982, Mock and Ullmann, 1993). This accumulation of cAMP may also aid the bacilli in evading host defenses, as cAMP is a potent inhibitor of the immune system (Barth *et al.*, 2004).

The smaller virulence plasmid, pXO2, encodes for the biosynthetic enzymes that produce a poly-γ-D-glutamate capsule which is covalently linked to the peptidoglycan of the bacillus (Green *et al.*, 1985, Uchida *et al.*, 1985, Thomas and Fouet, 2005). Transcription of capsule synthesis genes is controlled by AtxA, via positive regulation of the regulatory genes *acpA* and *acpB,* both located on pXO2 (Drysdale *et al.*, 2004, Drysdale *et al.*, 2005a). Capsule biosynthesis genes are encoded in the *capBCADE* operon (Makino *et al.*, 1988, Makino *et al.*, 1989). CapB, CapC, CapA, and CapE are all required for the formation of the capsule itself, while CapD is required for the anchoring of the capsule to peptidoglycan (Candela *et al.*, 2005, Thomas and Fouet, 2005). *In vivo*, this capsule is essential for virulence, as it aids in evasion of the immune response by protecting vegetative bacilli from phagocytosis (Makino *et al.*, 1989, Drysdale *et al.*, 2005b). Capsule is non-immunogenic *in vivo*, but some reports have suggested that small

glutamic acid polymers may illicit an immune response when conjugated to immunogenic molecules (Leonard and Thorne, 1961, Schneerson *et al.*, 2003)(Rhie *et al.*, 2003, Schneerson *et al.*, 2003, Taia *et al.*, 2004). This finding may provide improvements in vaccination strategies in the future

Together, these virulence factors exert their immunosuppressive effects on the mammalian host, allowing the bacilli to multiply to extremely high titers, resulting in septicemia, and ultimately death (Dixon *et al.*, 1999). In animals, death of the host results in a starvation state for the bacilli. This initiates a stress response triggering the process of sporulation, whereby bacilli return to their metabolically dormant state. Spores are released into the environment from the decomposing host, where they stay until contact with another suitable mammalian host occurs, initiating the disease cycle anew (Turnbull *et al.*, 1999).

Spore anatomy and formation

Spore resistance: Dehydration

To investigate the process of germination, we must better understand spore formation and composition. Bacterial spores are metabolically dormant and form in response to environmental stresses, like nutrient depletion or high cell density. Spores have a unique physical structure, which conveys resistance to many forms of environmental stress such as heat, pressure, pH changes, desiccation, radiation, and chemicals (Fox and Eder, 1969, Koike *et al.*, 1992, Knott *et al.*, 1995, Wuytack *et al.*, 1998, Nicholson *et al.*, 2000, Setlow, 2001). These resistance properties allow for spores to survive in the environment for years, or even millennia (Kennedy *et al.*, 1994, Cano

and Borucki, 1995). Resistance properties are largely due to the dehydration of the spore's central structure, the core, which houses its DNA, ribosomes, and tRNA (Figure 1.1A). It is this core, surrounded by the germ cell wall, which will emerge from the protective layers of the spore as a newly germinated bacillus. The core has lower water content (25-50% wet weight) than do the outer layers of the spore (about 80%). This is due to a large amount of pyridine-2,6-dicarboxylic acid, or dipicolinic acid (DPA), that takes its place (25% of core dry weight) (Setlow, 2007). DPA is synthesized in the mother cell, and chelated with divalent calcium ions in a 1:1 ratio. Loss of the ability to produce this molecule results in significantly decreased resistance to wet heat (Setlow *et al.*, 2006).

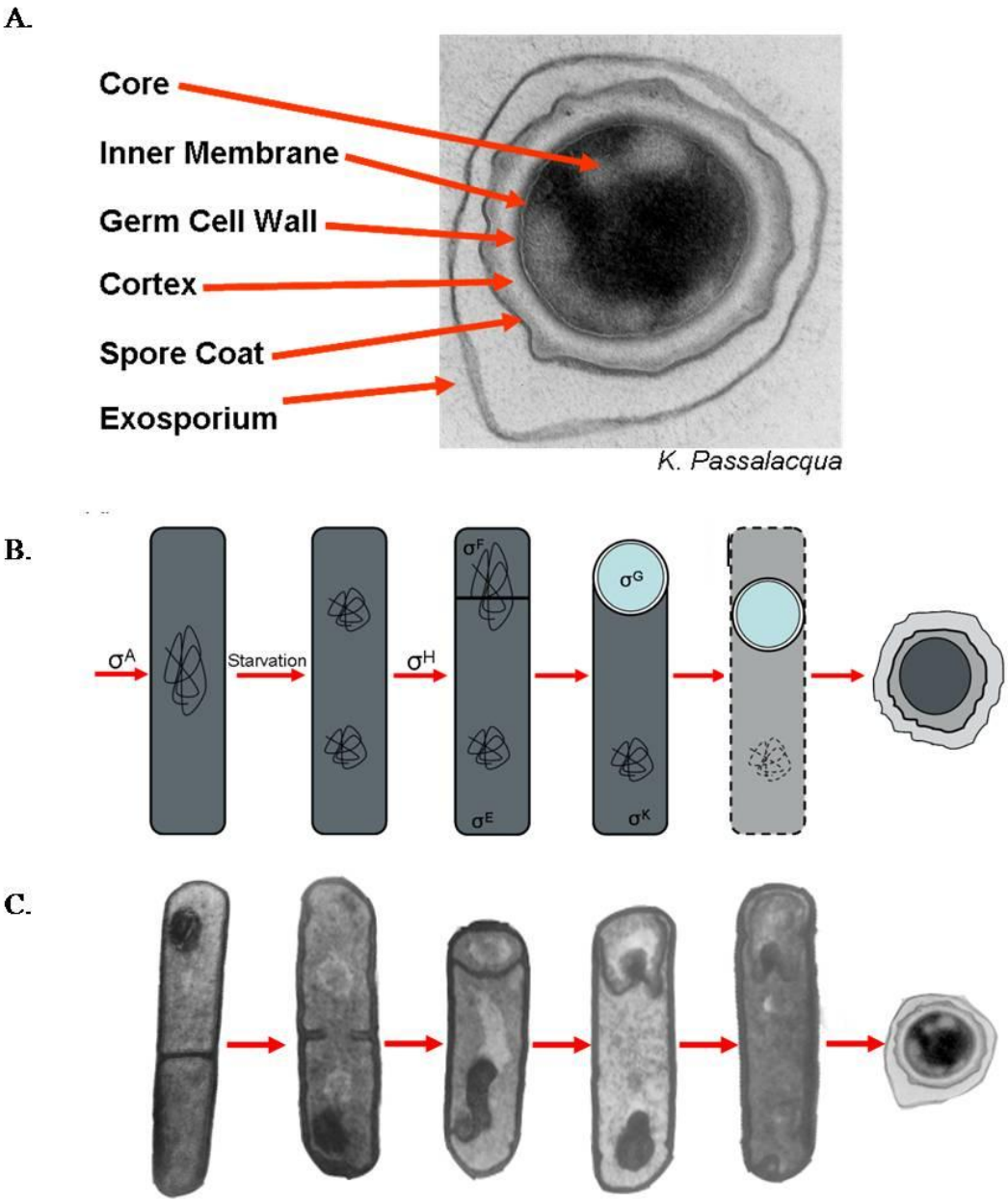

Figure 1. 1. Spore anatomy and sporulation. (A) Spore anatomy. At the center of the spore is the core, which houses DNA, RNA, and proteins. The core is surrounded by the inner membrane, which houses germinant receptors. The germ cell wall is a layer of peptidoglycan, which will become the cell wall of the germinated bacillus. The cortex is a layer of modified peptidoglycan that surrounds the germ cell wall. A proteinaceous coat is laid down upon the outer membrane. The final layer is the exosporium composed of glycoproteins. Additional information on assembly and function of each layer are included in the text. (B) Cartoon of sporulation, and the sigma factors involved. Starvation signals stimulate formation of metabolically dormant spores. See pages 13-16 for additional details. (C) Electron micrographs of sporulation in B. subtilis. Figure adapted from Illing *et al.* (Illing and Errington, 1991).

Spore resistance: SASPs

Spore stability can also be attributed to the α/β-type small, acid-soluble spore proteins (SASPs) that associate with DNA in the spore's core. These proteins range from 57-75 residues and provide protection from both UV radiation and wet heat (Nicholson *et al.*, 1991, Lee *et al.*, 2008). Additionally, SASPs are hydrolyzed during germination to provide nutrients to the newly emerged bacillus (Setlow, 1975a, Setlow, 1975b, Johnson and Tipper, 1981, Yuan *et al.*, 1981). SASPs represent 5-10% of total core protein in both *Bacillus* and *Clostridium* spores, which is enough to saturate the DNA. SASPs bind DNA nonspecifically in the minor groove by forming hydrogen bonds to the oxygen in the phosphate groups of the backbone (Lee *et al.*, 2008). The SASPs convert DNA from the B-like conformation found in most living cells to an A-like conformation, which tends to form in low water environments, including the spore core (Setlow, 1992). This conversion helps to protect the spore from UV radiation by enhancing the production of spore photoproduct (SP) (Setlow and Setlow, 1993). SP is a 5-thyminyl-5,6-dihydrothymine adduct that forms when DNA is exposed to UV radiation. This is in place of the typical cyclobutane-type thymine-thymine dimer (TT) produced by vegetative bacilli after UV exposure. SP is much easier to repair than TT upon germination, resulting in increased stability of the new bacillus. Like the SASPs, DPA also helps to protect the spore from DNA damage by enhancing the amount of spore photoproduct (SP) produced (Setlow and Setlow, 1993).

Spore formation

Bacterial spores form in response to starvation signals from their environment. With *B. anthracis*, starvation occurs after a host succumbs to infection and vegetative bacilli exhaust nutrients (Turnbull *et al.*, 1999). Bacilli receive the signal to sporulate, and become committed to the process of forming protective layers, and returning to metabolic dormancy. Sporulation is depicted in Figures 1.1B and 1.1C. Sporulation is initiated by a complex phospho-relay system initiated by the transcription factor Spo0A (Burbulys *et al.*, 1991, Levin and Losick, 1996). This process is highly regulated at the transcriptional level, in a growth phase dependant manner (Liu *et al.*, 2004). Initiation occurs about 15 minutes after chromosome replication, coordinated by a series of alternate sigma factors which regulate sporulation by altering promoter specificities of the RNA polymerase (Mandelstam and Higgs, 1974, Kroos and Yu, 2000). In addition to the vegetative sigma factor, σ^A, *B. anthracis* expresses at least five additional sigma factors, σ^H, σ^E, σ^F, σ^G, σ^K, that contribute to the temporally controlled expression of sporulation associated genes (Table 1.1) (Errington, 1993). Expression of the sigma factors themselves is also highly regulated, with each sigma factor controlling expression of the next one in the cascade (Lu *et al.*, 1990, Cutting *et al.*, 1991, Lu and Kroos, 1994, Liu *et al.*, 2004).

Table 1.1. Sigma factors of *Bacillus* species and their role in sporulation

Sigma Factor	Regulon[a]
σ^A	Vegetative growth
σ^H	Post-exponential growth
σ^E	Early mother cell
σ^F	Early prespore
σ^G	Late prespore
σ^K	Late mother cell

[a]Adapted from a figure in Errington, 1993.

During sporulation, the bacterial cell undergoes asymmetric division, resulting in a smaller prespore and a larger cell known as the mother cell. The mother cell engulfs the prespore shortly after division, protecting it from the outside environment. This engulfment causes a double membrane system in the spore, the inner membrane and outer membrane (Stragier and Losick, 1996).

The inner membrane surrounds the spore core and contains germinant receptors which sense germinant signals from the environment and stimulate the initiation of germination (Paidhungat and Setlow, 2001). DPA is synthesized in the mother cell, and taken up into the core during sporulation, facilitated by the SpoVA proteins (Errington, 1993). These proteins are encoded by a hexacistronic operon in *B. subtilis,* and are also predicted to localize to the inner membrane (Tovar-Rojo *et al.*, 2002, Vepachedu and Setlow, 2007a). The SpoVA proteins have been shown to interact with the germinant receptors of *B. subtilis,* suggesting that they may also facilitate Ca-DPA release upon germination (Vepachedu and Setlow, 2007b). The outer membrane of the spore acts as

an anchor point for initial assembly of the spore coat. Within this double membrane system, the prespore undergoes various morphological changes via highly regulated gene expression using sigma factors, namely σ^G in the prespore and σ^K in the mother cell (Haldenwang, 1995). These changes result in the production of a mature *B. anthracis* spore.

After the mother cell engulfs the prespore, two types of peptidoglycans are layered between the inner and outer membranes. The layer closest to the prespore, the germ cell wall, will eventually become the cell wall of the newly germinated bacillus. The outer layer of peptidoglycan, known as the cortex, is much thicker and helps maintain dehydration of the core, mineralization, and dormancy of the spore (Henriques and Moran, 2007). Cortex peptidoglycan contains several modifications which allow for lytic enzymes to distinguish it from cell wall peptidoglycan during germination. Most notably, about 50% of the *N*-acetylmuramic acid (NAM) residues are converted to muramic-δ-lactam, resulting in a decrease in cross-linking between the peptidoglycan strands (Warth and Strominger, 1969, Warth and Strominger, 1972, Atrih *et al.*, 1996). These modifications are important for cortex degradation during germination, as germination specific lytic enzymes (GSLE) recognize peptidoglycan containing muramic-δ-lactam (Popham *et al.*, 1996, Setlow *et al.*, 2001). This modification also appears to be conserved among various spore forming bacteria (Popham *et al.*, 1996, Atrih and Foster, 2001a).

A layer of protein is deposited around the outer membrane of the spore, forming the coat. Coat proteins are assembled into two layers in *B. subtilis*, known as the inner coat and outer coat. These layers are visible in electron microscopy images. Two coat

layers likely exist in *B. anthracis*, as well, although these layers are much more compact and therefore difficult to distinguish visually (Driks, 2002). Coat assembly is controlled by the morphogenetic proteins SpoIVA and SpoVID, which are expressed early during sporulation under the control of σ^E (Roels *et al.*, 1992, Stevens *et al.*, 1992, Beall *et al.*, 1993). These proteins provide the framework for the deposition of additional coat proteins during spore assembly. The coat is responsible for environmental resistance, as well as aiding in the response to germinants (Jenkinson *et al.*, 1980, Henriques and Moran, 2000, Bagyan and Setlow, 2002, Klobutcher *et al.*, 2006, Laaberki and Dworkin, 2008). Coat protein assembly is a function of the mother cell, and is dependent upon σ^K for the expression of many of the structural coat proteins.

Spores of some *Bacillus* species, including *B. anthracis* and *B. cereus*, have a final outer layer of glycoproteins known as the exosporium. The function of the exosporium is poorly understood, but it is thought to play a role in adhesion, survival within host cells, germination, and interaction with professional phagocytes (Kang *et al.*, 2005, Brahmbhatt *et al.*, 2007), (Todd *et al.*, 2003, Bozue *et al.*, 2007, Brahmbhatt *et al.*, 2007, Chesnokova *et al.*, 2009). After formation of the exosporium, sporulation is complete, and the mother cell undergoes autolysis, releasing the mature spore into the environment (Piggot and Hilbert, 2004) (Figure 1.1).

Spore Germination

Germination initiation

When *B. anthracis* spores encounter a mammalian host, they sense nutrients in the environment that signal for germination to begin. Germination is essential to initiate

disease, and propagate bacteria. Chapter III of this work will focus on examining the signals that stimulate germination *in vivo,* and the spore components required for detection of germinants. During this process spores shed their outer protective layers in order to return to metabolically active bacteria. As spores are metabolically dormant, all of the components necessary to facilitate germination must be pre-packaged into the spore during sporulation. The onset of germination is dependent upon an appropriate germinant/germinant receptor interaction, first defined in *Bacillus subtilis* (Moir *et al.*, 1979, Sammons *et al.*, 1981, Zuberi *et al.*, 1987, Corfe *et al.*, 1994b). Germinant receptors, located in the inner membrane of the spore, sense the presence of discrete germinant signals in their environment. This event commits spores to germinate, and begins the irreversible cascade of germination (Stewart *et al.*, 1981, Yi and Setlow, 2010).

Germinant receptors

The germinant receptors of *Bacillus* species are encoded by tricistronic operons, and are under the control of a σ^G promoter (Feavers *et al.*, 1990, Corfe *et al.*, 1994a, Liu *et al.*, 2004). Receptors are composed of three proteins, denoted A, B, and C (Figure 1.2A). Receptor proteins are localized to the inner membrane of the spore at very low levels (24-40 per spore), and it is likely that these proteins interact (Hudson *et al.*, 2001, Paidhungat and Setlow, 2001, Igarashi and Setlow, 2005) The "A" protein of the germinant receptors has five or six predicted membrane spanning domains, as well as a large hydrophilic N-terminal domain. The "B" proteins resemble integral membrane proteins, as they contain ten predicted transmembrane domains. Finally, the "C" proteins

have a predicted lipoprotein signal sequence, and are thought to be membrane anchored (Zuberi *et al.*, 1987, Moir *et al.*, 2002). Although the specific roles of each of these proteins are not well understood, all three are thought to be required for proper function of the receptor. Paidhungat and Setlow showed that point mutations in the GerBA or GerBB proteins could change germinant specificities in B. subtilis, suggesting that there may be some kind of physical interaction between receptor and ligand (Paidhungat and Setlow, 1999). It should be noted that, even though germinant ligands have not been shown to bind directly to these so-called germinant receptors, these proteins are commonly referred to as 'receptors' in the literature, and I will do so in this work.

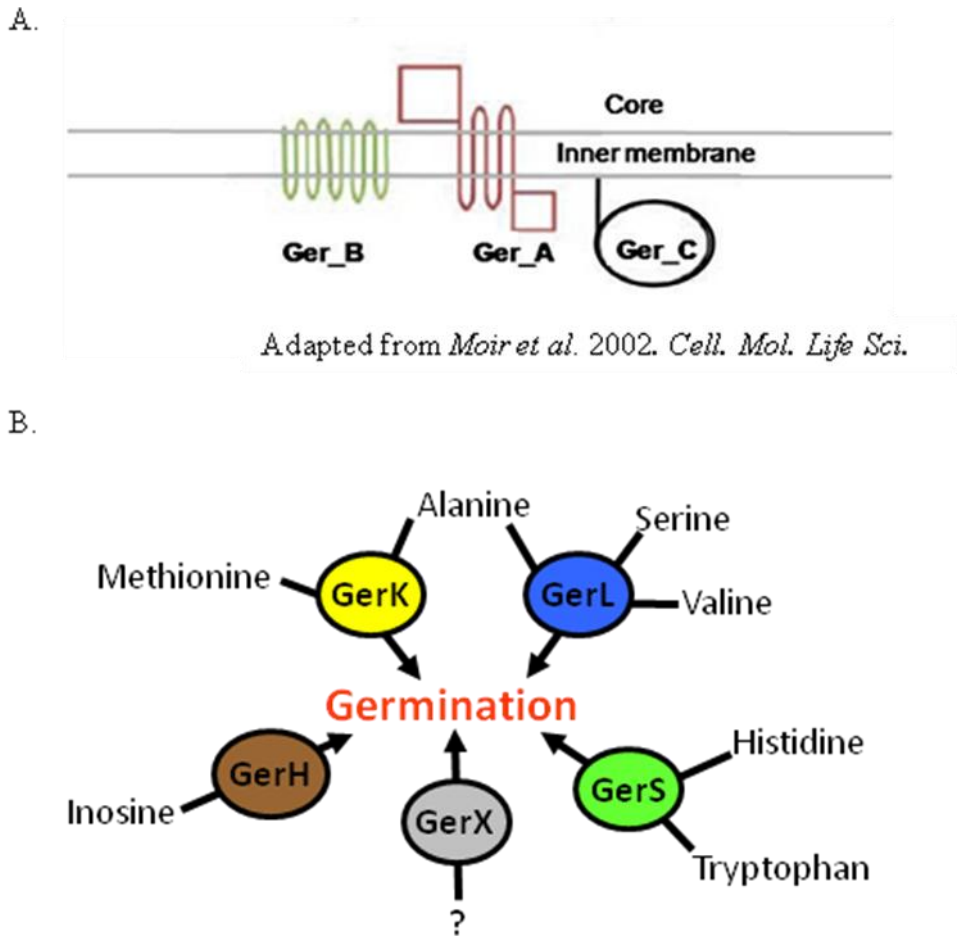

Figure 1. 2. The germinant receptors of *B. anthracis.*
(A) Model of membrane localization of germinant receptor proteins (Moir *et al.*, 2002). (B) Germinant specificities of *B. anthracis* germinant receptors.

Earlier work in *B. subtilis* showed that the gene products of the three germinant receptor operons of *B. subtilis*, $gerA_{ABC}$, $gerB_{ABC}$, and $gerK_{ABC}$, were responsible for responding to different defined germinants, and that disruption of these receptors resulted in a loss of the ability to respond to those cognate germinant ligands (Sammons *et al.*, 1981, Corfe *et al.*, 1994b, McCann *et al.*, 1996). The spores of *B. anthracis* have five known germinant receptor operons, $gerH_{ABC}$ (*gerH*), $gerK_{ABC}$ (*gerK*), $gerL_{ABC}$ (*gerL*), $gerS_{ABC}$ (*gerS*), and $gerX_{ABC}$ (*gerX*), each of which responds to a specific set of L-amino

acid or purine germinants (Ireland and Hanna, 2002a, Read *et al.*, 2003, Weiner *et al.*, 2003, Fisher and Hanna, 2005). These receptors were identified based on homology to receptors in other *Bacillus* species (Kunst *et al.*, 1997, Read *et al.*, 2003, Rasko *et al.*, 2004). The *gerH*, *gerK*, *gerL*, and *gerS* operons are present on the chromosome, while the *gerX* operon is located on the pXO1 virulence plasmid (Read *et al.*, 2003) (Figure 1.2B).

Two additional receptor-like operons exist in *B. anthracis*, named *gerA* and *gerY*. These operons are not expected to play a role in germination, as the *gerAA*, *gerYB*, and *gerYC* open reading frames contain frame-shift mutations (Read *et al.*, 2003). Fisher and Hanna performed transcriptional analyses on these operons, and showed that the *gerA* operon is not expressed, and the *gerY* operon is expressed, but at a greatly reduced level compared to the other *ger* operons. Additionally, disruption of these loci did not result in any measurable germination defect (Fisher and Hanna, 2005). Collectively, these data suggest that these two putative receptor-like operons do not play a role in germination.

Much work has been done in *B. anthracis* and other *Bacillus* species to characterize the germinant specificities of each germinant receptor complex. The five receptors of *B. anthracis* have distinct requirements, based on previous research (Figure 1.2B) (Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005). It is these specificities that Chapter II of this thesis will examine in more detail.

B. anthracis specific germinants consist of a purine nucleoside, notably adenosine or inosine, paired with an L-amino acid. (Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005). L-alanine was also shown to germinate spores in the absence of an additional germinant, but only at very high concentrations. Inosine, however, is

unable to function as a germinant without the presence of a co-germinant (Weiner *et al.*, 2003). A recent study also identified an additional germination pathway independent of nutrient ligands or germinant receptors, in which dormant spores sense peptidoglycan fragments released by other bacteria and stimulate germination (Shah *et al.*, 2008). Additional non-nutrient routes of germination also exist in the laboratory, including surfactants, and pressure. Exogenous Ca-DPA can also be used, as it directly stimulates the activation of the germination specific lytic enzyme CwlJ1, which then degrades the peptidoglycan of the cortex (Wuytack *et al.*, 1998, Paidhungat and Setlow, 2000, Paidhungat *et al.*, 2002, Setlow *et al.*, 2003, Setlow, 2003).

Germinant entry

Before germination can begin, germinants must somehow pass through the exosporium, coat, and cortex to reach these receptors. This process is not understood, although it is predicted somehow selective for specific small molecule germinants, acting as a checkpoint to ensure that spores are in an environment suitable for growth. A group of proteins encoded by the $gerP_{ABCDEF}$ (*gerP*) operon may play a role in germinant entry in *B. anthracis*. Studies of this operon in *B. cereus* and *B. subtilis* suggest that the GerP proteins may facilitate germinant entry or spore coat formation (Behravan *et al.*, 2000). Chapter IV of this work aims to determine the role that the *gerP* operon homologue plays in germination in *B. anthracis*.

Stage I germination

When receptors recognize appropriate germinant signals, a series of biophysical events, known as Stage I germination, begin in the spore (Figure 1.3). Stage I events are considered "biophysical", as they involve purely mechanical changes in the spores. After ligand-receptor interactions occur, H^+, K^+, Na^+ and other monovalent cations are released from the spore, resulting in an increase in core pH (Swerdlow *et al.*, 1981, Setlow, 2003). Next, large stores of dipicolinic acid and its associated calcium ions are released from the core, possibly facilitated by the germinant receptors and SpoVA proteins (Vepachedu and Setlow, 2007a, Vepachedu and Setlow, 2007b). This efflux allows for water to flow back into the core and begin rehydration (Moir, 2003). Rehydration results in a loss of heat resistance as well as a measurable decrease in refractility (Figure 1.3).

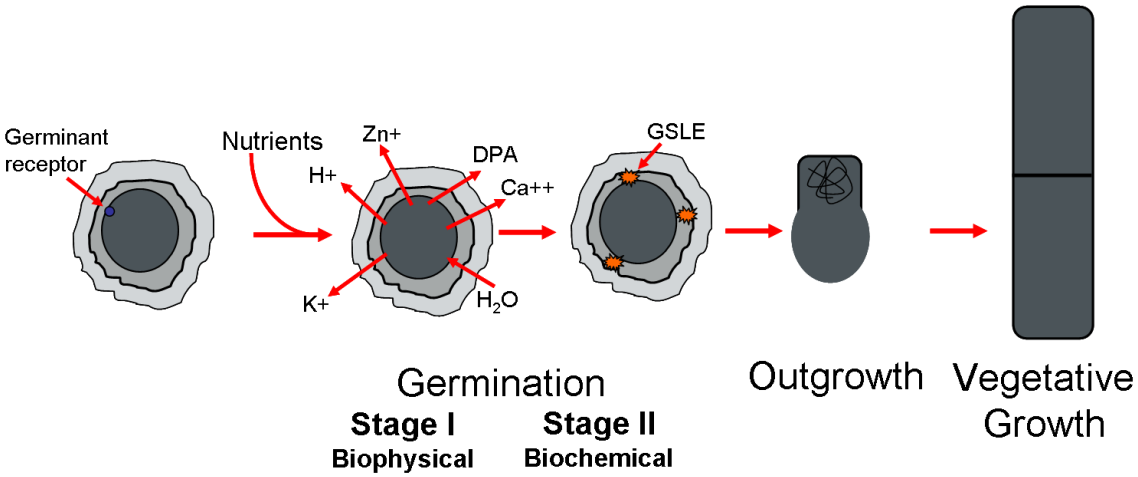

Figure 1. 3. Spore germination. Detection of nutrient germinants by germinant receptors stimulates the opening of channels and release of Ca-DPA and ions, allowing for rehydration in Stage I germination. Stage II germination begins upon activation of germination specific lytic enzymes (GSLEs), which degrade the modified peptidoglycan of the cortex, completing rehydration of the spore and allowing for the return of metabolic processes during outgrowth. This rapid process results in the emergence of a vegetative bacillus.

Stage II germination

Partial rehydration of *B. anthracis* spores stimulates the activation of the germination specific lytic enzymes (GSLEs) SleB, CwlJ1, and CwlJ2 in Stage II, biochemical germination. These enzymes are prepackaged in an inactive form, and are activated upon initiation of Stage I germination via an unknown mechanism. Activated GSLEs hydrolyze the modified peptidoglycan of the spore cortex, leading to full rehydration of the spore (Moriyama *et al.*, 1996, Chirakkal *et al.*, 2002). These enzymes are able to selectively degrade the modified muramic acid linkages in cortex peptidoglycan, while leaving the peptidoglycan of the germ cell wall intact. SleB is a lytic transglycosylase that, when activated, hydrolyzes the bond between N-acetyl muramic acid and N-acetyl glucosamine (Boland *et al.*, 2000). *sleB* is in a bicistronic operon with the gene *ypeB*, whose function is unknown, but YpeB is essential for SleB function (Boland *et al.*, 2000, Atrih and Foster, 2001b). No specific enzymatic activity has been attributed to CwlJ1 yet, but it shares a homologous catalytic domain with SleB, and its presence is required for full germination in *B. subtilis* (Chirakkal *et al.*, 2002). In *B. subtilis*, CwlJ is localized to the coat, and can be activated directly by Ca-DPA *in vitro* (Paidhungat *et al.*, 2001, Bagyan and Setlow, 2002). This likely occurs as well *in vivo*, whereby Ca-DPA released from the spore core during Stage I germination activates CwlJ1, allowing progression into Stage II germination. The *B. anthracis* genome contains two *cwlJ1* homologues, denoted *cwlJ1* and *cwlJ2*, in contrast to the single copy found in *B. subtilis* and *B. cereus* (Read *et al.*, 2003, Heffron *et al.*, 2009). Similar to *sleB*, the *cwlJ1* gene is in a bicistronic operon with the gene *gerQ*, while *cwlJ2* stands

alone on the chromosome (Heffron *et al.*, 2009). In *B. anthracis*, the presence of at least one of the GSLEs, SleB, CwlJ1, or CwlJ2, is essential for germination to occur, as degradation of the cortex is vital for full rehydration of the spore. There is some evidence of partial redundancies with these enzymes (Giebel *et al.*, 2009, Heffron *et al.*, 2009).

Full rehydration of the spore, via the actions of the GSLEs results in the activation of additional enzymes and the initiation of ATP synthesis. The SASPs in the spore core responsible for maintaining DNA stability are degraded, allowing transcription to resume (Setlow, 2007). The initiation of RNA, protein, and DNA synthesis during outgrowth results in a newly germinated, metabolically active bacillus. Although complex, this entire process is extremely efficient and rapid, with 95% of spores germinating within minutes in rich media. The process of germination is illustrated in Figure 1.3 above.

Germination assays

Currently, we have no way of actually detecting receptor-ligand interactions and, therefore, indirect methods of measuring germination must be employed. Physical and chemical properties of germinating spores can be measured and used as tools to assess germination. Ca-DPA release, loss of heat resistance, and decrease in absorbance are all frequently used as assays of germination (Paidhungat and Setlow, 2002). Each of these techniques assesses the ability of spores to initiate the process of germination. As germination is an irreversible event, we can directly correlate changes in the properties mentioned above to germination (Stewart *et al.*, 1981, Yi and Setlow, 2010). Combining

these techniques with genetic mutations in genes involved in germination allows us to score for the necessity of various spore components during the process of germination.

Scope of study

B. anthracis has been identified as a potential bioweapon, as well as a useful model organism for other spore forming bacteria. Current therapeutics may not be ideal for a large scale outbreak, and much more needs to be done to further our understanding of *B. anthracis* biology and pathogenesis in order to find better options. One important avenue is the process of spore germination. Germination is essential for the establishment of disease in a mammalian host. This process is unique to spore forming bacteria, but conserved among spore formers. Lessons learned from one spore forming species may provide valuable insights into other spore forming pathogens, such as *B. cereus* or *Clostridial* species, which are becoming increasingly more relevant, in terms of public health. Expanding our knowledge of germination may allow us to exploit this process for benefit to the host. This could be through developing therapeutics to prevent germination until suitable treatments or treatment facilities are available, as in a bioterrorist attack. It could also lead to measures of forced germination, during either decontamination of an environmental area, or as a therapeutic in humans. *B. anthracis* vegetative bacilli are susceptible to antibiotics, while spores are not. Promoting germination in the host would allow antibiotics to be more effective in fully clearing the infection.

The overall goal of this study was to expand our understanding of the earliest moments of germination. Extensive work has been conducted on the topic, and we have

gained a much greater understanding of this process in recent years. Much more remains to be understood, however. The work presented here was initiated with the overarching goal of better understanding the relationship between nutrient germinants and the spore: both the mechanisms of recognition, as well as the mechanism for germinant entry into the spore. The data presented here contribute significant progress to the field of *B. anthracis* spore germination. Two major questions pertaining to germinant receptors were asked during the course of these studies. I also focused on a putative germinant entry complex residing within the outer layers of the spore that may facilitate contact between small molecule nutrient ligands and these receptors.

The first question aimed to expand our knowledge of germinant receptor specificities. Previous work on the germinant specificities of *B. anthracis* germinant receptors provided an excellent basis for this. A panel of mutant strains lacking one or more germinant receptors was created and used to assess which germinant receptors were necessary for germination with defined germinants (Chapter II). In creating strains lacking multiple receptors, I also asked which germinant receptors were sufficient to stimulate germination in a defined germinant mixture. In using these mutant strains, I began to better understand the roles each receptor plays in germination. I also began to recognize a significant amount of promiscuity and redundancy between receptors that were previously believed to have more defined germinant specificities.

In addition to the *in vitro* work trying to further characterize germinant specificities, I used these mutant strains to understand the role each germinant receptor may play during anthrax infection (Chapter III). Using a mouse model of infection, and strains lacking one or multiple germinant receptors, I tested which germinant receptors

were essential for a virulent infection, and if any were dispensable. These mutant strains, with varying germinant specificities, also provided a powerful tool for us to begin understanding how potential germinants might be distributed in various microenvironments in the body. Using multiple routes of infection, I was able to determine the germinant receptors required for germination and virulence in a few different *in vivo* environments. These experiments also allowed for examination of the germinants encountered during these infections that may contribute to germination and disease initiation *in vivo*.

The second goal of this work was to characterize a group of proteins thought to play a role in germinant access into the spore's multi-layered structure (Chapter IV). While some aspects of germination are beginning to be understood, the mechanism by which germinants gain access to the inner layers of the spore, where the germinant receptors are located, remains unclear. This event must be at least semi-selective, as spores can remain dormant in the environment for many years, but contact with appropriate germinants rapidly stimulates the germination cascade. Previous work in *B. subtilis* and *B. cereus* point to a hexacistronic operon termed $gerP_{ABCDEF}$, or *gerP* (Behravan *et al.*, 2000). This operon has been implicated in other *Bacillus* species to be important for germination, and evidence suggests that it is localized to the coat. These two features led to speculation that the GerP proteins may be involved in germinant passage through the spore coats and/or cortex. We sought to examine the role of a homologous operon in germination of *B. anthracis* by creating deletion mutants of each of the *gerP* genes. With these mutants we were able to provide evidence that the GerP

proteins are indeed important for germination, and that their role may be to provide nutrient and non-nutrient germinants the ability to flow both in and out of the spore.

Collectively, these studies expand our understanding of *B. anthracis* as a pathogen, and as a model spore-forming organism. Gaining a better appreciation for how germination is initiated will further our ability to combat this pathogen, and its relatives in the future.

Chapter II

In vitro analysis of *Bacillus anthracis* Germinant Receptor Specificities

ABSTRACT

Nutrient-dependent germination of *Bacillus anthracis* spores is stimulated when receptors located in the inner membrane detect combinations of L-amino acid and purine nucleoside germinants. *B. anthracis* produces five distinct germinant receptors, GerH, GerK, GerL, GerS, and GerX. Otherwise isogenic mutant strains expressing only one of these receptors were created and tested for germination. The GerH receptor was necessary and sufficient for wild-type levels of germination with inosine-containing germinants in the absence of other receptors. All germinant receptors, with the exception of GerX, exhibited a larger amount of promiscuity in their germinant specificities than previously appreciated. A mutant lacking all five germinant receptors exhibited a severe germination defect *in vitro*. A similar defect was seen in a strain expressing only the GerX receptor, which was unable to germinate in any conditions tested. Additionally, purine nucleoside germinants appeared to compete for the same or overlapping recognition sites on germinant receptors. Together, these data give us a greater understanding of the earliest moments of germination, and provide a more detailed picture of the signals required to stimulate this process.

INTRODUCTION

The Gram positive bacterium *Bacillus anthracis* exists in two morphologically distinct forms, the metabolically active bacillus and the dormant spore. Bacterial spores form in response to nutrient depletion to protect the organism from environmental stresses such as heat, desiccation, chemicals, and radiation (Nicholson *et al.*, 2000, Piggot and Hilbert, 2004). Spore formation enables *B. anthracis* to survive dormant in the environment for years, until it comes in contact with an appropriate mammalian host, germinates, and outgrows, resulting in the disease anthrax (Dixon *et al.*, 1999). Although dormancy can last for an extensive period of time, germination is a very rapid and efficient process, allowing *B. anthracis* to multiply and spread quickly (Dixon *et al.*, 1999).

Anthrax can present in a variety of ways, depending upon route of infection. The most severe of these presentations is inhalational anthrax. Upon entering the lungs of a host, spores are taken up into phagocytes, where they germinate and are transported to the mediastinal lymph nodes (Guidi-Rontani *et al.*, 1999b). Here, they can disseminate systemically and cause disease, and oftentimes, death. Other forms of the disease also exist, including cutaneous and gastrointestinal anthrax (Dixon *et al.*, 1999).

The onset of germination is dependent upon appropriate germinant/germinant receptor interactions (Dixon *et al.*, 1999). For *B. anthracis*, nutrient germinants are primarily L-amino acids and purine nucleosides, which interact with their specific receptors located in the inner membrane of the spore (Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005). L-alanine or inosine typically serve as primary germinants, with a separate amino acid functioning as a co-germinant. L-alanine can

trigger germination of spores by itself, although only at very high concentrations (Ireland and Hanna, 2002a). Inosine is the more potent of the primary germinants, stimulating a greater germination response at a lower concentration than L-alanine. Inosine can be paired with a variety of L-amino acids, including L-histidine, L-serine, L-valine, L-tryptophan, and L-methionine, as well as with the primary germinant L-alanine. Inosine alone at any concentration is not sufficient, however, to germinate *B. anthracis* spores (Weiner *et al.*, 2003).

After receptors recognize their specific germinants, the cascade of germination events is initiated. Stores of dipicolinic acid and its associated calcium ions (Ca-DPA) are released from the spore core, allowing for water to flow back into the core and begin rehydration (Moir, 2003). Activation of lytic enzymes that hydrolyze peptidoglycan in the spore cortex then leads to further rehydration of the spore (Setlow *et al.*, 2001). RNA, protein, and DNA synthesis then resumes in the outgrowth phase, resulting in a vegetative bacillus. This entire process is very rapid, nearing 100% within minutes under optimal conditions.

The five germinant receptors of *B. anthracis* are encoded by the tricistronic operons, *gerH*, *gerK*, *gerL*, and *gerS*, located on the chromosome, and *gerX*, located on the pXO1 virulence plasmid (Read *et al.*, 2003). Two additional receptor-like operons exist, named *gerA* and *gerY*, but both contain frame shift mutations, and were shown to not play a role in germination (Fisher and Hanna, 2005). The five functional germinant receptors have been characterized previously using single receptor operon mutations (Guidi-Rontani *et al.*, 1999a, Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005). In this study we have used markerless deletions to create isogenic

quadruple mutants lacking all but one germinant receptor, as well as a null strain lacking all five functional germinant receptors. These mutants, as well as isogenic single receptor mutants, were used to further elucidate the roles of these receptors during germination *in vitro*.

MATERIALS AND METHODS

Strains and culture conditions

Strains used in this study are listed in Table 2.1. Strains were cultured in Brain Heart Infusion (BHI, Difco) broth or solid media containing 15g agar per liter. Spores were prepared by growth in Modified G medium (Kim and Goepfert, 1974) for three days at 37° C with shaking. Spores were prepared as previously described (Passalacqua *et al.*, 2006). Spores were stored at room temperature in sterile water and titered by counting phase-bright particles using a hemacytometer (spores/mL) and/or by plate count (cfu/mL), as indicated in the text.

Mutant construction

Each of the mutant strains used in this work were created using allelic exchange, resulting in markerless deletions (Figure 2.1). Each mutant allele was designed to contain the first 10 codons of the first gene in the operon, (*gerHA, gerKA, gerLA, gerSA,* or *gerXB*), a short insert sequence of three stop codons and restriction sites for the restriction endonucleases BamHI and SmaI, followed by the final 10 codons of the last gene in the operon, including the putative stop codon (*gerHC, gerKC, gerLC, gerSC,* or *gerXC*). The constructs used to create each mutant were isolated by PCR (primer sequences available upon request). In addition to the mutant allele described above, each PCR product contained approximately 500 bp of DNA sequence homologous to the upstream and downstream region of the *ger* operon, flanked by the recognition sequence for the restriction endonuclease NotI. Each PCR product was cloned into the

pCR®8/*GW*/*TOPO* vector (Invitrogen) according to manufacturer's instructions. The DNA sequence of each construct was verified to ensure no additional mutations were present due to PCR error. The NotI fragment was then cloned into the allelic exchange vector pBKJ258-kan. This vector was identical to the previously described pBKJ258, with the exception that a kanamycin resistance cassette was exchanged for the original erythromycin cassette (Lee *et al.*, 2007). Allelic exchange was performed essentially as described previously (Janes and Stibitz, 2006). The quadruple mutants and Δger_{null} strain were isolated by performing allelic exchange sequentially, knocking out the appropriate *ger* allele. All mutant alleles were verified using PCR, with primers designed to anneal outside of the sequences used for homologous recombination. Additionally, expression of the *gerX* operon was verified via reverse transcription PCR (RT PCR) in the $gerX^+$ strain, as its phenotype resembled that of a Δger_{null} strain *in vitro* and *in vivo*. RNA isolation was performed as previously described (Carlson *et al.*, 2009). The primers to verify the Δ*gerX* mutant, via PCR, and expression in the $gerX^+$ strain, via RT PCR, were specific to approximately 200 bp of the first gene of each receptor operon.

Germination assays

Germination was measured by two assays, loss of heat resistance and decrease in optical density of spore suspensions after exposure to germinants. For the loss of heat resistance experiments, spores were first heat activated by incubating at 65°C for 20 minutes. $5x10^3$ spores were mixed with 2 mL of germinant in 1x phosphate buffered saline (PBS), pH 7.4 (Gibco). Germinant concentrations were as follows: Inosine, 1 mM; L-tryptophan, 5 mM; L-serine, L-valine, L-methionine, and L-histidine, 50 mM; L-

alanine was at either 50 mM or 0.5 mM, as noted in Tables 2.2 to Table 2.4. For purine competition, spores were incubated in 0.5 mM L-alanine plus inosine and guanosine at varying concentrations, as noted in the text. Germinants for the purine competition experiments were prepared in PBS, pH 10, as guanosine is only soluble at high pH. Samples were vortexed briefly and 50 μL were plated on a BHI plate. Samples were incubated at 37°C for 30 minutes. At 30 minutes, 200 μL aliquots were moved to a fresh tube and heat treated at 65°C for 20 minutes, after which 100 μL was plated on BHI. Plates were incubated overnight at 37°C and colonies were counted. A sample that was 100% sensitive to a 65°C incubation was considered 100% germinated. Standard error of the mean (SEM) was calculated as (standard deviation/$\sqrt{n}$).

To assay decrease in optical density, heat activated spores (see above) were added to 400 μL of germinant, giving a starting OD_{600} of 0.3 using a Genesys 10UV spectrophotometer (Spectronic Unicam, Rochester, NY). The reaction was incubated at 37°C while shaking at 200 RPM for 30 minutes. After this time the OD_{600} of the germinated spore mixture was measured. It has been previously established that a loss of 60-70% of the starting OD_{600} value corresponds with complete germination (Fisher and Hanna, 2005). In all cases the parental $34F_2$ Sterne strain was used as the positive control.

Colony Forming Efficiency

Colony forming efficiencies were determined by comparing the number of spores/mL to the number of cfu/mL. Spore stocks were titered by counting phase bright

particles using an Improved Neubauer hemacytometer. The same spore stock was then diluted and plated, and colonies were counted after an overnight incubation at 37°C.

RESULTS

In vitro characterization of germinant receptor mutants

Mutants in which individual germinant receptor operons were disrupted have been studied previously (Guidi-Rontani *et al.*, 1999a, Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005). However, the use of antibiotic resistance markers in these strains prevented the subsequent isolation of strains containing more than a single deletion. To examine the role of the individual germinant receptors more thoroughly, we created markerless deletion mutants targeting each operon in our parental $34F_2$ strain (Table 2.1).

Table 2.1. *Bacillus anthracis* strains used in this study

Strain	Mutant Name	Relevant Characteristics[a]	Reference
$34F_2$	--	Wild-type ($pXO1^+$, $pXO2^-$)	(Sterne, 1939)
SL110	Δ*gerH*	$34F_2$, Δ*gerH*	This work
SL111	Δ*gerK*	$34F_2$, Δ*gerK*	This work
SL112	Δ*gerL*	$34F_2$, Δ*gerL*	This work
SL113	Δ*gerS*	$34F_2$, Δ*gerS*	This work
SL114	Δ*gerX*	$34F_2$, Δ*gerX*	This work
SL115	$gerH^+$	$34F_2$, Δ*gerK* Δ*gerL* Δ*gerS* Δ*gerX*	This work
SL116	$gerK^+$	$34F_2$, Δ*gerH* Δ*gerL* Δ*gerS* Δ*gerX*	This work
SL117	$gerL^+$	$34F_2$, Δ*gerH* Δ*gerK* Δ*gerS* Δ*gerX*	This work
SL118	$gerS^+$	$34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerX*	This work
SL119	$gerX^+$	$34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerS*	This work
SL120	Δger_{null}	$34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerS* Δ*gerX*	This work

[a]Mutants alleles consist of markerless deletions of nearly the entire tricistronic germinant receptor operon (see Methods).

The three genes for each tricistronic germinant receptor operon were removed, resulting in strains lacking one receptor operon each; these were named Δ*gerH*, Δ*gerK*, Δ*gerL*, Δ*gerS*, and Δ*gerX*, respectively. A schematic of a typical germinant receptor operon and our deletion scheme is depicted in Figure 2.1A and 2.1B. This mutagenesis method allowed the deletion of additional germinant receptor operons in these strains, eventually leading to the creation of five distinct quadruple mutants, each containing only a single germinant receptor operon. These were named $gerH^+$, $gerK^+$, $gerL^+$, $gerS^+$, and

gerX^{+}, to denote the remaining functional germinant receptor operon. For example, the *gerH*$^{+}$ strain retained the *gerH* operon but contained the deleted forms of the operons *gerK*, *gerL*, *gerS* and *gerX*. Additionally, a mutant was constructed that lacked all five germinant receptors (Δger_{null}). Creation of these mutant strains allowed us to directly assay the function of each individual receptor in the absence of the other four, and alleviated complications from the potential cooperativity between different germinant receptor proteins described in other *Bacillus* species (Atluri *et al.*, 2006).

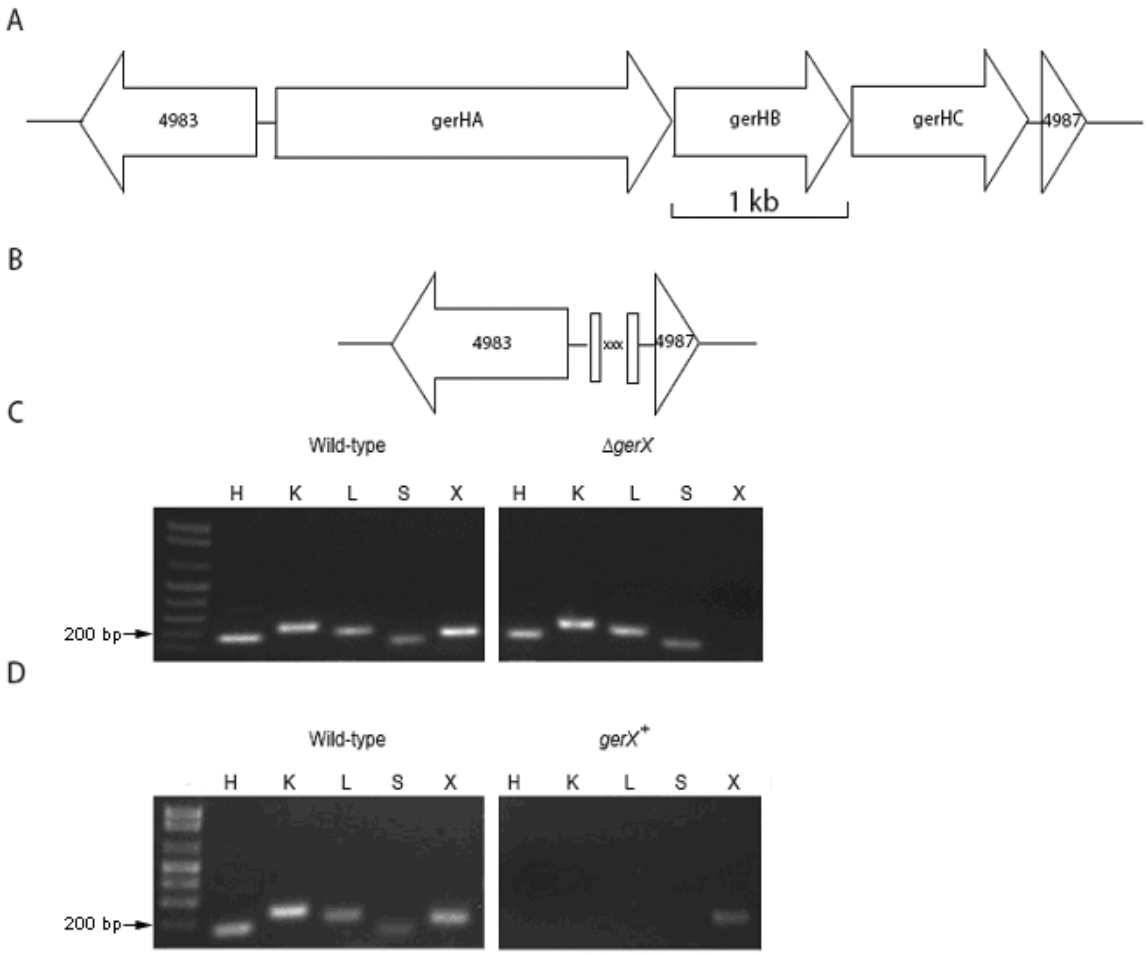

Figure 2. 1. Germinant receptor operon organization and mutagenesis.
(A) Gene map of the *gerH* operon. The other *ger* operons discussed in this work have the same gene order, with the exception of *gerX*, in which the *gerXB* and *gerXA* genes are transposed. (B) Gene map of the Δ*gerH* allele. The mutant allele was designed as a markerless deletion of nearly the entire operon. Non-native sequences are denoted by "X" (see Methods). This general scheme was used to construct each of the *ger* operon deletions. (C) Verification of the Δ*gerX* operon genotype. PCR was performed using primers for each locus that amplified approximately 200bp of the first gene of each receptor operon. Letters denote the germinant receptor being tested. Additional PCR reactions were performed with primers flanking the loci, to verify the deleted allele (data not shown). (D) Expression of germinant receptor operons in the *gerX*$^{+}$ strain. RT PCR was performed using the same primers as in Figure 2.1C.

To determine the specific germinant recognized by each receptor, *in vitro* germination profiles were generated for each receptor mutant using a variety of germinant mixtures defined previously (Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005). Germinant mixtures used in this study included either the amino acid L-alanine or the purine nucleoside inosine as the "primary" germinant, and an additional L-amino acid as a "co-germinant." Co-germinants used included L-tryptophan, L-histidine, L-serine, L-valine, or L-methionine, and a mixture of both primary germinants (L-alanine/inosine) as well. L-alanine was also tested in the absence of a co-germinant because it has previously been shown to function without a co-germinant, but only at high concentrations (Fisher and Hanna, 2005). Where possible, two separate assays were performed to quantify the germination of each mutant in the individual germination mixtures. These assays take advantage of measurable physical changes that occur in the germinating spore. One assay measured the hallmark loss of heat resistance associated with rehydration of the spore's core (Paidhungat and Setlow, 2002). The other assay measured the decreased optical density (OD) associated with a loss in refractility in a germinating spore suspension (Paidhungat and Setlow, 2002). Both assays are described in detail in the Methods section.

Spores lacking all germinant receptors

As predicted, the spores from the strain lacking all five germinant receptors (Δger_{null}) exhibited a severe germination defect, even when plated on the rich medium brain heart infusion agar (BHI). In order to quantify the severity of this defect, colony

forming efficiency was determined. Colony forming efficiency was defined as the number of colony forming units (cfu) formed, when compared to the number of phase-bright spore particles plated. Spores were titered by visual counting via hemacytometer, then diluted and plated on BHI to measure cfu. Wild-type spores had a colony forming efficiency of approximately one cfu per spore particle plated (Figure 2.2). In contrast, the Δ*ger*$_{null}$ strain had a reduced colony forming efficiency of one cfu per 1,000 spore particles plated. The low level of germination and outgrowth seen with this mutant is similar to that seen in an analogous receptor deficient strain of *B. subtilis* (Paidhungat and Setlow, 2000). When tested more directly for germination, none of the previously defined germinant combinations were able to germinate these spores, as measured by decrease in OD_{600} (Table 2.2). The Δ*ger*$_{null}$ strain was not tested in our heat sensitivity assay, as its defect in colony forming efficiency precluded direct comparisons between it and the wild-type strain.

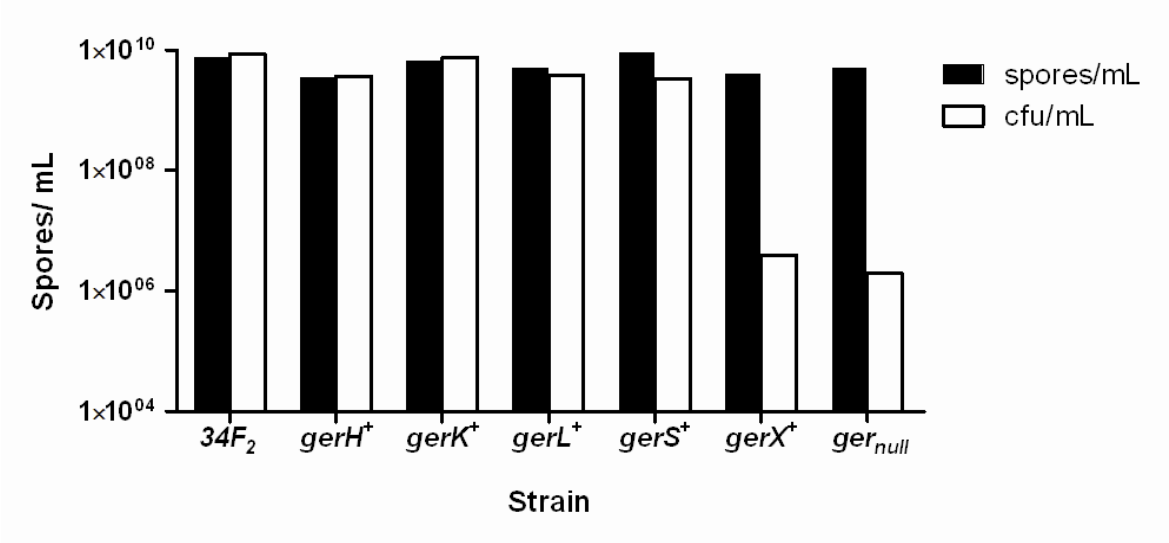

Figure 2. 2. Colony forming efficiency of germinant receptor mutants.
Colony forming efficiency is reported as the number of spore particles/mL, as determined visually by hemacytometer, or via cfu when plated on rich media (BHI). Data shown are the average of two independent experiments with independent spore preparations.

Table 2.2. Decrease in optical density of strains expressing only one germinant receptor after exposure to germinants.

	Mean % loss of OD_{600} after 30 minutes[a]						
Germinant[b]	$34F_2$	*gerH*$^+$	*gerK*$^+$	*gerL*$^+$	*gerS*$^+$	*gerX*$^+$	Δ*ger*$_{null}$
PBS, pH 7.4	0	0	0	0	0	0	0
L-ala (50 mM)	61	19	60	44	30	6	2
L-ala/ inosine	66	72	9	49	30	11	6
L-ala/ L-trp	34	17	22	19	16	15	3
Inosine/ L-trp	66	57	8	8	16	8	8
Inosine/ L-his	40	60	4	5	5	9	9
Inosine/ L-met	72	58	9	0	2	7	3
Inosine/ L-ser	72	60	4	38	21	11	1
Inosine/ L-val	65	55	9	8	4	6	9

[a]Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤10% of the mean in all instances.
[b]Germinant concentrations were 0.5 mM L-alanine, 1 mM inosine, 5 mM L-tryptophan, and 50 mM of other amino acids. ~ 65% decrease in OD_{600} represents ~100% germination.

GerX

The strain lacking only GerX (Δ*gerX*) was tested via heat sensitivity and scored for a germination deficiency in our panel of germinants. Although PCR analysis of genomic DNA confirmed that our Δ*gerX* mutant had the correct genotype (Figure 2.1C), we could not detect any notable germination defect for Δ*gerX* in response to any germinant mix, with the exception of a mild decrease in germination in L-alanine and L-

tryptophan (Table 2.3). Like the Δ*ger*$_{null}$ strain, spores of the *gerX*$^{+}$ strain showed a germination defect when plated on BHI. Indeed this quadruple mutant, expressing only the *gerX* germinant receptor operon, behaved identically to Δ*ger*$_{null}$ in all *in vitro* assays tested (Figure 2.2, Table 2.2). RNA expression of the five receptor operons was verified in the *gerX*$^{+}$ strain in order to ensure that *gerX* was, indeed, expressed (Figure 2.1D).

Table 2.3. Loss of heat resistance of strains lacking one germinant receptor after exposure to germinants.

	Mean % loss of heat resistance after 30 minutes[a]					
Germinant[b]	34F$_2$	Δ*gerH*	Δ*gerK*	Δ*gerL*	Δ*gerS*	Δ*gerX*
PBS, pH 7.4	0	0	0	0	0	0
L-ala (50 mM)	86	88	44	60	43	71
L-ala/ inosine	100	86	99	79	99	100
L-ala/ L-trp	63	20	39	28	42	42
Inosine/ L-trp	76	32	72	60	55	92
Inosine/ L-his	52	23	35	25	31	42
Inosine/ L-met	78	19	21	44	52	72
Inosine/ L-ser	100	24	98	28	99	99
Inosine/ L-val	95	4	62	24	74	86

[a]Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤10% of the mean in all instances.
[b]Germinant concentrations were 0.5 mM L-alanine, 1 mM inosine, 5 mM L-tryptophan, and 50 mM of other amino acids.

GerS

The GerS receptor was sufficient for germination in rich media, as the strain expressing only this receptor (*gerS*$^+$) exhibited a colony forming efficiency similar to the wild-type level of one cfu for every spore plated (Figure 2.2). In addition, the *gerS*$^+$ strain exhibited some level of germination from many of the germinant mixes tested (Tables 2.2 and 2.4), although none were as high as wild-type levels. Previous studies have implicated GerS in germination in inosine, with L-histidine or L-tryptophan as co-germinants (Ireland and Hanna, 2002a, Fisher and Hanna, 2005), and our Δ*gerS* mutant, missing only *gerS*, mimicked these results (Table 2.3). However, the *gerS*$^+$ strain, missing all *ger* operons other than *gerS*, was unable to germinate to wild-type levels in the presence of either of these co-germinants (Tables 2.2 and 2.4). Additionally, our Δ*gerS* mutant exhibited a defect in inosine/L-methionine, as well as L-alanine. As was the case above, the *gerS*$^+$ strain could not germinate to wild-type levels in the presence of these germinant mixes either. Together, these data suggest that the GerS receptor can respond moderately to a wide variety of germinants, but that it needs another receptor present to trigger full germination.

Table 2.4. Loss of heat resistance of strains expressing only one germinant receptor after exposure to germinants.

	Mean % loss of heat resistance after 30 minutes[a]				
Germinant[b]	$34F_2$	*gerH*$^+$	*gerK*$^+$	*gerL*$^+$	*gerS*$^+$
PBS, pH 7.4	0	0	0	0	0
L-ala (50 mM)	86	43	53	22	50
L-ala/ L-trp	63	52	7	35	9
L-ala/ inosine	100	99	11	32	51
Inosine/ L-trp	76	84	16	19	11
Inosine/ L-his	52	45	21	21	9
Inosine/ L-met	78	60	21	12	31
Inosine/ L-ser	100	100	22	25	37
Inosine/ L-val	95	91	37	20	40
Inosine	0				

[a]Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤10% of the mean in all instances.
[b]Germinant concentrations were 0.5 mM L-alanine, 1 mM inosine, 5 mM L-tryptophan, and 50 mM of other amino acids.

GerK and GerL

As with the strain containing only GerS, strains that had either GerK or GerL as the only germinant receptor (*gerK*$^+$ and *gerL*$^+$, respectively) had colony forming efficiencies identical to wild-type, suggesting that they could recognize some germinant combination present in rich media (Figure 2.2). Earlier work implicated a role for GerK and GerL in response to L-alanine (Fisher and Hanna, 2005). Indeed, the *gerK*$^+$ strain responded strongly when L-alanine was the sole germinant, nearly as well as wild-type

with respect to loss of heat resistance (Table 2.4), and indistinguishable from wild-type as scored by drop in absorbance (Table 2.2). The *gerL*$^+$ strain also exhibited an ability to respond to 50 mM L-alanine, when measured by drop in absorbance (Table 2.2), although it appeared less sufficient when measured by a loss in heat resistance (Table 2.4). Based on these data, it is likely that these two receptors act in a concerted effort to stimulate L-alanine based germination.

A role has also been suggested for GerK in response to L-methionine when inosine was the primary germinant, and for GerL in response to L-serine or L-valine with inosine as the primary germinant (Fisher and Hanna, 2005). Additionally, we saw a slight defect in L-histidine mediated germination with our Δ*gerK* or Δ*gerL* mutants, as well as a defect in L-methionine with the Δ*gerL* strain (Table 2.3). When expressed as the sole germinant receptor, roles for these proteins appeared less clear. As measured by heat sensitivity, a *gerL*$^+$ strain appeared to respond weakly to all germinants tested, with the possible exception of inosine paired with methionine (Table 2.4). When germination was measured by a loss of absorbance at 600 nm, this same strain exhibited some greater discrimination, failing to germinate well with inosine as the primary germinant, with the exception of the case where serine or alanine was the co-germinant. However, none of these responses were as strong as that of wild-type (Table 2.2). A *gerK*$^+$ strain appeared less promiscuous than *gerL*$^+$, when germination was assessed by loss of absorbance at 600 nm, failing to respond well to any germinant combination (Table 2.2). When measured by a loss of heat resistance, however, the *gerK*$^+$ strain exhibited a low level response to inosine pared with several co-germinants, including L-histidine, L-methionine, L-serine, and L-valine (Table 2.4). As with the GerS receptor, GerK and

GerL both appear to recognize a wider variety of germinants that previously realized, but germination is likely more efficient when multiple receptors are present.

GerH

Previous reports have shown that GerH is important for purine nucleoside-mediated germination (Weiner and Hanna, 2003). Indeed, our Δ*gerH* mutant, missing *gerH* but maintaining all other *ger* operons, had a severe germination defect in the presence of nearly all germinant mixes containing inosine, with the exception of L-alanine/inosine (Table 2.3), consistent with the previous report (Weiner *et al.*, 2003). The *gerH*$^{+}$ strain, expressing only the GerH receptor, exhibited wild-type colony forming efficiency (Figure 2.2). The germination profile of the *gerH*$^{+}$ mutant mirrored wild-type spores when inosine was used as the primary germinant in either assay used (Tables 2.2 and 2.4). It should be noted, however, that although GerH can trigger germination without the need for other receptors, it requires both inosine and a co-germinant to do so, as wild-type spores could not respond to inosine alone (Table 2.4). None of the other quadruple mutants were able to germinate to a similar level in the germinant mixtures containing inosine as the primary germinant. Together, these data suggest that not only is GerH required for inosine-dependent responses, but that it is also able to trigger a full response in the absence of all other receptors.

Competition of purine germinants

Purine nucleosides are powerful germinants of *B. anthracis.* Inosine, adenosine, and guanosine have all been shown to mediate germination, when in the presence of an L-amino acid co-germinant (Weiner *et al.*, 2003). The GerH receptor has been implicated in the germination response to purines in this study and others (Weiner *et al.*, 2003). As one receptor can respond to a variety of purines, we hypothesized that purines may interact with their receptor in a similar manner. Guanosine stimulates germination much less efficiently than inosine. In fact, wild-type spores only achieved about 25% germination when incubated in guanosine and L-alanine, compared to 100% germination when inosine was present (Figure 2.3). This inefficient guanosine-based germination was exploited in order to determine whether inosine and guanosine competed for interaction with their receptor *in vitro*. Spores were incubated in L-alanine and varying concentrations of the two purines, using heat sensitivity as a marker for germination. When inosine and guanosine were incubated together in the presence of L-alanine, a decrease in germination efficiency was seen, when compared to L-alanine and inosine alone (Fig 2.3). As the concentration of guanosine increased, germination decreased, with saturation of the inosine response at 4 mM guanosine and 1 mM inosine. At this concentration of germinants, germination was identical to what was seen with guanosine and L-alanine alone. This suggests that the weak germinant guanosine is able to inhibit germination mediated by the more efficient germinant inosine. A similar experiment was attempted to determine whether L-amino acids competed for binding in a similar manner. No germinant competition was detected with any L-amino acids, at any concentration tested (data not shown).

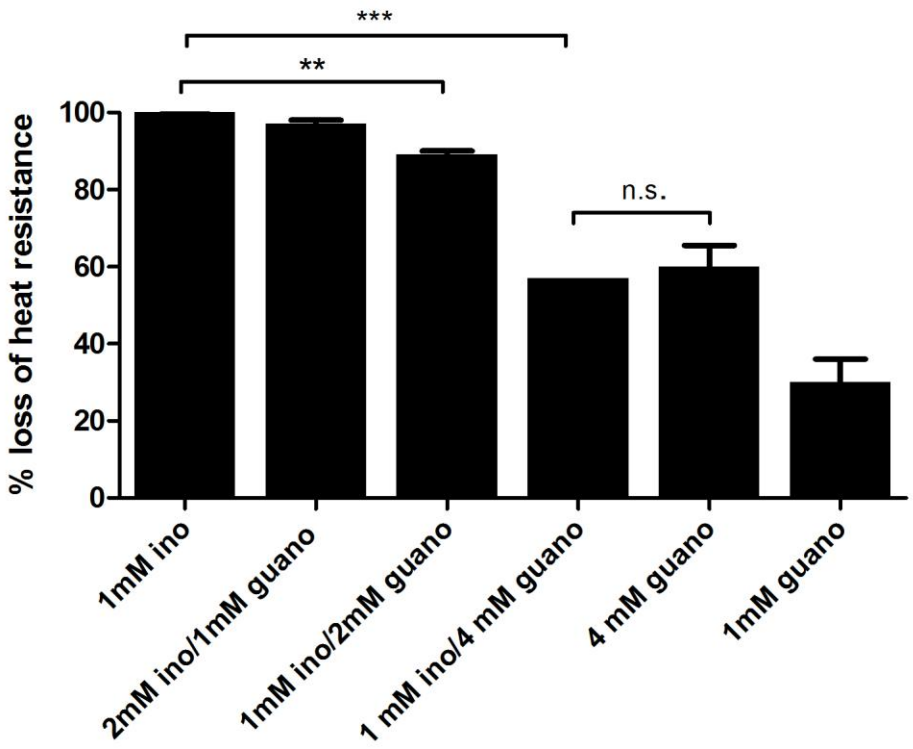

Figure 2. 3. Purine competition.
Wild-type spores were incubated in 0.5 mM L-alanine and the purine germinant listed, pH 10, at 37°C for 30 minutes. Germination was measured as a loss in heat resistance. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean. *** $p \leq 0.0001$. ** $p = 0.0004$. n.s.: not significant. Ino: inosine. Guano: guanosine.

DISCUSSION

B. anthracis may have evolved a variety of germinant receptors in order to optimize its germinant recognition capabilities, thereby increasing chances of efficient germination and survival in various niches. The presence of five distinct receptors, coupled with the possibility of beneficial cooperation between receptors or receptor components, may increase the array of germinants spores respond to. Theoretically, this may increase chances of spore germination upon inoculation into various sites within a host, or increase the variety of possible hosts. The inability of strains expressing only GerK, GerL, or GerS to fully germinate in their previously characterized germinants, with the exception of L-alanine-specific germination in the *gerK*$^+$ and *gerL*$^+$ strains, suggests that there is at least some level of cooperativity between receptors of *B. anthracis*, and that the presence of more than one receptor family member is typically required for a full, robust response to certain germinants. Cooperativity among these various receptors has been speculated to play a role in germinant detection in *B. anthracis* (Fisher and Hanna, 2005). Conversely, the observation that these quadruple receptor mutants responded to germinants in addition to those described in the literature, suggests a high level of promiscuity among the germinant specificities of these receptors.

The finding that the *gerH*$^+$ strain could germinate to wild-type levels in inosine-based germinants independent of all other receptors, however, suggests that not all receptors absolutely need cooperative partners in order to germinate (Tables 2.2 and 2.4). It appears that the GerH receptor, in the absence of all others, can recognize and respond to not only inosine, but also the amino acid co-germinants required for inosine-dependent germination. Whether this involves individual GerH receptors binding discrete

germinant species and interacting in some beneficial manner or one individual receptor complex binding two or more germinant species simultaneously remains unclear. While it is tempting to speculate that the GerH receptor can interact with both inosine and its co-germinants, our data does not preclude the possibility that an additional unknown spore component could be contributing to this germination response.

The *in vitro* data from the Δ*ger*$_{null}$ strain, lacking all of its germinant receptors, further enforces the importance of a germinant detection system for germination. A *B. subtilis* strain lacking all known germinant receptors exhibited a decrease in colony forming efficiency similar to the 1,000 fold defect seen with our Δ*ger*$_{null}$ strain (Paidhungat and Setlow, 2000). The residual growth seen with the *B. subtilis* mutant was attributed to a small percentage of these spores that were still able to germinate, based on a low level of "spontaneous" germination over time. This is likely the case with our Δ*ger*$_{null}$ strain as well. Together, these data suggest that germinant receptors are an essential component to efficient, productive germination.

Curiously, the *gerX*$^{+}$ mutant (expressing only the *gerX* receptor operon) exhibited the same phenotypes as the Δ*ger*$_{null}$ mutant, with a severe defect in colony forming efficiency and germination. This observation was somewhat surprising, as a previous study implicated the GerX receptor in germination with L-alanine (Hu *et al.*, 2007). That the Δ*ger*$_{null}$ and *gerX*$^{+}$ strains behaved identically in all conditions tested suggests that the GerX receptor, when present by itself in the spore, is not capable of promoting germination *in vitro*. It is possible, perhaps even likely, however, that all *B. anthracis* germinant receptors, including GerX, have additional germinant specificities not tested in this work.

The method by which nutrient germinants stimulate their receptors is still unknown. Receptors could potentially bind their appropriate amino acid or purine germinant, stimulating a conformational shift that opens channels in the spore, beginning the process of ion and Ca-DPA release. The data suggesting that purines may compete for interaction with germinant receptors may provide some evidence for this binding process (Figure 2.3). The purine nucleosides guanosine and inosine have very different abilities to stimulate germination; inosine is a potent germinant, while guanosine only stimulates germination 25% as efficiently. It is unclear why guanosine is less effective at initiating germination. Guanosine may not bind germinant receptors as strongly as inosine, resulting in fewer stimulation events. Alternatively, guanosine may bind effectively, but may be less able to stimulate germinant receptors to begin germination. When spores were exposed to alanine and inosine, with increasing amounts of guanosine, germination efficiency was markedly decreased. In fact, at a ratio of 1:4 inosine: guanosine, germination was identical to L-alanine and guanosine alone. These data suggest that guanosine likely binds germinant receptors efficiently, but that it is less able to stimulate germination once bound.

In contrast to the response seen with purine germinants, L-amino acid germinants did not exhibit the same ability to compete with one another for binding. This may be due to more discrete binding sites of these L-amino acid germinants. It could also result from L-amino acids binding less strongly, resulting in a more transient interaction between germinant and receptor. This would lead to more opportunities for more efficient L-amino acid germinants to reach their receptors, even in the presence of large amounts of weaker varieties.

In order to understand the role that germinant receptors play in disease we must first understand the signals that stimulate them. Using our set of mutants allowed us to better characterize the receptor-ligand interactions necessary for germination in defined germinants. Additionally, we were able to determine which receptors were sufficient for germination, *in vitro*, independent of any other kind of germinant receptor. Understanding the complete set of germinants that *B. anthracis* spores have evolved to recognize will give us a guide for future therapeutics aimed at either blocking, or exploiting germination during an active infection.

Chapter III

Analysis of *Bacillus anthracis* Germinant Receptor Specificities and their Contributions to Virulence

ABSTRACT

Germination is an essential step in the establishment of the disease anthrax, and is initiated upon detection of nutrient germinants by germinant receptors. *B. anthracis* produces five distinct germinant receptors, GerH, GerK, GerL, GerS, and GerX. Mutant strains lacking one or more of these receptors were created and tested for virulence in subcutaneous or intratracheal infections in a murine model of infection. When mutants were inoculated intratracheally into DBA/2 mice any receptor, except for GerX, was sufficient to stimulate germination and allow for a fully virulent infection. In contrast, when inoculated subcutaneously only the GerH receptor was able act alone to facilitate a fully virulent infection. These results suggest that route of infection determines germinant receptor requirements. A mutant lacking all five germinant receptors was severely attenuated in both routes of infection. *In vivo* imaging of subcutaneous infection with the Δ*gerH* strain showed that spores must be able to germinate at the site of infection, without trafficking to a secondary body site, to cause disease. The GerH receptor was also necessary and sufficient for germination in a variety of mammalian bloods, as well as macrophages. Germination under these conditions could be inhibited

by either an L-amino acid or purine inhibitor, suggesting that both of these germinant classes were required for germination *in vivo*. Together, these data give us a greater understanding of the earliest moments of germination, and provide a more detailed picture of the signals required to stimulate this process in the host.

INTRODUCTION

Bacillus anthracis spore germination is an essential step in disease initiation. Anthrax can present in a variety of ways, depending upon route of infection, but the infectious particle is always the spore (Dixon *et al.*, 1999). The most severe of these presentations is inhalational anthrax. Upon entering the lungs of a host, spores are taken up into alveolar phagocytes, where they germinate and are transported to the mediastinal lymph nodes (Guidi-Rontani *et al.*, 1999b, Cleret *et al.*, 2007). *B. anthracis* spores detect germinants in their environment by the germinant receptors GerH, GerK, GerL, GerS, and/or GerX. As shown by the *in vitro* data in Chapter II, germination is dependent upon germinant recognition by one or more of these receptors (Ireland and Hanna, 2002a, Weiner and Hanna, 2003, Fisher and Hanna, 2005). Independent studies from our lab and others showed that disruption of the *gerH, gerS* or *gerX* operons resulted in decreased germination in macrophages in cell culture, implicating them in disease establishment (Guidi-Rontani *et al.*, 1999a, Ireland and Hanna, 2002b, Weiner and Hanna, 2003). In addition to inhalation anthrax, spores can also cause disease when introduced cutaneously or via the gastrointestinal tract, and phagocytic cells may also play a role in spore germination in these routes of infection (Dixon *et al.*, 1999). Upon germination in these cells, bacilli produce lethal toxin and edema toxin, facilitating escape from the trafficking macrophage (Guidi-Rontani *et al.*, 1999b). Once freed from these immune cells, bacilli disseminate systemically, causing disease, and potentially death.

Anthrax pathogenesis has been studied in various animal backgrounds, using multiple routes of inoculation. Murine models of infection have been well studied, and

two mouse strains have been classified as susceptible to infection with the *B. anthracis* Sterne strain, which lacks the pXO2 plasmid encoding the capsule biosynthesis genes (Sterne, 1939, Welkos *et al.*, 1986). Both the DBA/2 and A/J mouse strains are susceptible to anthrax, due to a similar defect in complement factor 5 (C5) production (Welkos and Friedlander, 1988). As mentioned, anthrax pathogenesis is also studied via a variety of routes of infection, including aerosol and intratracheal inoculations, which mimic an inhalation infection (Heffernan *et al.*, 2006, Loving *et al.*, 2007). Subcutaneous injection is also often used, which bypasses germination requirements that may be located in the airways of the animal (Welkos and Friedlander, 1988).

In this study, we sought to determine the contribution each germinant receptor makes to germination and, therefore, virulence following different routes of inoculation. We again used our isogenic quadruple mutants lacking all but one germinant receptor, as well as a null strain lacking all five functional germinant receptors. These *in vivo* studies were used to correlate virulence in mice with the *in vitro* data described in Chapter II, in an attempt to determine defined germinant specificities in a mammalian host. We also used an *in vivo* imaging system (IVIS) in order to compare differences in dissemination of these *B. anthracis* strains during subcutaneous infection. Additionally, germination of the receptor mutants was also tested in mammalian blood and macrophages. These represent two potential environments that the spore may encounter in the host. Understanding the germinant receptor requirements at these sites, as well as during murine infection, will allow for a more complete understanding of the specificities required to cause disease.

MATERIALS AND METHODS

Strains and culture conditions

Strains in addition to those described in Chapter II are listed in Table 3.1. Spores containing the bioluminescence cassette *luxCDABE* were prepared as previously described (Finlay *et al.*, 2002, Pickering and Merkel, 2004). Spores were stored at room temperature in sterile water and titered by counting phase-bright particles using a hemacytometer (spores/mL).

Table 3.1. *Bacillus anthracis* strains used in this study

Strain	Name	Relevant Characteristics[a]	Reference
$34F_2$	$34F_2$-*lux*	Wild-type ($pXO1^+$, $pXO2^-$) + *luxCDABE*	This work
SL110L	Δ*gerH-lux*	$34F_2$, Δ*gerH* + *luxCDABE*	This work
SL120L	ger_{null}-*lux*	$34F_2$, Δ*gerH* Δ*gerK* Δ*gerL* Δ*gerS* Δ*gerX* + *luxCDABE*	This work

[a]Mutants alleles consist of markerless deletions of nearly the entire tricistronic germinant receptor operon. The *luxCDABE* operon was introduced stably on the chromosome (see Methods).

Introduction of bioluminescent genes into *ger* mutants

Bioluminescent versions of the germinant receptor mutants were created in the laboratories of Tod Merkel and Scott Stibitz at the Center for Biologics Evaluation and Research at the Food and Drug Administration, as previously described (Loving *et al.*, 2007). Briefly, the plasmid pSS4530 was introduced into wild-type or mutant strains of *B. anthracis* via conjugation. This plasmid contains the *luxCDABE* operon from

Photorhabdus luminescens under control of a constitutive promoter under metabolically active conditions. Isolates were selected in which the plasmid had integrated into the chromosome behind GBAA 1951, an open reading frame driven by the constitutive P_{ntr} promoter. Isolates were serially passaged to obtain a highly luminescent strain (Figure 3.1). This method was used to create the bioluminescent strains WT-*lux*, Δ*gerH-lux*, and Δger_{null}-*lux* (Table 3.1).

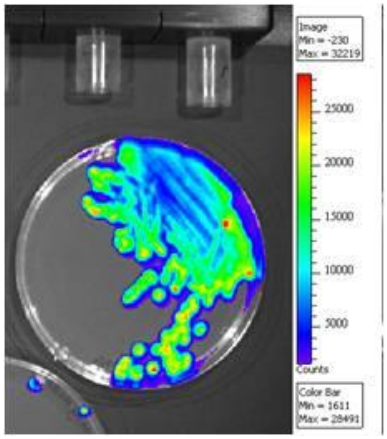

Figure 3.1. Bioluminescent bacteria. Vegetative wild-type bacilli on a rich media BHI plate. Image was obtained using the IVIS 100 imaging system (Xenogen) with a five second exposure time.

Germination assays

Germination was measured by loss of heat resistance after exposure to germinants, as described in Chapter II. Blood germination assays were performed in whole mouse blood treated with the anti-coagulant EDTA (Lampire), defibrinated whole sheep blood (Lampire), defibrinated whole rabbit blood (Lampire), and defibrinated whole cow blood (Hemostat). Experiments were started with $5x10^4$ spores, and then diluted 1:10 in PBS before heat treatment to prevent clotting. Samples were incubated at

37°C for 30 minutes, then heat treated at 65°C for 20 minutes, and plated on BHI. Plates were incubated overnight at 37°C and colonies were counted. Standard error of the mean (SEM) was calculated as (standard deviation/√n).

For inhibitor studies, 10 mM D-alanine or 10 mM 6-Thioguanosine (6-TG) were added to either a defined germinant mixture (as denoted in Figures 3.7 and 3.8) or cow serum isolated from whole cow blood (Hemostat). 6-TG was only soluble at pH 10, and serum pH was adjusted for this. Serum was isolated by centrifuging blood in an Eppendorf 5810R tabletop centrifuged at 4° C for 20 minutes at 2800 rpm. The serum fraction was transferred to fresh centrifuge tubes and centrifuged at 4° C for an additional 20 minutes at 18,000 rpm in a Sorvall RC5B Plus centrifuge. Pure serum was removed and used to assay germination by measuring loss of heat resistance.

Macrophage germination

RAW 264.7 cells were cultured in media containing D-MEM, 10% FBS, 1x Glutamax (Gibco), and 25 mM HEPES (Gibco) at 37° C with 5% CO_2. To assay germination, cells were harvested, washed once in phosphate buffered saline pH 7.4 (Gibco) and titered using Trypan blue staining and counting with a hemacytometer. $5x10^4$ macrophages resuspended in infection media, consisting of MEM, 1x Glutamax, and 25 mM HEPES, were added to the wells of a polystyrene 96-well plate. Spores were added to wells at an MOI of 10. Plates were incubated at 37°C with 5% CO_2 for 30 minutes to allow for cell adherence, spore uptake, and germination. Following incubation, wells were washed once with warm PBS and then incubated with 0.5 mM alanine, 1 mM inosine, and 5 μg/ mL gentamycin for 20 minutes in order to germinate

and kill any remaining extracellular spores. Wells were washed three times in warm PBS to remove the antibiotic/germinant mixture. Macrophages were lysed with 2% saponin in infection media with 2 mM D-alanine to prevent germination of spores upon lysis. Lysates were serially diluted and plated to determine total number of spores that infected the macrophages. Samples were also heat treated at 65°C for 20 minutes, then plated to determine the number of ungerminated spores. For germination inhibition studies, either 10 mM 6-thioguanosine (pH 10) or 2 mM D-alanine were added to infection media. Macrophages were preincubated in the inhibitor for 10 minutes to allow for uptake into the cells. Spores were then added and germination was assayed as described above.

Murine Challenges

Intratracheal infections of six week old female DBA/2 mice (Jackson Laboratories) were performed as previously described (Heffernan *et al.*, 2006). Groups of eight mice were infected with either mutant or wild-type spores at a variety of doses ranging from $1.5x10^3$ to $1.5x10^7$ spores per mouse. Mice were monitored for disease for a period of 14 days. For subcutaneous infections, mice were infected in the scruff of the neck with doses ranging between $5x10^3$ and $5x10^6$ spores per mouse. LD_{50} values were calculated using the methods of Reed and Muench (Reed and Muench, 1938). All mouse experiments were performed using protocols approved by the University of Michigan University Committee on the Use and Care of Animals.

IVIS Based Murine Challenges

Subcutaneous challenges were performed as described above. Briefly, groups of A/J or DBA/2 (National Cancer Institute) mice were injected with $5x10^4$ spores in the scruff of the neck. Mice were monitored for a period of 10 days. All mice for these studies were housed and maintained at the Center for Biologics Evaluation and Research animal facility at the Food and Drug Administration under the approval of the Institutional Animal Care and Use Committee.

Imaging of mice was performed using the IVIS 100 *in vivo* imaging system (Xenogen). Mice were anesthetized with 2.5% isoflorane mixed with oxygen using the XGI-8 gas anesthesia system supplied with the IVIS 100 (Xenogen). Images were analyzed using Living Image 2.5 software (Xenogen). Images were acquired at various time points post infection, as noted in the text.

RESULTS

Virulence of germinant receptor mutants in mice

As the spore's ability to germinate upon entering a potential host is essential for disease, we sought to determine the requirement for specific germinant receptors in different inoculation routes in murine models of anthrax infection. Two separate sites of inoculation were used, intratracheal injection to simulate an inhalational method of infection, and subcutaneous injection that bypasses uptake through the lungs. Six week old female DBA/2 mice were used, due to their susceptibility to the $34F_2$ Sterne strain of *B. anthracis* (Sterne, 1939, Harvill *et al.*, 2005). Results from these infections, and how disruption of individual germinant receptors impacted virulence, are described below.

In the *in vitro* studies described in Chapter II, the *gerS*$^+$ strain, containing only the GerS receptor, was unable to germinate fully in any of the germinant mixes tested (Tables 2.2 and 2.4). It was somewhat surprising, therefore, that the *gerS*$^+$ strain was as virulent as wild-type spores when inoculated intratracheally, with all mice succumbing to infection by day four (Figure 3.2). Likewise, each of the *gerH*$^+$, *gerK*$^+$, and *gerL*$^+$ strains were sufficient to cause fully virulent infections (Figure 3.2). Together, these data suggest that any one of these receptors is sufficient for initiation of disease via an intratracheal route of infection, so long as one of them is present. Because of the level of virulence seen with each of these quadruple mutants, none of the single receptor mutants were tested.

As described in Chapter II, both the Δger_{null} and *gerX*$^+$ strains exhibited a severe, and nearly identical, germination defect in colony forming efficiency when compared to wild-type spores. When either of these strains was inoculated into mice intratracheally, a

strong attenuation was observed, with both strains causing only a single fatal infection through the duration of the experiment (Figure 3.2).

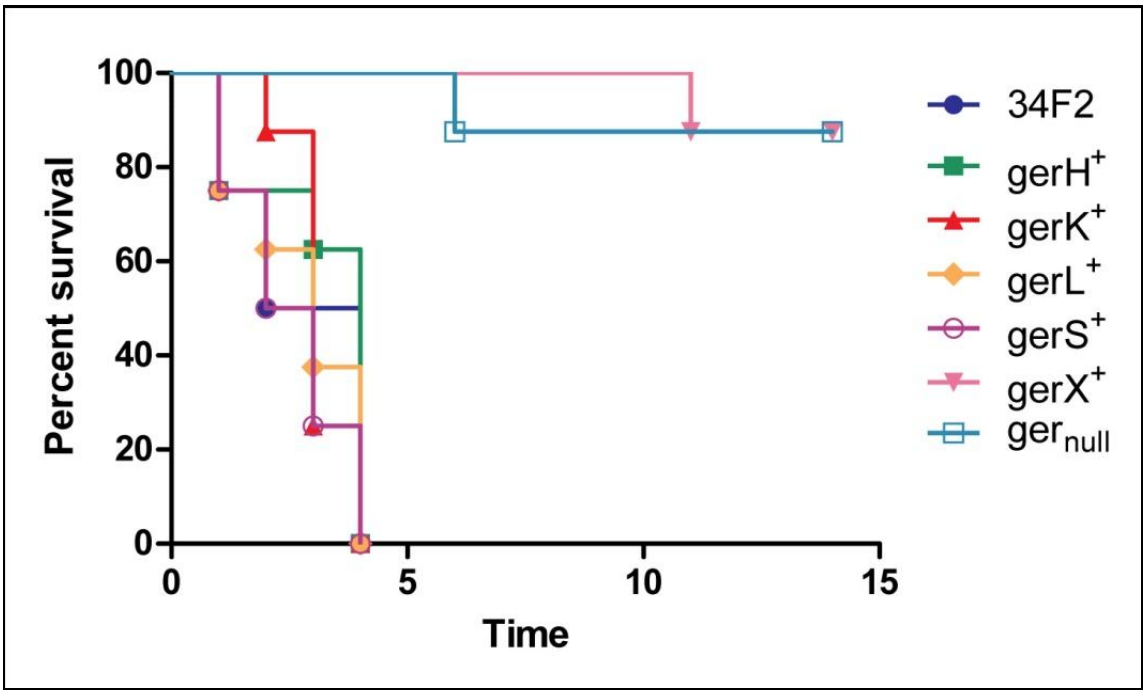

Figure 3.2. Virulence of germinant receptor mutants after intratracheal inoculation. DBA/2 mice were inoculated intratracheally with either the parent or mutant strain at a dose of $1.5x10^6$ spores/mouse. The initial group size was 8, and the percentage of each group surviving is shown over time. Survival curves of the *gerX*$^+$ and Δ*ger*$_{null}$ strains were statistically significant when compared to wild-type, with p=0.0001, as calculated by the log-rank (Mantel-Cox) test.

To further understand the severity of this attenuation, LD_{50} values were determined for the wild-type, *gerX*$^+$ and Δ*ger*$_{null}$ strains. Both mutant strains were greater than 700-fold attenuated when compared to wild-type (Table 3.2).

Table 3.2. The LD_{50} values of germinant receptor mutants.

	LD_{50} value[a]	
Strain	IT[b]	SubQ[b]
$34F_2$	1.50×10^4	1.03×10^4
Δ*gerH*	ND	3.52×10^4
gerS$^+$	ND	3.07×10^5
gerX$^+$	1.06×10^7	3.50×10^6
Δ*ger*$_{null}$	1.20×10^7	5.00×10^6

[a]LD_{50} values were calculated using the methods of Reed and Meunch (Reed and Muench, 1938) and were based on survival data from three groups of mice (n=8) at three different doses of each of the strains assayed; ND: not determined
[b]IT: Intratracheal inoculation; SubQ: Subcutaneous inoculation.

To examine germinant receptor requirements at different inoculation sites, mice were also inoculated with mutant or wild-type spores subcutaneously into the scruff of their necks. Animals inoculated with wild-type spores showed a median time to death of three days (Figure 3.3). Similar to the survival patterns seen above via intratracheal infections, the *gerX*$^{+}$ and Δ*ger*$_{null}$ strains were highly attenuated in this route of infection. LD_{50} values were also calculated for these mutants in this route of infection, and were found to be about 400 fold greater than wild-type (Table 3.2). It was previously reported that a Δ*gerX* mutant was attenuated in a subcutaneous infection (Guidi-Rontani *et al.*, 1999a). Our findings contradict that report, as our Δ*gerX* strain appeared just as lethal as wild-type (Figure 3.3A).

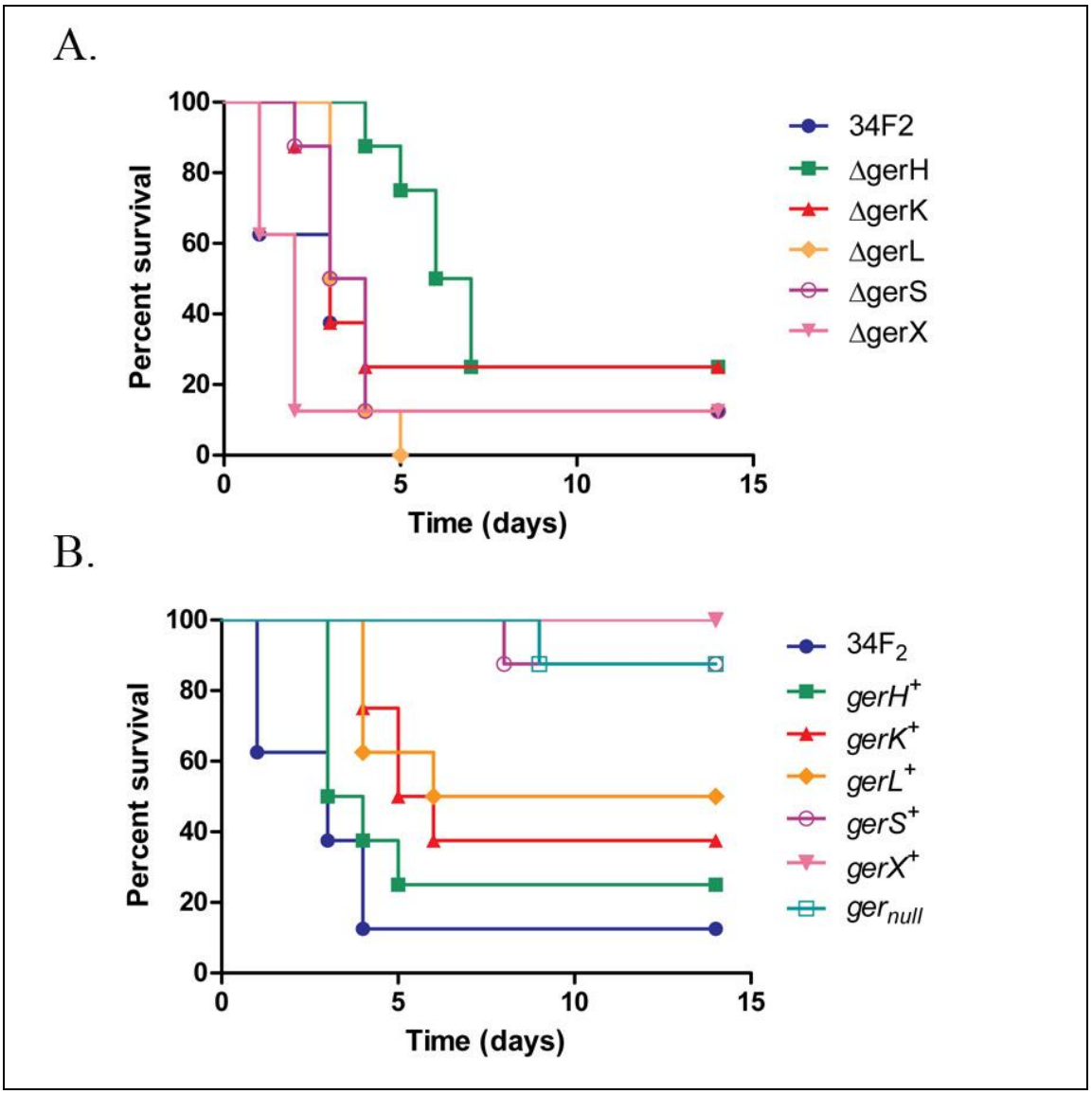

Figure 3.3. Virulence of germinant receptor mutants in mice infected subcutaneously.
DBA/2 mice were injected in the scruff of the neck with either the parent or mutant strain at a dose of $5x10^4$ spores/mouse (n=8). The percentage of each group surviving is shown over time. (A) Survival curves of the single mutants, lacking one receptor. The survival curve of the Δ*gerH* strain was statistically significant when compared to wild-type, with p=0.033, as calculated by the log-rank (Mantel-Cox) test. (B) Survival curves of the quadruple mutants expressing only one receptor. Survival curves of the $gerK^+$ and $gerL^+$ strains were statistically significant when compared to wild-type, with p=0.026 and p=0.018, respectively. The survival curves of $gerS^+$, $gerX^+$, and Δger_{null} strains were also statistically significant when compared to wild-type, with $p \leq 0.001$ for each.

Although intratracheal inoculations with the strains expressing only *gerH, gerK, gerL,* or *gerS* (*gerH*$^+$, *gerK*$^+$, *gerL*$^+$, or *gerS*$^+$, respectively) showed that all were as virulent as wild-type, by the subcutaneous route these same strains exhibited a wider array of virulence phenotypes (Figure 3.3B). By this route of infection the LD_{50} value of the *gerS*$^+$ strain was 30 fold higher than that of wild-type (Table 3.2). When the Δ*gerS* strain, lacking only *gerS,* was inoculated subcutaneously, it showed no attenuation when compared to wild-type, suggesting that GerS was not necessary at this site of infection (Figure 3.3A). Collectively these data indicate that the ability of the GerS receptor to stimulate germination, and thereby cause disease, is dependent upon the site of inoculation. This also suggests that the ligand specific for this receptor was not present at sufficient concentration to stimulate germination at the site of subcutaneous inoculation.

The *gerL*$^+$ strain, containing only the GerL receptor, showed moderate attenuation when inoculated subcutaneously, suggesting that having this receptor alone may be sufficient to trigger a low level of germination at this site of infection, but not enough to elicit a fully virulent infection (Figure 3.3B). Likewise, the *gerK*$^+$ strain also exhibited a slight attenuation when injected subcutaneously, with a significant delay in median time to death (five days), as compared to wild-type strain (three days) (Figure 3.3B). In contrast, the *gerH*$^+$ strain more closely resembled wild-type spores in terms of lethality, as well as median time to death. These data suggested that the GerH receptor alone is sufficient for the spore to be able to respond and germinate at this site of infection. GerH was also necessary for germination at this site, as the Δ*gerH* strain exhibited a significant delay in median time to death (six days) when compared to the wild-type strain (three days) (Figure 3.3A). In contrast, the other single receptor mutants, Δ*gerK,* Δ*gerL,* Δ*gerS,*

and Δ*gerX*, were all as virulent as wild-type, suggesting that these receptors were not required for germination, and ultimately virulence, in this route of infection (Figure 3.3A). Together, these data suggest that GerH is the primary receptor required for germination and establishment of disease in a subcutaneous route of infection.

In vivo imaging of DBA/2 and A/J mouse strains

It was hypothesized that the delay in median time to death exhibited by the Δ*gerH* strain could be due to inefficient germination at the site of inoculation (Figure 3.3A). Another possibility was that these spores may traffic upon inoculation until they find an *in vivo* environment hospitable for germination via one of the remaining four germinant receptors. To test these hypotheses, we used *in vivo* imaging to more precisely determine the site of germination encountered by this mutant. *In vivo* imaging allowed us to visualize the site of metabolic growth, and therefore germination, in an actively infected live animal. We used DBA/2 and A/J mice in these studies, two strains known to be susceptible to infection with *B. anthracis* spores (Welkos and Friedlander, 1988).

Bioluminescent strains of our germinant receptor mutants were generated by introducing the *luxCDABE* operon of *Photorhabdus luminescens* onto the chromosome of our strains (Figure 3.1) (see Materials and Methods). This technique has been used previously and was shown to have no impact on the virulence of wild-type *B. anthracis* strains (Loving *et al.*, 2007). Wild-type and Δ*gerH* strains expressing this bioluminescence cassette were inoculated into both DBA/2 and A/J mice subcutaneously (WT-*lux* and Δ*gerH-lux*, respectively). Dissemination (luminescence) and survival were monitored for a period of seven days.

WT-*lux* or Δ*gerH-lux* spores were inoculated into the scruff of the neck of DBA/2 mice and images were obtained. At 12 hours post infection, luminescence was clearly detected near the site of inoculation and appeared to be contained within punctate areas (Figure 3.4A). This luminescent signal expanded over the course of infection, as the bacterial load increased, but it remained within the same area of the mouse without disseminating systemically. Post mortem analysis was unable to detect bacteria in the spleen, lungs, or liver.

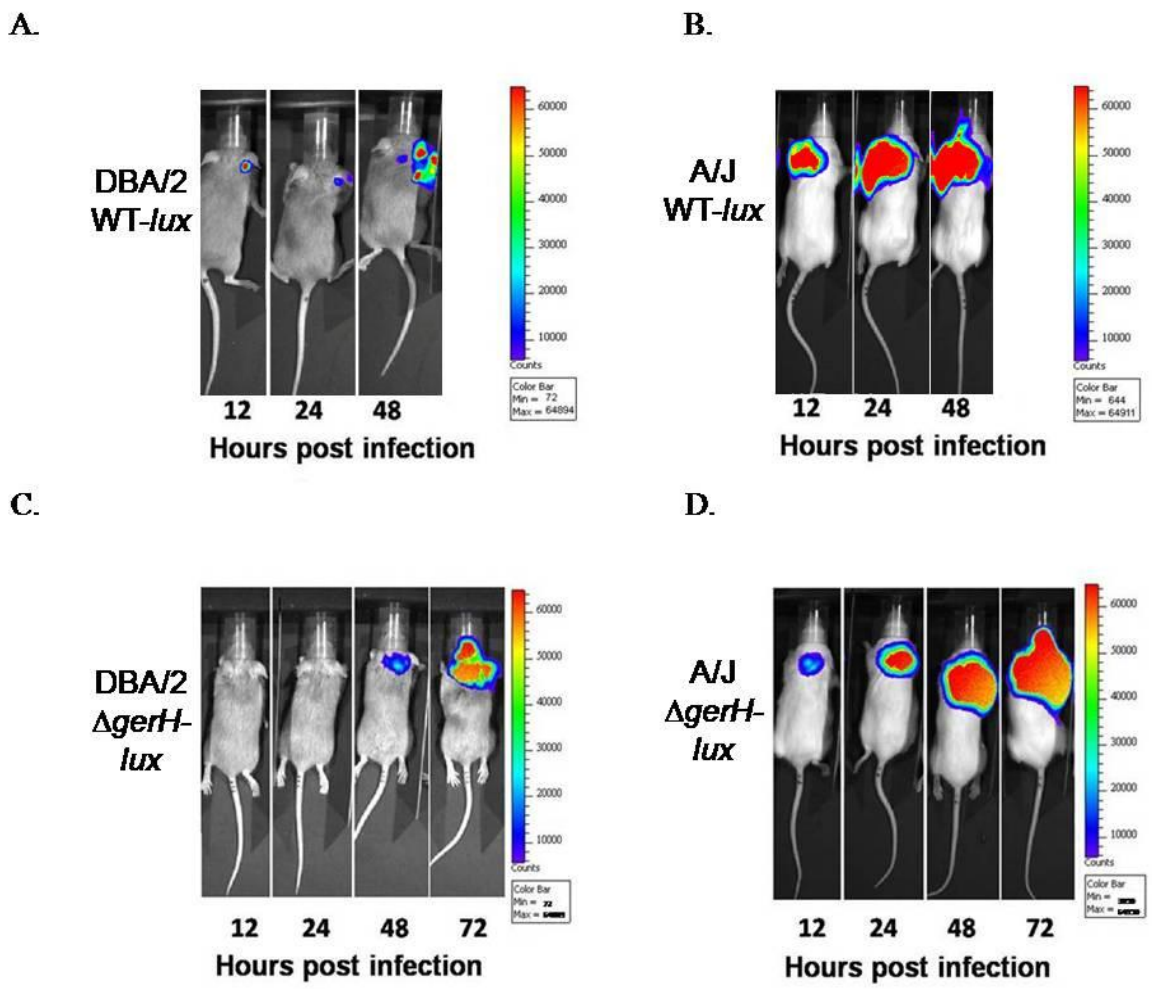

Figure 3.4. Dissemination of subcutaneously inoculated spores.
WT-*lux* (A and B) or Δ*gerH-lux* (C and D) spores were injected into the scruff of the neck in DBA/2 (A and C) and A/J (B and D) mice. Animals were imaged at the first 12 hours, then once per day afterward. Each panel shows disease progression of one representative animal. Images are shown up to the day the animal succumbed to infection.

In order to determine the germination site *in vivo* when the GerH receptor is absent, Δ*gerH-lux* spores were inoculated into DBA/2 mice. In stark contrast to the WT-*lux* infection, no luminescence signal could be detected until 48 hours post infection (Figure 3.4C). As with the WT-*lux* infection, the luminescent signal remained near the

injection site, without disseminating to organs or other sites of the body. This suggested that, although germination appears to occur much later in subcutaneously inoculated spores lacking the GerH receptor, these spores did not traffic away from the injection site in order to find germinants. Instead, a low level of germination at the site of infection was likely the cause of delayed disease presentation.

A/J mice are also classified as susceptible to $34F_2$ *B. anthracis* spores. Luminescent wild-type and Δ*gerH* spores were inoculated into these mice, and bacterial dissemination was monitored. Similar to DBA/2 mice, luminescence was detected at 12 hours in the A/J mice infected with WT-*lux* spores (Figure 3.4B). This signal appeared to be more diffuse than what was seen in the DBA/2 mice, which appeared more sequestered near the injection site. As disease progressed in the A/J strain, luminescence spread away from the inoculation site, covering the chest of the animals. A/J mice were also inoculated with the Δ*gerH-lux* strain. As with the DBA/2 infections, A/J mice infected with these spores exhibited delayed luminescence, but only at very early time points after infection. At the 12 hour time point, luminescence was detected near the injection site, but at a lower level than in the WT-*lux* infected animals (Figure 3.4D). Again, these data suggest that spores do not traffic after subcutaneous inoculation, but either germinate slowly, or germinate when appropriate signals are encountered near the injection site. These data also suggest that there may be some differences between susceptible DBA/2 and A/J mouse strains, when challenged with *B. anthracis* spores. Appendix I will further explore the differences between these strains, and attempt to understand what the basis for these may be.

Germination of receptor mutants in blood

The observation that the GerH receptor was required for full, rapid disease progression via subcutaneous infection led us to speculate that the GerH receptor was fully capable of responding to germinants at the site of inoculation, in the absence of other receptor family members. As spores inoculated subcutaneously may come in direct contact with blood, we tested whether the GerH receptor was capable of responding specifically to factors contained within blood. To test this, *in vitro* germination experiments were performed using whole mouse blood as the source of germinants. Loss of heat resistance was used as a measure of germination. After 30 minutes in mouse blood, 54% of wild-type spores became heat sensitive (Figure 3.5A). Likewise, the single receptor mutants that maintained a functional copy of *gerH*, specifically Δ*gerK*, Δ*gerL*, Δ*gerS*, and Δ*gerX*, all germinated as efficiently as wild-type spores. In contrast, the Δ*gerH* strain, which lacks only the *gerH* operon, was unable to germinate to any significant level after 30 minutes. Furthermore, the quadruple mutants expressing only one receptor, $gerK^+$, $gerL^+$, and $gerS^+$, were unable to germinate to a substantial degree. This finding was in contrast to the $gerH^+$ mutant that germinated nearly as well as wild-type spores, suggesting that not only is the GerH receptor necessary for germination in blood, but that it is also sufficient in the absence of all other receptors. It should be noted that the $gerX^+$ and Δger_{null} strains could not be tested in this assay, due to their severe colony forming defects (Figure 2.2) This assay was repeated in the presence of other mammalian bloods including sheep (Figure 3.5B), rabbit (Figure 3.5C), and cow (Figure 3.5D). The trend that the GerH receptor was both necessary and sufficient for germination was consistent in all mammalian bloods tested.

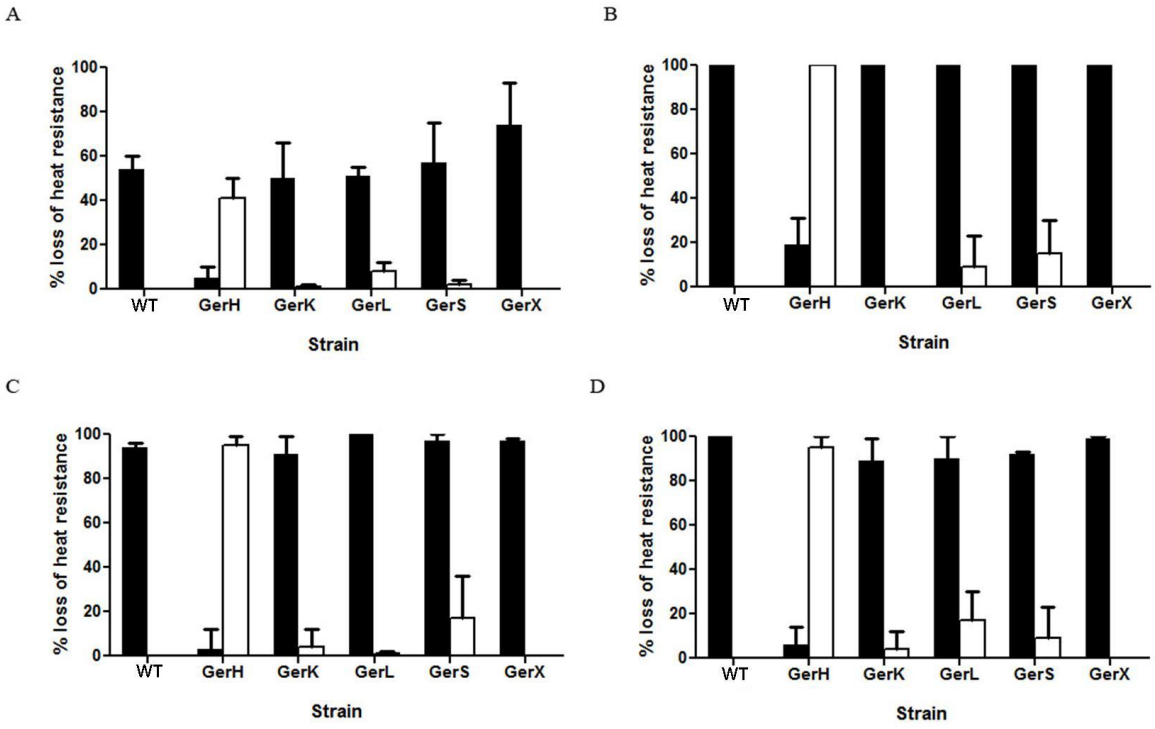

Figure 3.5. GerH is necessary and sufficient for germination in mammalian blood. Wild-type or mutant spores were incubated in whole (A) mouse, (B) sheep, (C) rabbit, or (D) cow blood at 37°C for 30 minutes, and germination was measured as a loss in heat resistance. Dark bars represent the Δger strains (lacking only one germinant receptor). White bars denotes the ger+ strains (encoding only one germinant receptor). Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean.

Germination in macrophages

As germination within the macrophage may be essential for disease initiation in some routes of infection, we sought to determine the role the germinant receptors of *B. anthracis* played in this environment. RAW 264.7 macrophages were used to test the

germination efficiency of our panel of germinant receptor mutants. Previous work in our lab, and others, examining germination of single receptor mutants of *gerH, gerS,* and *gerX*, found that each of these receptors were necessary for efficient germination within macrophages (Guidi-Rontani *et al.*, 1999a, Ireland and Hanna, 2002b, Weiner and Hanna, 2003). Indeed, germination was severely defective in Δ*gerH* spores in our experiments (Figure 3.6). Conversely, the *gerH*$^{+}$ strain germinated to near wild-type levels, suggesting that the GerH receptor is both necessary, and sufficient for germination in macrophages. Although previous reports suggested that GerS and GerX were important for germination, our data suggested the opposite. Our strains lacking either *gerS* or *gerX* both germinated to near wild-type levels, suggesting that neither receptor was essential for germination in macrophages. Similarly, the Δ*gerK* and Δ*gerL* spores also exhibited a strong germination response. Collectively, these data suggest that, as in blood, the GerH receptor is required for full germination in macrophages.

Unlike blood based germination, where GerH was essential for any germination to occur, macrophage-based germination appeared to be less stringent. This was exemplified by the fact that all receptor mutants germinated at least a small amount in this macrophage model. In contrast to blood based germination, where the *gerH*$^{+}$ strain was the only quadruple mutant that could germinate to a significant level, the quadruple mutants *gerK*$^{+}$, *gerL*$^{+}$, and *gerS*$^{+}$ were all able to germinate to at least a moderate degree in these cells. It should be noted, however, that neither the *gerX*$^{+}$ nor the Δ*ger*$_{null}$ strain could germinate at all in macrophages, again supporting the importance of functional germinant receptors in germination and virulence.

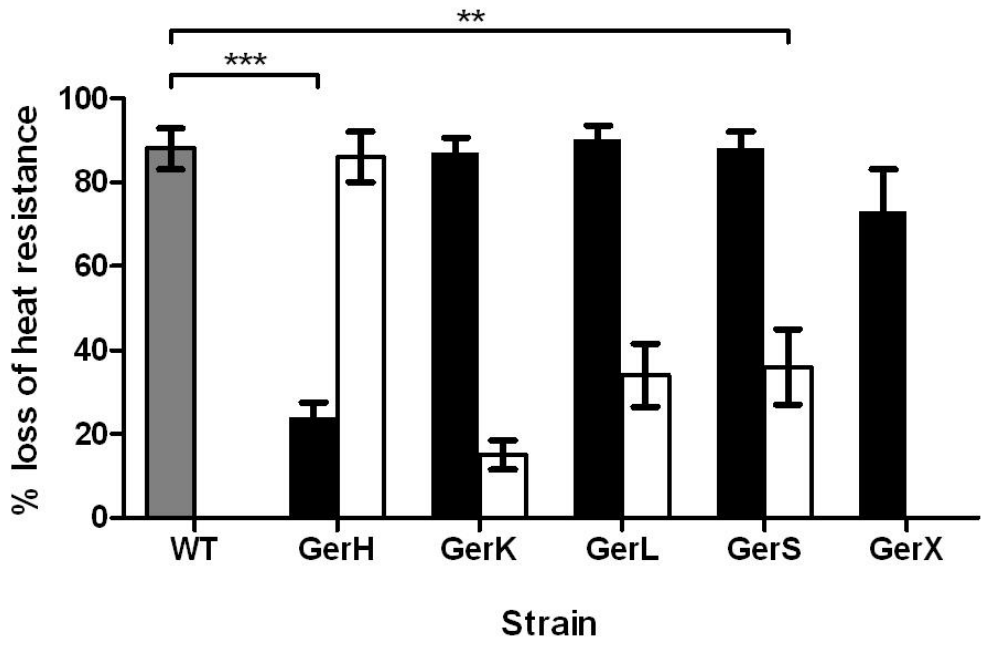

Figure 3.6. GerH is required for germination of *B. anthracis* spores in macrophages. RAW 264.7 macrophages were infected at an MOI 10 with wild-type or mutant spores for 30 minutes and germination was measured as a loss in heat resistance. Dark bars represent the Δ*ger* strains (missing only the germinant receptor indicated). White bars denote the *ger*$^{+}$ strains (expressing only the germinant receptor indicated). Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean. ***p<0.0001. **p=0.0018.

Inhibition of germination

Blood based germination: inhibition of L-amino acids

As germinant receptors play an essential role in blood based germination, we sought to identify components of mammalian blood that may mediate germination events. As GerH responds specifically to L-amino acids and purines, known inhibitors of these germinants were used to indirectly determine whether these germinant signals were present in blood. D-alanine has long been known to be a potent inhibitor of L-amino acid based germination (Fey *et al.*, 1964). Indeed, D-alanine effectively inhibited germination in all inosine/L-amino acid germinant combinations tested, as measured by loss of heat resistance (Figure 3.7). In order to determine whether L-amino acids were required for germination in blood, D-alanine was added to cow serum, and germination of wild-type spores was measured. Cow serum was used as the germinant, as it can germinate spores as efficiently as whole blood (Figures 3.5D and 3.7). D-alanine potently inhibited germination of spores in cow serum, suggesting that L-amino acids are essential for germination. D-alanine itself is able to stimulate a low level of germination, which may explain the residual germination seen in the presence of this inhibitor. It should be noted that several other D-amino acids were tested for the ability to inhibit germination, including D-histidine and D-tryptophan, but no inhibition was seen with these conditions (data not shown). The wide range of L-amino acid inhibition mediated by D-alanine precluded us from identifying specific amino acids present at sufficient amounts to stimulate germination.

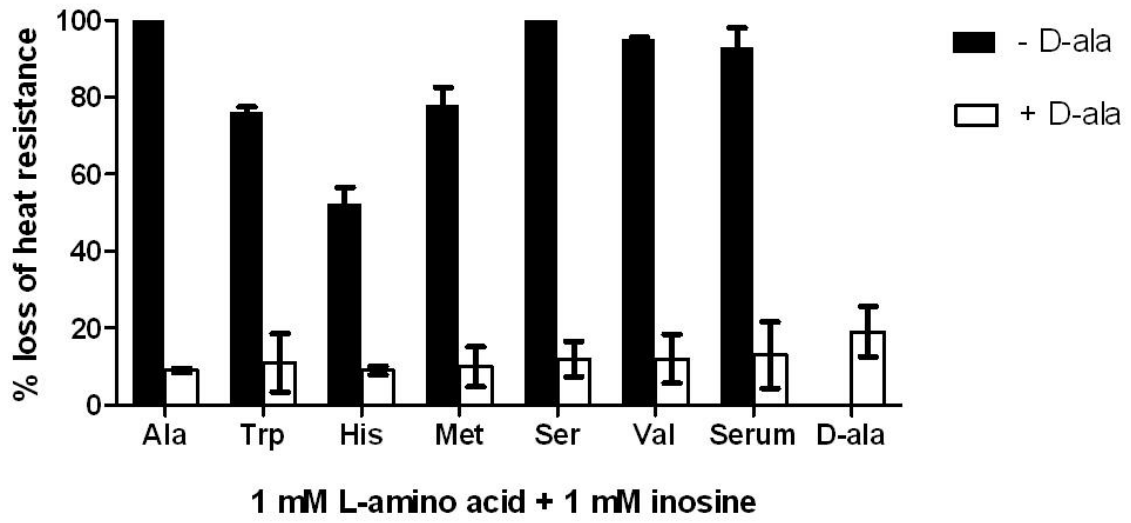

Figure 3.7. D-alanine can inhibit L-amino acid mediated germination in cow serum. Inhibition of L-amino acid mediated germination was achieved using 10 mM D-alanine. Wild-type spores were incubated in 1 mM inosine and 1 mM of the L-amino acid as indicated on the graph at 37°C for 30 minutes. Germination was measured as a loss in heat resistance. Cow serum was also used as a germinant. Dark bars represent germination without the inhibitor D-alanine. White bars represent germination in the presence of D-alanine. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean. $P \leq 0.0001$ in all instances.

Blood based germination: inhibition of purine nucleosides

As mentioned, germinant responses require a purine nucleoside as well as an L-amino acid. In the studies presented thus far, inosine has been used as the purine nucleoside, although adenosine and guanosine are also able to stimulate germination when paired with L-alanine (Weiner *et al.*, 2003). A previous study identified a purine nucleoside analog, 6-thioguanosine (6-TG), which could inhibit inosine-based germination of *B. anthracis* spores (Akoachere *et al.*, 2007). This analog was used in this

study to assay the contribution of purine nucleosides to germination in mammalian blood. When added to defined germinant mixes of purine/L-alanine, 6-TG specifically inhibited inosine based germination, while having little effect on the germination in the presence of adenosine or guanosine (Figure 3.8). When added to cow serum, 6-TG was also able to significantly inhibit the ability of spores to germinate, suggesting that purines are, indeed, important for germination in blood. Similar to D-alanine, 6-TG was able to stimulate a low level of germination itself, when in the presence of L-alanine. It should be noted that germinant mixes in this experiment were at pH 10, due to the solubility of 6-TG, contributing to the decreased level of germination seen in these experiments. Since germination inhibition with 6-TG appears to be specific for inosine, and since 6-TG was also able to inhibit germination in serum, these data suggest that a key germinant in mammalian serum may be inosine. This does not eliminate the possibility that other purines contribute to germination *in vivo*, but these data suggest that inosine plays a significant role.

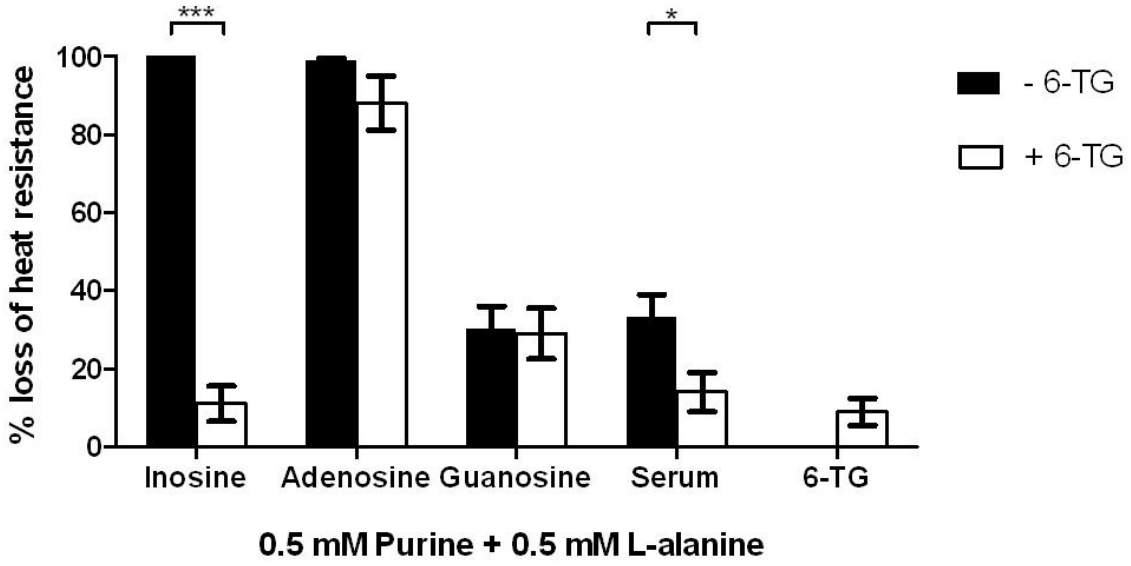

Figure 3.8. 6-Thioguanosine can inhibit inosine mediated germination in cow serum. Wild-type spores were incubated in 0.5 mM L-alanine and 0.5 mM purine nucleoside (as indicated on the graph) pH 10 at 37°C for 30 minutes and germination was measured as a loss in heat resistance. Cow serum was also used as a germinant. Dark bars represent germination without the inhibitor 6-TG. White bars represent germination in the presence of 10 mM 6-TG. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean. *P=0.023 ***P=0.0001

Germination inhibition in macrophages

Germination inhibition in macrophages was also tested using D-alanine and 6-TG as described above. As was the case in blood, these germinant analogs were able to significantly inhibit germination of wild-type spores in RAW 264.7 macrophages. Macrophages were preincubated with the inhibitors to allow for uptake into the cells before the addition of spores. Germination efficiency was cut to about 50%, when compared to untreated cells, in the presence of either D-alanine or 6-TG (Figure 3.9). 6-TG specifically inhibits inosine based germination, suggesting that inosine may be

important in macrophage germination. This is supported by the fact that the GerH receptor is necessary for wild-type level germination in these cells, and GerH is also required for inosine based germination (Figure 3.6 and Table 2.3). Experiments with 6-TG, including the untreated control group, were conducted at pH 10 to ensure solubility of the inhibitor. This did not appear to affect the viability of the macrophages. D-alanine has been used previously to inhibit germination in macrophages, and these data support those experiments (Hu *et al.*, 2007). Together these data suggest that L-amino acids and inosine are important for macrophage based germination of *B. anthracis* spores.

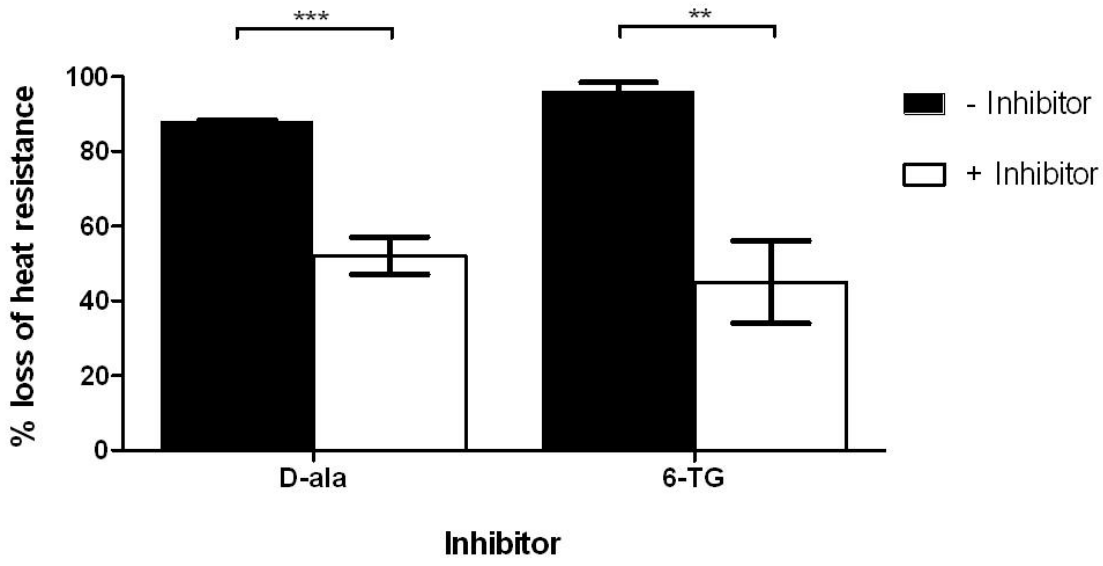

Figure 3. 9. Macrophage germination inhibition by D-alanine and 6-thioguanosine. RAW 264.7 macrophages were preincubated with either 2 mM D-alanine or 10 mM 6-thioguanosine for 10 minutes then infected at an MOI 10 with wild-type spores for 30 minutes. Germination was measured as a loss in heat resistance. Dark bars represent the untreated strains. White bars denote the treated strains. Experiments with 6-TG, including the untreated control, are at pH 10 to ensure solubility of the inhibitor. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean. ***p=0.0007. **p=0.004.

DISCUSSION

Germination of *B. anthracis* spores is an essential step in disease initiation. The germinant receptors described in this study function to detect germinants in mammalian hosts. These experiments aimed to better understand the role each receptor plays during an infection. We also sought to identify the germinant signals present at the site of infection that may stimulate the germination process.

The severe attenuation of the Δ*ger*$_{null}$ strain in the DBA/2 mouse model was consistent with the colony forming defect seen *in vitro*, as described in Chapter II. As expected, the 1,000-fold decrease in colony forming efficiency (Figure 2.2) translated into a 500-800 fold decrease in virulence in our DBA/2 mouse model, depending on route of infection (Figures 3.2 and 3.3B). The remaining low level of virulence seen with the Δ*ger*$_{null}$ strain was likely due to the corresponding low level of spontaneous germination events described *in vitro* (Paidhungat and Setlow, 2000). It is likely that a small fraction of receptor-deficient spores germinate after inoculation into the host, independent of the germinant receptors described in this work. Together, these data show that germinant receptors are an essential component for efficient, productive germination and directly contribute to virulence in our mouse model.

As mentioned in Chapter II, the *gerX*$^{+}$ mutant (expressing only the *gerX* receptor operon) exhibited the same phenotypes as the Δ*ger*$_{null}$ mutant in colony forming efficiency and germination (Figure 2.2 and Table 2.2). When this strain was tested in the DBA/2 mouse model, attenuation similar to the Δ*ger*$_{null}$ strain was observed. The attenuation seen *in vivo* with our *gerX*$^{+}$ strain differs from a previous report implicating GerX in virulence (Guidi-Rontani *et al.,* 1999a), but several experimental differences

between our study and the previous study may explain the disparity between our results. These include different parental *B. anthracis* strains ($34F_2$ *vs*. 7702), differences in mouse strains (DBA/2 *vs*. Swiss), mutagenesis methods (markerless deletion *vs*. spectinomycin resistance cassette insertion), and inoculation sites (scruff of neck *vs*. groin). Although a role for GerX remains to be elucidated, possible functions for GerX remain, including contributing to a cooperative germination response with one or more of the other receptors, inhibiting germination in unsuitable environments by acting as a check point for the presence of suitable germinants, or a role in germination in other host species yet to be tested. Further work is needed to better understand what, if any, role this receptor is playing, as its presence on a virulence plasmid leads to speculation of its importance during infection.

In contrast to GerX, the four remaining germinant receptors were fully responsive to germinants *in vivo*. The wild-type level of virulence seen upon intratracheal challenges of DBA/2 mice with the strains expressing only GerH, GerK, GerL, or GerS show that the presence of any one of these germinant receptors is sufficient to cause lethal disease in the intratracheal mouse model of infection. This suggests that the site of germination after an intratracheal inoculation contains many discrete germinant signals and is able, therefore, to fulfill the broad specificities of these different receptors. In contrast, only the *gerH*$^+$ strain (expressing only the GerH receptor) retained wild-type level virulence when inoculated subcutaneously (Figure 3.3B). This suggests that GerH plays a greater role than the other receptors in germination during a subcutaneous infection. Additional support for the importance of GerH comes from data showing that the Δ*gerH* strain was the only single mutant strain to exhibit any significant defect in

disease. It should be noted that the delay in the survival curve seen with this mutant did not substantially impact its LD_{50} value (Table 3.2). The delayed appearance of disease was likely due to germination triggered by one of the other four germinant receptors still present in the mutant.

These delayed germination events could be explained by two possible mechanisms; Perhaps, as the Δ*gerH* strain traveled through the body, it eventually found a site rich in a germinant that either the GerK, GerL, GerS, or GerX receptors recognized, stimulating germination, and ultimately resulting in disease. An equally like possibility exists that spores do not traffic at all, but instead lie dormant until natural circulation brings a germinant-containing body fluid or cell-type into contact, facilitating germination. Alternatively, spores may remain near the injection site and undergo an inefficient, yet constant, level of germination mediated by the germinant receptors still present, eventually leading to a sufficient number of vegetative bacilli to cause disease. This may be due to low levels of GerK, GerL, GerS, or GerX specific germinants at this site. In order to differentiate between these possibilities, *in vivo* imaging was used to visualize the germination and dissemination of bacilli in the host. Upon inoculation of Δ*gerH-lux* spores expressing the *luxCDABE* operon, it became clear that spores injected subcutaneously do not traffic from the injection site. When WT-*lux* spores were inoculated subcutaneously into DBA/2 mice, luminescence was detected within four hours. This luminescence signal stayed near the injection site, and appeared to traffic to regional lymph nodes shortly after detection. In contrast, the detection of luminescence was delayed by several days with Δ*gerH-lux* spores (Figures 3.4A and 3.4C). The pattern of luminescence that eventually appeared, however, mimicked that of WT-*lux*. This

luminescent signal, like that of wild-type, appeared only near the site of injection, suggesting that spores did not traffic until after germination. The trafficking seen post germination may have been due to the host immune response, and was likely not a passive migration of bacilli. This suggests that spores do not traffic to find germinants, and that circulation of *in vivo* fluids or cells, or inefficient germination mediated by the remaining receptors may explain the delay in median time to death seen.

In addition to DBA/2 mice, A/J mice were also inoculated with our luminescent spores. Δ*gerH-lux* spores also exhibited a delay in germination in this mouse strain, as the luminescent signal was much less intense than was seen in WT-*lux* spores at 12 hours post inoculation (Figures 3.4B and 3.4D). It should be noted, however, that the delay was much less prolonged than what was seen in the DBA/2 mice. Although both are classified as susceptible to infection with $34F_2$ spores, DBA/2 and A/J mice exhibited much different patterns of luminescence after infection, with bacilli appearing to traffic to lymph nodes in DBA/2 mice and spreading diffusely away from the injection site in A/J mice. Appendix I further investigates the differences between these mouse strains and provides evidence that A/J mice are actually more susceptible to anthrax than are DBA/2 mice. These data implicate additional innate immunity factors in host defenses against anthrax infection, than just the previously established complement component C5 (Harvill *et al.*, 2005).

Collectively, these data support the notion that different routes of infection require different germinant receptors for efficient, rapid germination and, therefore, full virulence. They also suggest that a specific ligand at the site of subcutaneous inoculation is able to rapidly, and specifically, trigger germination in a GerH-dependent manner. The

data from spores germinated in blood *in vitro* further support this hypothesis, as efficient germination, in all mammalian blood species tested, was dependent on the presence of GerH (Figure 3.5). Therefore, the GerH receptor is likely stimulated specifically by a component in the blood, resulting in the virulence seen in our mouse experiments. This may also be true of other species, as suggested by the *in vitro* analysis of the additional mammalian bloods, although this remains to be tested. The blood component that stimulates germination via the GerH receptor has yet to be definitively identified, but purine nucleosides and L-amino acids are likely involved as evidenced in this study, and others have implicated GerH in purine nucleoside based germination (Weiner *et al.*, 2003).

In an attempt to better characterize the germinants present in blood, a potential germination site *in vivo*, germinant inhibitors were used to identify blood components necessary for germination. An amino acid inhibitor, D-alanine, and a purine inhibitor, 6-TG, were both able to inhibit germination in serum (Figures 3.7 and 3.8). Of the purines tested, 6-TG was only able to efficiently inhibit germination with inosine, suggesting that inosine may be a key germinant in the host. In contrast, D-alanine inhibited germination with all L-amino acids tested. It is unclear why it was able to inhibit all these L-amino acids, but its small size and simple structure may allow it to interfere with the ability of a wide variety of germinants to effectively reach their receptors. The broad range of inhibition by D-alanine precludes us from identifying specific L-amino acids involved in germination *in vivo*. It is unlikely, however, that only one amino acid is responsible for the germination of *B. anthracis* spores, as blood is nutrient rich and likely contains many of the L-amino acids tested in this work. These data do allow us to speculate that

germination *in vivo* requires two germinants, an L-amino acid and a purine, similar to the germinant requirements we see with defined germinant mixes *in vitro*. Closer examination of the specific components of blood will likely narrow down the nutrients that spores may encounter in blood and other body fluids.

Our panel of germinant receptor mutants also allowed us to examine which receptors were important for germination in macrophages. In contrast to the staunch GerH requirement for germination in mammalian bloods, germinant specificities were less defined in RAW 264.7 macrophages. Although the *gerH*$^+$ strain was still the only quadruple mutant to exhibit wild-type level germination when expressed alone, the other quadruple mutants, *gerK*$^+$, *gerL*$^+$, and *gerS*$^+$, exhibited moderate amounts of germination as well (Figure 3.6). This low level of germination was similar to what was seen in the Δ*gerH* strain lacking the GerH receptor, suggesting that although GerH is important for wild-type level germination, the other receptors are capable of stimulating a small amount of germination. These data contradict earlier reports that GerS and GerX were also important for germination (Guidi-Rontani *et al.*, 1999a, Ireland and Hanna, 2002b). Different *B. anthracis* strains (34F$_2$ *vs.* 7702) as well as mutagenesis methods (deletion *vs.* insertion mutations) may explain the differences seen in the data presented here. Additionally, these data are consistent with the data from the intratracheal inoculations of DBA/2 mice, wherein the presence of any one germinant receptor was sufficient for full virulence (with the exception of GerX). This suggests that even the low level of germination supported by GerK, GerL, or GerS in macrophages, or possibly other phagocytes, is enough to initiate a full infection.

As with the blood based germination, we used two inhibitors, D-alanine and 6-TG, to identify potential germinants present in macrophages. These two inhibitors both effectively reduced germination of wild-type spores, but not to the extent that was seen in blood (Figure 3.9). This suggests that additional nutrient signals exist in macrophages that may facilitate germination, or that germinant concentrations were higher at this site leading to inefficient germination inhibition. These data are consistent with the observation that GerH, GerK, GerL, and GerS are all able to stimulate at least a low level of germination when expressed alone. These receptors have a wide range of germinant specificities and all germinants able to stimulate these receptors may not be inhibited by the two germinant analogs used in this study. Much more work remains in order to fully appreciate the germinants present at this important site of germination *in vivo*. An important first step is to fully characterize the prevalence of L-amino acids and purines in blood, macrophages, and other cell types and body fluids that spores may encounter after inoculation.

The ultimate goal of this study was to provide a more complete characterization of germinant/germinant receptor specificities in *B. anthracis.* Additionally, we determined which receptors were sufficient for germination *in vivo*, independent of any other kind of germinant receptor. The diversity of germinant/receptor interactions may play an important role in the ability of *B. anthracis* to exploit subtle chemical differences presented at a variety of anatomical sites within a host, allowing for the multiple routes by which disease can be established. Gaining a better understanding of how spores sense germinants in their environment and what those germinant specificities are may aid in the development of therapeutics, and allow for a better understanding of disease progression.

Chapter IV

Role of the *gerP* operon in germination and outgrowth of *Bacillus anthracis* spores

ABSTRACT

Germination of *Bacillus anthracis* spores occurs when nutrients, such as amino acids or purine nucleosides, stimulate specific germinant receptors located in the spore inner membrane. The $gerP_{ABCDEF}$ operon has been suggested to play a role in facilitating the interaction between germinants and their receptors in spores of *Bacillus subtilis* and *Bacillus cereus.* *B. anthracis* mutants containing deletions in each of the six genes belonging to the orthologue of the $gerP_{ABCDEF}$ operon, or containing a deletion of the entire operon, were tested for their ability to germinate. Deletion of the entire *gerP* operon resulted in a significant delay in germination in response to nutrient germinants. These spores eventually germinated to levels equivalent to wild-type, suggesting that an additional entry point for nutrient germinants exists. Deletions of each individual gene resulted in a similar phenotype, with the exception of Δ*gerPF* which showed no obvious defect. The removal of two additional *gerPF*-like orthologues was necessary to achieve the germination defect observed for the other mutants. Upon physical removal of the spore coat, the mutant lacking the full *gerP* operon no longer exhibited a germination defect, suggesting that the GerP proteins play a role in spore coat permeability. Additionally, each of the *gerP* mutants exhibited a severe defect in calcium-dipicolinic

acid (Ca-DPA) dependent germination, suggesting a role for the GerP proteins in this process. Collectively, these data implicate all GerP proteins in the early stages of spore germination.

INTRODUCTION

The gram positive bacterium *Bacillus anthracis* exists in two morphologically distinct forms, the metabolically active cell, and the dormant spore. Bacterial spores form in response to nutrient depletion as a means of protection until they enter into a hospitable environment (Piggot and Hilbert, 2004). At the center of the spore is the core which contains a high concentration of dipicolinic acid and its associated calcium ions (Ca-DPA) that helps to protect DNA from damage, and takes the place of water inside the spore (Setlow *et al.*, 2001). This dehydrated state is also essential for spore stability and contributes to the hallmark heat resistance that spores possess (Setlow, 1995). Outside the core lies the cortex, a layer of highly modified peptidoglycan which contributes to the resistance properties of the spore by maintaining a high level of dehydration in the core (Betty and Murrell, 1970, Gould, 1977, Popham, 2002). The cortex is surrounded by the proteinaceous spore coat, which provides a protective barrier, and also plays a role in the process of germination (Jenkinson *et al.*, 1980, Bagyan and Setlow, 2002, Klobutcher *et al.*, 2006, Henriques and Moran, 2007). The coat and cortex are both believed to be permeable to water, while still maintaining the spore's resistance to environmental stresses and various chemicals (Betty and Murrell, 1970, Gould, 1977). *B. anthracis* also has an additional outermost layer, the exosporium, which may contribute to adhesion to host cells and communication with the host environment (Todd *et al.*, 2003, Kang *et al.*, 2005, Bozue *et al.*, 2007, Brahmbhatt *et al.*, 2007, Henriques and Moran, 2007). The spore structure enables *B. anthracis* to survive dormant in the environment for years, until it encounters an appropriate mammalian host, germinates, and initiates disease. This process can also be triggered *in vitro* using chemical germinants. Although

dormancy can last for extended periods of time, the germination process, once initiated, is very rapid. This may contribute to the abilities of *B. anthracis* to multiply and spread quickly once spores enter a host (Dixon *et al.*, 1999).

The onset of germination is dependent upon appropriate germinant-receptor interactions. Germinants are typically amino acids and purines for *B. anthracis*, although other nutrient and non-nutrient related pathways exist, such as the stimulation of cortex lytic enzymes by exogenous Ca-DPA (Paidhungat and Setlow, 2000, Paidhungat *et al.*, 2001, Ireland and Hanna, 2002a, Weiner *et al.*, 2003, Fisher and Hanna, 2005).

Before germination can begin, germinants must somehow pass through the exterior layers of the spore to reach their receptors located in the inner membrane (Paidhungat and Setlow, 2001). Next, Ca-DPA and cations are released from the spore core. This efflux allows for water to flow back into the core and begin rehydration (Moir, 2003). Activated lytic enzymes hydrolyze the cortex peptidoglycan leading to full rehydration (Setlow *et al.*, 2001). The entire germination process can occur within minutes under optimal conditions.

Although the overall process of germination has been elucidated in a general sense, much remains to be understood, including how germinants are able to gain access to their receptors in the inner membrane. A six-gene operon, termed $gerP_{ABCDEF}$ (*gerP),* first identified in *Bacillus cereus* and *Bacillus subtilis*, may be involved in this process. The *gerP* operon is under control of a σ^K promoter, characteristic of spore coat related genes transcribed in the mother cell during sporulation (Behravan *et al.*, 2000). GerP proteins encoded within this operon are not predicted to have enzymatic activity, and yet *gerP* mutants have severe germination defects in other *Bacillus* species, suggesting that

they may play a structural role instead (Behravan *et al.*, 2000). The *B. anthracis* GerP proteins share an average of 97% amino acid identity to the GerP proteins of *B. cereus* (Read *et al.*, 2003, Rasko *et al.*, 2004) (Figure 4.1A). As *B. cereus* GerP gene products are predicted to play a role in coat structure or formation, and mutation of the operon results in a germination defect, it is possible that the GerP proteins help facilitate the interaction between germinants and their receptors, either by contributing to spore coat permeability, or directly facilitating the entry of germinants into the spore. Here we have created markerless deletions of the *gerP* operon, and each individual gene within the operon, in order to assess the role the GerP proteins play in the rapid germination of *B. anthracis* spores.

MATERIALS AND METHODS

Strains and culture conditions

The strains used in this study are listed in Table 4.1. Strains were cultured with brain heart infusion (BHI, Difco) broth or solid media containing 15g agar per liter. Strains were grown in modified G medium (Kim and Goepfert, 1974) for three days at 37° C with shaking, or for five days at room temperature for complementation, and spores were prepared as previously described (Passalacqua *et al.*, 2006). Spores were stored at room temperature in sterile water and titered by hemacytometer (spores/mL) or by colony forming units (cfu/mL), as indicated in the text.

Table 4.1. Strains used in this study

Strains	Mutant Name	Relevant Characteristics[a]	Reference
$34F_2$	-	Wild-type ($pXO1^+$, $pXO2^-$)	(Sterne, 1939)
KC101	Δ*gerPA*	$34F_2$, Δ*gerPA*	This work
KC102	Δ*gerPB*	$34F_2$, Δ*gerPB*	This work
KC103	Δ*gerPC*	$34F_2$, Δ*gerPC*	This work
KC104	Δ*gerPD*	$34F_2$, Δ*gerPD*	This work
KC105	Δ*gerPE*	$34F_2$, Δ*gerPE*	This work
KC106	Δ*gerPF*	$34F_2$, Δ*gerPF*	This work
KC107	$\Delta gerPF_{null}$	$34F_2$, Δ*gerPF* Δ*gerPF2* Δ*gerPF3*	This work
KC108	$\Delta gerP_{null}$	$34F_2$, $\Delta gerP_{ABCDEF}$	This work
KC109	Δ5052	$34F_2$, Δ5052	This work
KC110	Δ5053	$34F_2$, Δ5053	This work
KC111	Δ5054	$34F_2$, Δ5054	This work

[a]Mutants alleles consist of markerless deletions of nearly the entire tricistronic germinant receptor operon (see Methods).

gerP mutant construction

Each of the mutant strains used in this work were created using allelic exchange, resulting in markerless deletions (Figure 4.1A). Each mutant allele was designed to contain the first 10 codons of the target gene, a short insert sequence of three stop codons and restriction sites for the restriction endonucleases BamHI and SmaI, followed by the final 10 codons of the gene, including the putative stop codon. The constructs used to

create each mutant were isolated by PCR (primer sequences available upon request). In addition to the mutant allele described above, each PCR product contained approximately 500 bp of DNA sequence homologous to the upstream and downstream region of the *gerP* gene, flanked by the recognition sequence for the restriction endonuclease NotI. Each PCR product was cloned into the pCR®8/*GW*/*TOPO* vector (Invitrogen) according to manufacturer's instructions. The DNA sequence of each construct was verified to ensure no additional mutations were present due to PCR error. The NotI fragment was then cloned into the allelic exchange vector pBKJ258-kan. This vector was identical to the previously described pBKJ258 (Lee *et al.*, 2007), with the exception that a kanamycin resistance cassette was exchanged for the original erythromycin cassette. Allelic exchange was performed essentially as described previously (Janes and Stibitz, 2006). The $\Delta gerP_{null}$ mutant was isolated by fusing the first 10 codons of *gerPA* to the last 10 codons of *gerPF*, with stop codons and restriction sites inserted in between, as described above. All mutant alleles were verified using PCR, with primers designed to anneal outside of the sequences used for homologous recombination.

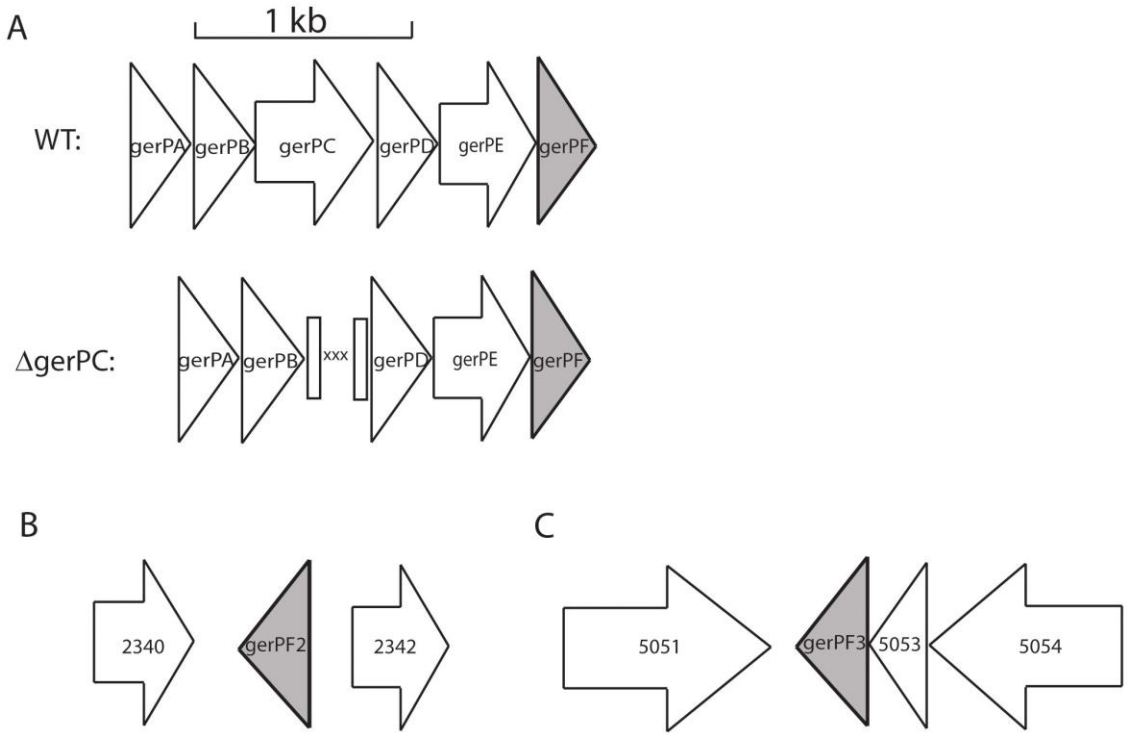

Figure 4. 1. The genes of the *gerP* operon and orthologues.
(A) Gene map of the *gerP* operon and example of construction of deletion mutants. Mutants were created by allelic exchange, removing the gene of interest (Δ*gerP*C shown here). For each deletion the first and last 30 nucleotides of the gene of interest were fused together, creating markerless deletions. Non-native sequences are denoted by an "X" above. (B) Gene map of the *gerPF* orthologue *gerPF2*. (C) Gene map of the *gerPF* orthologue *gerPF3*. *gerPF*- family genes are shown in gray.

For complementation studies, each gene was constructed under the expression of the native *gerP* promoter using PCR (primers available upon request). Each construct was moved into pBKJ401, a modified version of pBKJ258 containing sequence homology with the *B. anthracis* pXO1 virulence plasmid, and transferred into the mutant of choice via conjugation (Janes and Stibitz, 2006). Spores were produced by incubating at room temperature for 5 days, as the plasmid carrying the complementary allele was temperature sensitive. Wild-type spores prepared at room temperature showed no difference in germination ability when compared to wild-type spores prepared at 37°C, suggesting that this was a reasonable method of complementation (data not shown).

Germination assays

Germination was assayed by measuring the loss of heat resistance of spore suspensions after exposure to germinants. Spores were first heat activated by incubating at 65°C for 20 minutes. $5x10^3$ spores were mixed with 2 mL of germinant, either 0.5 mM L-alanine with 1 mM inosine in phosphate buffered saline (PBS) pH 7.4 (Gibco), or a solution of 60 mM $CaCl_2$ and 60 mM dipicolinic acid (pH 8, adjusted with NaOH) in water. Samples were vortexed very briefly and 50 μL were plated on a BHI plate in order to obtain the total number of spores in the sample. Samples were incubated at 37°C for 30 minutes. At 2, 5, 10, 15, 20, and 30 minutes, 200 μL aliquots were removed and heat treated at 65°C for 20 minutes, after which time 100 μL were plated on BHI. Plates were incubated overnight at 37°C and cfu/mL determined.

To assay decrease in optical density, heat activated spores (see above) were added to 400 μL of germinant, giving a starting OD_{600} of 0.3 using a Genesys 10UV spectrophotometer (Spectronic Unicam, Rochester, NY). The reaction was incubated at 37°C while shaking at 200 RPM. At 5 and 30 minutes the OD_{600} of the germinated spore mixture was measured. It has been previously established that a loss of 60-70% of the starting OD_{600} value corresponds with complete germination (Fisher and Hanna, 2005). In all cases the parental $34F_2$ Sterne strain was used as the positive control.

Growth experiments

Bacterial growth was measured in a 96 well plate in a Molecular Devices M2 plate reader. 100 μL of a non heat-activated $2.5x10^8$ spores/mL stock were added to 100 μL 2x BHI. Plates were secured with Parafilm and incubated with shaking for 10 hours, with OD_{600} being measured every 10 minutes.

Spore coat removal

Spore coats were removed using a method adapted from Brown *et al.* (Brown *et al.*, 1982). $2.5x10^5$ spores were added to 500 μL UDS buffer (5 mM 2-(*N*-cyclohexylamino) ethanesulfonic acid (CHES) buffer, pH 8.6, containing 8 M urea, 70 mM dithiothreitol, and 1% (wt/vol) sodium dodecyl sulfate (SDS)) and incubated at 37°C for 90 minutes to remove the spore coats. Treated spores were then washed 5 times with cold sterile water prior to heat activation. Loss of heat resistance was used as a measure of germination (see above). Coat removal was verified by testing sensitivity to lysozyme

mediated germination. Germination assays were performed as described above, using 100 μg/mL egg white lysozyme in 0.05 M Tris-HCl, pH 8 and 5 mM EDTA as a germinant. Coat-depleted spores exhibited the expected enhanced sensitivity to lysozyme (ranging from 40-70%), when compared to non-stripped spores (about 10%) (data not shown). Although full coat removal may not have been obtained, it was sufficient to test whether the germination defect could be alleviated with this technique.

RESULTS

Germination phenotypes of Δ*gerP* mutants.

In order to test the role of the GerP proteins during germination, markerless deletions were made in each of the six *gerP* genes, *gerPA-gerPF*, as well as a full operon deletion, denoted as Δ$gerP_{null}$ (Figure 4.1A). Two separate assays were performed to assess germination ability for each mutant in an L-alanine/inosine mixture. One assay measured the hallmark loss of heat resistance associated with the earliest moments of germination (Paidhungat and Setlow, 2002). The other assay measured the decreased optical density (OD) associated with a germinating spore suspension (Paidhungat and Setlow, 2002). Both assays are described in detail in the Methods section. Wild-type spores germinated rapidly, as measured by loss of heat resistance, reaching nearly full germination by five minutes (Figure 4.2). Δ$gerP_{null}$ spores, however, exhibited a significant delay in germination rates, especially at early time points during the assay (Figure 4.2, Table 4.2). While wild-type spores had already reached 92% heat sensitivity at two minutes, only 19% of the Δ$gerP_{null}$ spores were sensitive at the same time point. Over the course of the 30-minute assay, mutant spores germinated to a certain extent, but had still not reached 100%. Similar results were seen when germination was scored as a loss in OD_{600} (Table 4.3).

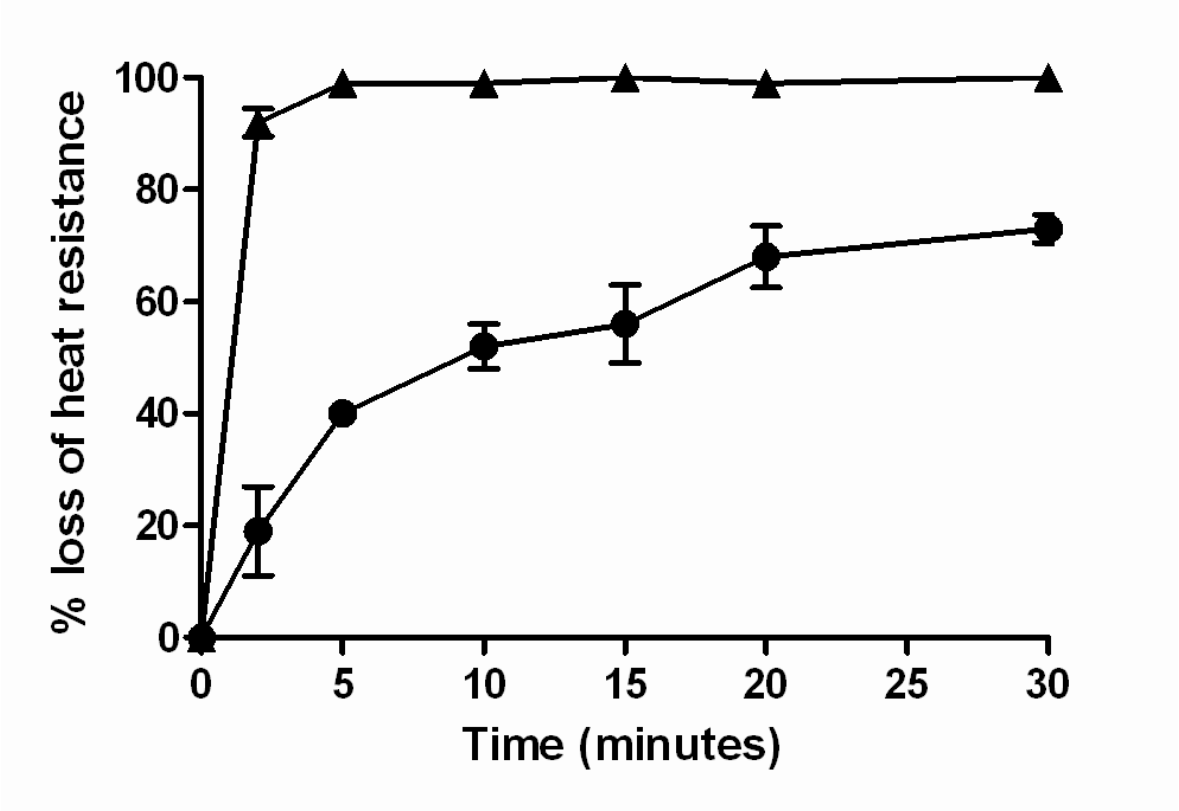

Figure 4. 2. The *gerP* operon is important for germination in L-alanine and inosine. Wild-type (▲) or Δ$gerP_{null}$ (●) spore suspensions were incubated in 0.5 mM L-alanine and 1 mM inosine at 37°C for 0-30 minutes, and germination was measured as a loss in heat resistance. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean.

Table 4.2. Loss of heat resistance of *gerP* mutants in L-alanine and inosine

	5 minutes[a]			30 minutes[a]		
Strain		+ $gerP_n$ [b]	+ $gerP_{ABCDEF}$ [c]		+ $gerP_n$ [b]	+ $gerP_{ABCDEF}$ [c]
34F2	99%			100%		
Δ*gerPA*	41%	77%	83%	70%	86%	93%
Δ*gerPB*	36%	80%	80%	62%	91%	93%
Δ*gerPC*	36%	83%	85%	62%	94%	88%
Δ*gerPD*	36%	56%	81%	72%	88%	95%
Δ*gerPE*	48%	77%	79%	71%	95%	89%
Δ*gerPF*	84%	ND	ND	97%	ND	ND
Δ$gerPF_{null}$	36%	88%	84%	73%	93%	92%
Δ$gerP_{null}$	40%		88	73%		91%

[a]Spores were incubated in 0.5 mM L-alanine and 1 mM inosine. Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤8% of the mean in all instances.

[b] +$gerP_n$ denotes a mutant complemented *in trans* with its respective *gerP* gene.

[c] +$gerP_{ABCDEF}$ denotes a mutant complemented *in trans* with the full *gerP* operon.

Table 4.3. Decrease in optical density of *gerP* mutants in L-alanine and inosine

	5 minutes[a]			30 minutes[a]		
Strain		+ $gerP_n$ [b]	+ $gerP_{ABCDEF}$ [c]		+ $gerP_n$ [b]	+ $gerP_{ABCDEF}$ [c]
34F2	38%			68%		
Δ*gerPA*	8%	30%	24%	18%	61%	59%
Δ*gerPB*	14%	32%	17%	45%	65%	55%
Δ*gerPC*	14%	36%	26%	52%	57%	56%
Δ*gerPD*	10%	23%	19%	45%	68%	58%
Δ*gerPE*	7%	32%	27%	28%	68%	58%
Δ$gerPF_{null}$	10%	28%	24%	31%	62%	59%
Δ$gerP_{null}$	8%		32%	29%		62%

[a]Spores were incubated in 0.5 mM L-alanine and 1 mM inosine. ~ 65% decrease in OD_{600} represents ~100% germination. Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤9% of the mean in all instances.

[b] +$gerP_n$ denotes a mutant complemented *in trans* with its respective *gerP* gene.

[c] +$gerP_{ABCDEF}$ denotes a mutant complemented *in trans* with the full *gerP* operon.

To better understand defects caused by deleting the *gerP* operon, growth curves were performed. Wild-type and mutant *B. anthracis* spores were tested for their ability to germinate, outgrow, and reach logarithmic growth in BHI medium. Spores were incubated in a 96-well plate with shaking overnight. Absorbance at OD_{600} was measured every 10 minutes in order to capture the characteristic loss in refractility during the first few minutes of germination (McCormick, 1965). In this germinant-rich environment, wild-type spores germinated and lost their refractility by 10 minutes, followed by outgrowth and exponential growth within one hour (Figure 4.3). Over the same period, $\Delta gerP_{null}$ *spores* exhibited no measurable decrease in optical density. They outgrew eventually and reached exponential growth, about 4 hours later than wild-type spores.

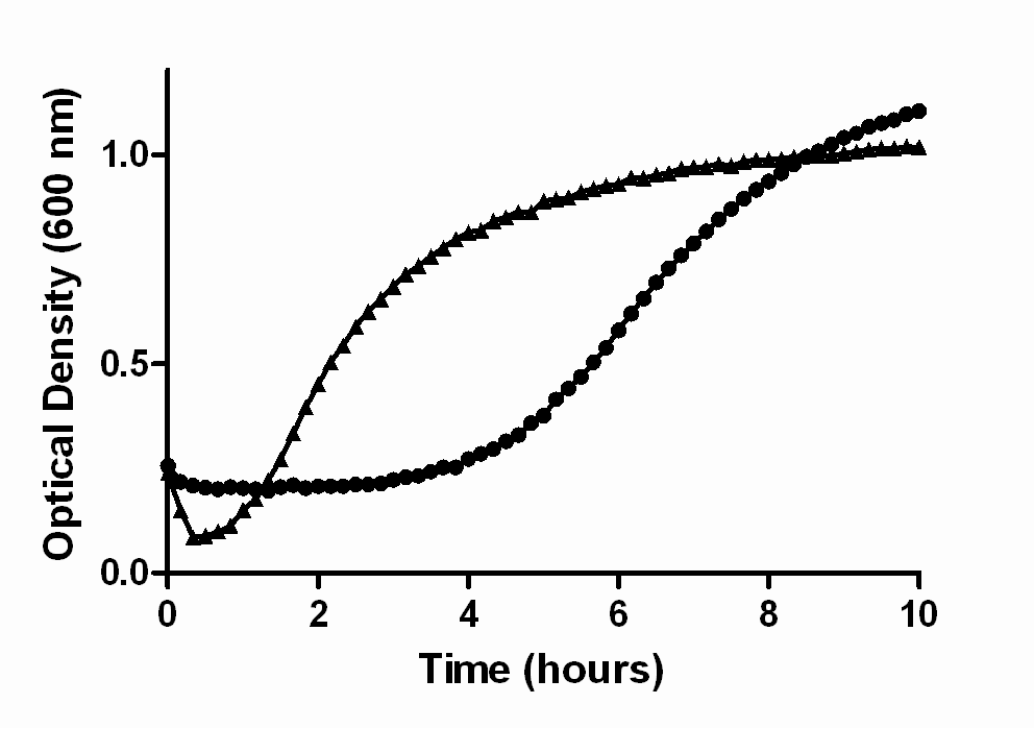

Figure 4. 3. Outgrowth phenotype of the $\Delta gerP_{null}$ mutant in rich media.
Wild-type (▲) or $\Delta gerP_{null}$ (●) spores in BHI. Spores were grown overnight at 37°C in a 96-well plate with shaking. Cultures were inoculated to a starting OD_{600} of 0.3. Data presented are representative of three independent experiments.

To further understand the role of the individual GerP proteins in germination, each of the individual *gerP* genes were removed, resulting in the strains Δ*gerPA,* Δ*gerPB,* Δ*gerPC,* Δ*gerPD,* Δ*gerPE,* and Δ*gerPF*. These mutants were also tested for the ability to germinate when exposed to L-alanine and inosine, as described above. All single deletion mutants exhibited a germination defect similar to the Δ$gerP_{null}$ strain, with the exception of the Δ*gerPF* strain, which behaved much more like wild-type (Tables 4.2 and 4.3). Analysis of the *B. anthracis* genome sequence identified two putative *gerPF* orthologues (Figures 4.1B and 4.1C) (Read *et al.*, 2003). These genes, GBAA 2341 (*gerPF2)* and GBAA 5052 (*gerPF3,* annotated as *gerPF-like),* had 79% and 59% nucleotide sequence identity and 95% and 52% amino acid similarity, respectively, to the *gerPF* gene located in the *gerP* operon. The two *gerPF* orthologues were deleted in the Δ*gerPF* background creating a triple *gerPF*-family mutant (Δ$gerPF_{null}$*)*. This triple mutant allowed us to better understand the role *gerPF* and its orthologues were playing during germination. Eliminating the contribution of all three *gerPF* orthologues resulted in a germination phenotype similar to those scored from other single *gerP* gene deletion mutants, suggesting that these three GerPF-family proteins may have overlapping functions (Tables 4.2 and 4.3).

Growth in BHI was also measured for the single gene deletion mutants Δ*gerPA,* Δ*gerPB,* Δ*gerPC,* Δ*gerPD,* Δ*gerPE,* and Δ$gerPF_{null}$. Delays into logarithmic growth for these mutants, when compared to wild-type, ranged between one and four hours (Figure 4.4). Though the severity of the growth delay varied from mutant to mutant, none of the single mutants exhibited the initial drop in OD_{600} characteristic of wild-type germinating

spores. These data further support that each of the *gerP* genes is important for proper timing of spore germination and outgrowth.

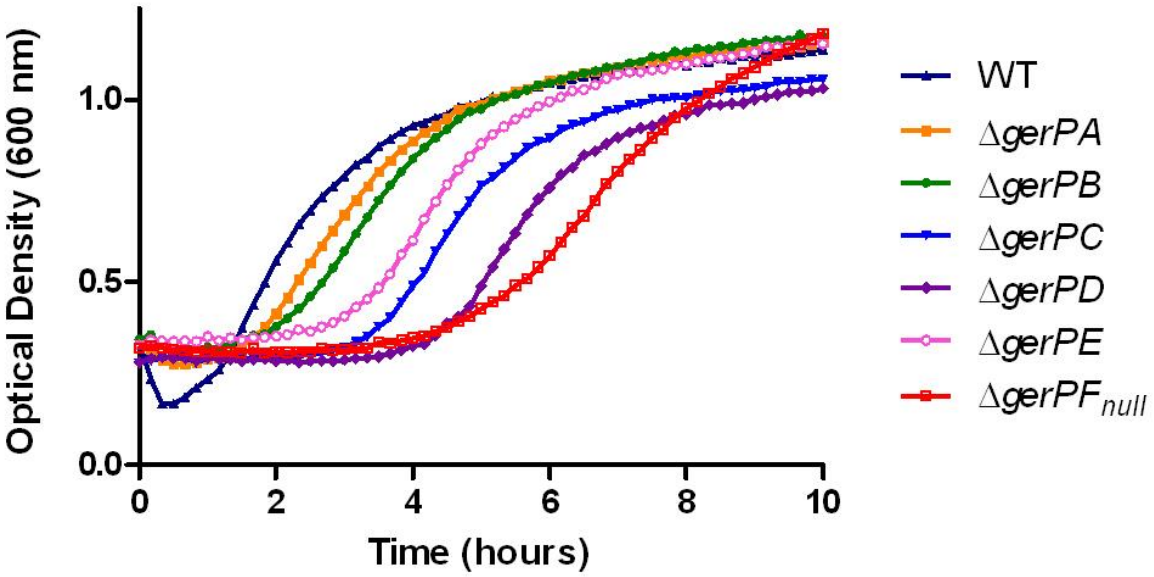

Figure 4. 4. The *gerP* genes are all important for rapid germination and outgrowth. Wild-type (▲) or Δ*gerP* single mutant spores (as noted) in BHI. Spores were grown overnight at 37°C in a 96-well plate with shaking. Cultures were inoculated to a starting OD_{600} of 0.3. Data presented are representative of three independent experiments.

Complementation of mutants was achieved *in trans* by placing a wild-type copy of the gene on plasmid pBKJ401, under control of the native *gerP* promoter (see Methods). The construct was then introduced into the mutant strain of interest. Complemented strains were tested for their ability to germinate in L-alanine and inosine. All of the mutants (Δ*gerPA*, Δ*gerPB*, Δ*gerPC*, Δ*gerPD*, Δ*gerPE*, Δ$gerPF_{null}$, and Δ$gerP_{null}$) showed an increased ability to germinate when the deleted gene was supplied

in trans (Tables 4.2 and 4.3). Additionally, a construct that carried the full length *gerP* operon was introduced into each mutant strain. Complementation with the full *gerP* operon was no better than complementation with the relevant individual gene (as measured by either germination assay: Tables 4.2 and 4.3), suggesting that the generation of these Δ*gerP* single mutants did not result in unintended polar effects. Collectively, these data suggest that each of the GerP proteins play a role in the process of germination, and that each appear essential to ensure rapid, wild-type level germination responses, as measured by these assays.

Germination behavior of Δ*gerP* mutant spores after coat removal

As initially reported by Behravan *et al.*, *B. cereus* GerP proteins were suggested be involved in spore coat structure and/or formation (Behravan *et al.*, 2000). It is hypothesized that GerP proteins facilitate trans-coat transport of germinants and, therefore, that disruption of these proteins may impede this. Chemical removal of the selective spore coat layer in *gerP* mutant strains of *B. subtilis* and *B. cereus* resulted in an increased ability of these spores to germinate (Behravan *et al.* 2000). In our study, wild-type and mutant spores were chemically treated to remove their spore coats, and tested for the ability to germinate in L-alanine/inosine. It should be noted that wild-type spores exhibited a reduced ability to germinate after the treatment to remove coats, suggesting that the treatment itself may inhibit a subpopulation from germinating (Figure 4.5). However, Δ$gerP_{null}$ spores that were treated for coat removal germinated more rapidly than untreated Δ$gerP_{null}$ spores, especially at the early time points of this assay. In fact, treated Δ$gerP_{null}$ spores mimicked the germination profile of treated wild-type spores.

The single gene deletion mutants in each of the first four genes of the operon, Δ*gerPA-gerPD,* also exhibited an increased ability to germinate after treatment, while Δ*gerPE* and Δ$gerPF_{null}$ did not show as much of an improvement (Table 4.4). These data suggest that at least some of the GerP proteins are important in spore coat permeability, and that their proper association with the coat is important for rapid spore germination.

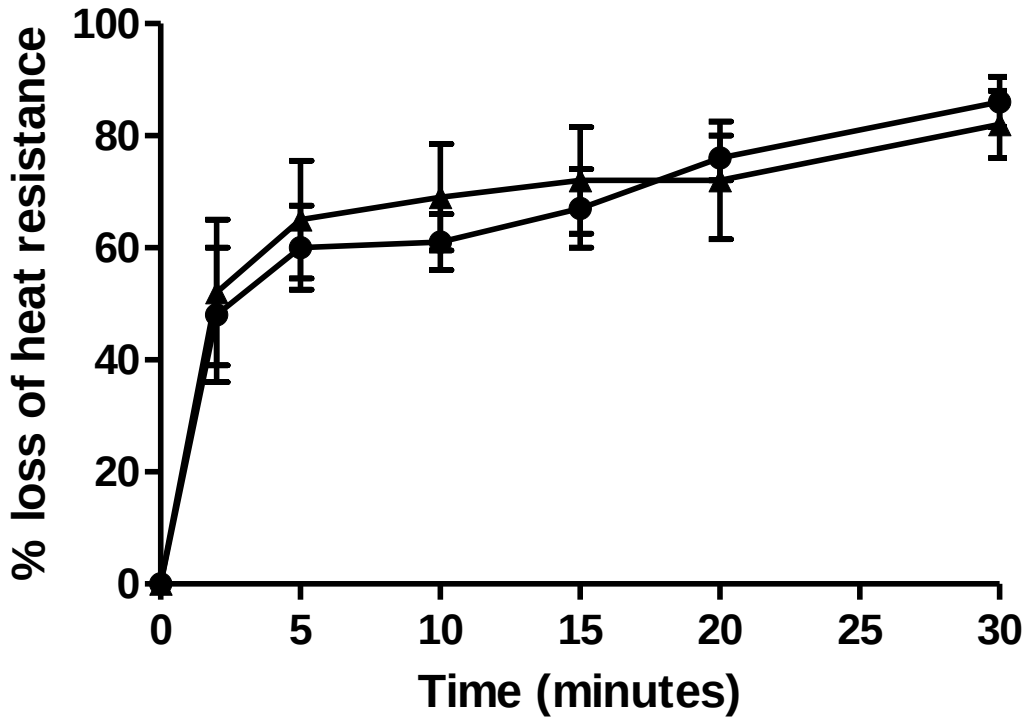

Figure 4. 5. Germination behavior of spores after removal of spore coats.
Wild-type (▲) or Δ$gerP_{null}$ (●) spores were treated in UDS in order to remove the spore coat. Germination was then assessed in 0.5 mM L-alanine and 1 mM inosine at 37°C for 0-30 minutes. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean.

Table 4.4. Loss of heat resistance of Δ*gerP* mutants after removal of spore coats.

Strain	2 minutes[a]	30 minutes[a]
34F2	52%	82%
Δ*gerPA*	56%	76%
Δ*gerPB*	48%	73%
Δ*gerPC*	48%	75%
Δ*gerPD*	47%	71%
Δ*gerPE*	40%	75%
$\Delta gerPF_{null}$	33%	82%

[a]Spores were incubated in 0.5 mM L-alanine and 1 mM inosine. Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤11% of the mean in all instances.

Germination in Ca-DPA

Exogenous Ca-DPA bypasses the need for germinant receptor stimulated germination of *B. subtilis* by directly activating the cortex lytic enzyme CwlJ. Active CwlJ degrades the spore cortex, allowing full germination (Paidhungat *et al.*, 2001). Wild-type *B. anthracis* spores readily germinated in the presence of Ca-DPA (Figure 4.6). The $\Delta gerP_{null}$ strain, however, exhibited a germination defect in this solution with only a 30% loss of heat resistant spores after 30 minutes (Figure 4.6). This defect was not just due to a slowed rate of germination, in contrast to the defect seen in amino acid germinants, as $\Delta gerP_{null}$ spores never fully germinated after exposure to the Ca-DPA.

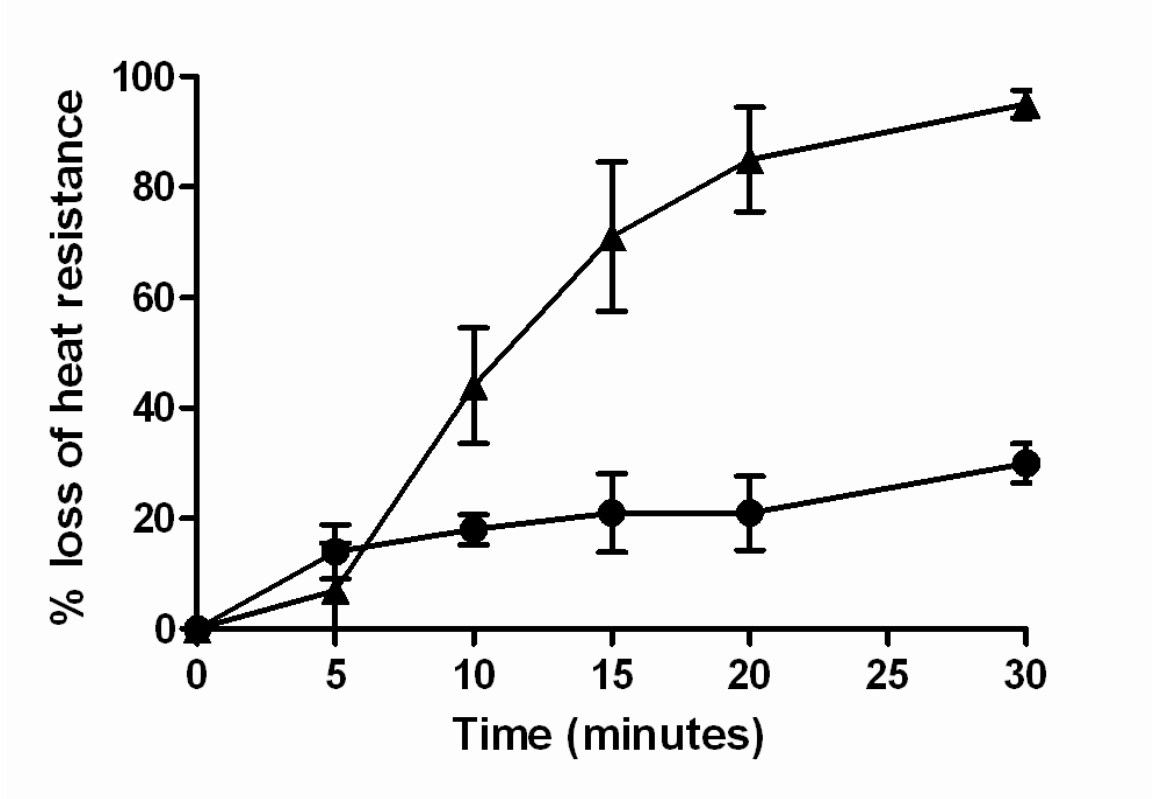

Figure 4. 6. Germination of the Δ*gerP*$_{null}$ mutant in response to Ca-DPA.
Wild-type (▲) or Δ*gerP*$_{null}$ (●) spore suspensions were incubated in 60 mM Ca-DPA at 37°C for 0-30 minutes, and germination was measured as a loss in heat resistance. Data presented are mean values of 4 independent experiments with 2 different spore preparations. Error bars represent ± standard error of the mean.

All of the mutants lacking a single *gerP* gene (or lacking the three *gerPF* family members) also exhibited this germination defect when compared to wild-type (Table 4.5). These data suggest that each of the *gerP* genes is required for Ca-DPA-mediated germination. They also suggest that, in addition to facilitating access of germinants to their receptors, the GerP proteins may be playing a different, and more essential, role in germination with exogenous Ca-DPA.

Table 4.5. Loss of heat resistance of Δ*gerP* mutants in Ca-DPA

Strain	% heat sensitive[a]
Wild-type	95%
Δ*gerP* $_{null}$	30%
Δ*gerPA*	19%
Δ*gerPB*	20%
Δ*gerPC*	20%
Δ*gerPD*	30%
Δ*gerPE*	35%
Δ*gerPF*$_{null}$	44%
Δ5052	99%
Δ5053	92%
Δ5054	70%

[a]Spores were incubated in 60 mM Ca-DPA for 30 minutes at 37° C. Results are the average of four experiments with two independent spore preparations. Standard error of the mean was ≤12% of the mean in all instances.

As mentioned above, the *B. anthracis* genome encodes for three *gerPF* orthologues. One of these, *gerPF3* (GBAA5052), is predicted to be in an operon with the genes GBAA5053 and GBAA5054 (Figure 4.1C) (Bergman *et al.*, 2007). Interestingly, gene GBAA5054 has a putative Excalibur calcium-binding domain and multiple S-layer homology (SLH) domains, which are typically anchored to the cell surface (Mesnage *et al.*, 1999, Daniel *et al.*, 2003). Gene expression of GBAA5054 is upregulated late during vegetative growth, and may be present in mature spores (Bergman *et al.*, 2006). The

presence of this calcium-binding protein in an operon with a *gerP*-like gene orthologue was intriguing, and led to speculation that it may play a role in Ca-DPA mediated germination. Markerless deletions of the genes GBAA5052 (the *gerPF* orthologue), GBAA5053, and GBAA5054 were made and each strain was tested for the ability to germinate. Δ5054 germinated to wild-type levels in L-alanine and inosine, suggesting that this gene does not play a role in nutrient based germination (data not shown). When tested for germination in Ca-DPA, however, this mutant showed a minor germination defect (Table 4.5). Although not as severe as the defect seen with the Δ*gerP* strains, it was significant (p=0.0097), suggesting that GBAA5054 may, indeed contribute to Ca-DPA mediated germination. Neither Δ5052 nor Δ5053 had a defect in Ca-DPA or nutrient germinants (Table 4.5, data not shown). This was unsurprising for Δ5052, as GBAA5052 is a *gerPF* analog and two additional copies remained on the chromosome. GBAA5053 is a hypothetical protein, and no evidence exists that it has a role in germination, except for its presence in a germination-associated operon.

DISCUSSION

Upon entry into a host, the spores of *B. anthracis* must germinate in order to cause disease (Dixon *et al.*, 1999). The ability of germinants to reach their receptors in the inner spore membrane is essential for germination, and yet the mechanism for this is still not well understood. The experiments described in this study have aimed to further our understanding of this process by characterizing the *B. anthracis* orthologue of the *gerP* operons from *B. subtilis* and *B. cereus* (Behravan *et al.*, 2000).

The GerP proteins in other *Bacillus* species have been shown to play an integral role in the process of germination. Strains that contain insertion mutations in the *gerPC* and *gerPE* genes of *B. subtilis* and *B. cereus* have severe germination defects when incubated in L-alanine or inosine. If the spore coats from these mutants are removed, the germination defect is alleviated (Behravan *et al.*, 2000). It therefore appears that a role of GerP is to facilitate germinant access to receptors, or to contribute to spore coat permeability. We have extended the study of GerP to *B. anthracis.* Deletion of the full *gerP* operon resulted in a delay in germination, but was not as severe as what was reported for other *Bacillus* species (Figure 4.2). Additionally, the data from the growth experiments suggested that in *B. anthracis* the *gerP* operon is important, but not essential, for rapid germination and outgrowth (Figure 4.3). The Δ$gerP_{null}$ mutant did not exhibit the characteristic decrease in optical density associated with germinating wild-type spores, likely because Δ*gerP* mutant spores germinated slowly, and in a non-synchronous manner. It is unlikely that this delay was simply due to a subpopulation of spores that were able to rapidly germinate, as wild-type and mutant spores had similar titers when plated on rich media (data not shown). These data suggest that the interaction of

germinants with their receptors may be delayed in the absence of the GerP proteins in *B. anthracis*, but that once this occurs, germination proceeds normally. Additionally, the Δ*gerP*$_{null}$ strain exhibited no attenuation compared to wild-type spores, when inoculated intratracheally in our DBA/2 mouse model (data not shown). Like the other *Bacillus* species examined, *B. anthracis* also showed increased germination after coat removal. Together these data suggest that the GerP proteins of *B. anthracis* are playing similar, but not identical roles, to the previously characterized *Bacillus* species, and that *B. anthracis* may possess another route of access for its nutrient germinants.

An additional phenotype not examined in other *Bacillus* species was the role GerP played in germination via exogenous Ca-DPA. This compound has been shown to directly activate the coat localized cortex lytic enzyme CwlJ in *B. subtilis*, alleviating the need for germinant receptors to initiate germination (Paidhungat *et al.*, 2001). As the GerP proteins are likely located in the spore coat, we hypothesized that the GerP proteins might also provide Ca-DPA access to CwlJ. Indeed, the GerP proteins appeared to be essential for germination with exogenous Ca-DPA (Figure 4.6 and Table 4.5). In *B. subtilis*, endogenous Ca-DPA released from the spore's core during the initial moments of germination activates CwlJ (Paidhungat *et al.*, 2001). It is possible that the GerP proteins are involved in this release of Ca-DPA, perhaps facilitating its passage through the spore, to activate CwlJ1 in the coat. When exogenous Ca-DPA was used as a germinant only a subpopulation of the mutant spores germinated, as no additional germination was seen over time. The low level of germination remaining in mutant strains may have been due to passive diffusion of Ca-DPA into the spore, or uptake via a nonspecific spore channel or mechanism. Together these data suggest that a GerP

independent route exists to allow in amino acid germinants, but Ca-DPA passage may be primarily dependent on the GerP proteins.

For this study we also determined the contribution of each individual *gerP* gene to the germination phenotype. These analyses have not been performed for other *Bacillus* species. In nutrient germinants, five of the single-gene mutants (Δ*gerPA*-Δ*gerPE*) had the same germination phenotype as the Δ$gerP_{null}$ strain (Tables 4.2 and 4.3), although only four (Δ*gerPA*-Δ*gerPD*) exhibited rescued germination upon coat removal (Table 4.4). Coat-removed Δ*gerPE* and Δ$gerPF_{null}$ spores did not exhibit increased germination, implying a role beyond the spore coat. They may play another role in germination, perhaps by providing a link between the receptors in the inner membrane and the remaining GerP proteins in the coat.

The Δ*gerPF* germination phenotype, which closely resembled that of wild-type, was due to the presence of two *gerPF* orthologues on the chromosome. These additional copies of the *gerPF* gene may be playing additional, or complementary, roles in the germination pathway. One of these orthologues, *gerPF3* (GBAA5052) is predicted to be in an operon with the genes GBAA5053 and GBAA5054 (Bergman *et al.*, 2007). Conserved domains in the protein sequence of GBAA5054, including an Excalibur calcium binding domain suggested that it may play a role in Ca-DPA mediated germination (Mesnage *et al.*, 1999, Daniel *et al.*, 2003). Indeed, the deletion strain exhibited a mild, but significant germination defect in Ca-DPA (Table 4.5). Although no precise role was determined for this putative calcium binding protein, the possibilities are intriguing. *B. anthracis* likely encodes for additional proteins with redundant functions, however, as deletion of GBAA5054 only mildly disrupted Ca-DPA. Additional studies

examining whether this protein is, in fact, involved in binding Ca-DPA will be needed to determine the relevance (if any) this protein has to germination. *B. subtilis* and *B. cereus* also have two *gerPF* orthologues located on their chromosomes, although their roles in germination have yet to be tested (Kunst *et al.*, 1997, Rasko *et al.*, 2004).

Collectively, the germination profiles of the individual Δ*gerP* mutants and the Δ$gerPF_{null}$ triple mutant suggest that each GerP protein is playing a crucial role in facilitating proper, rapid, germination. The GerP proteins play an important role in the early events of germination in all *Bacillus* species tested thus far. This role may involve allowing nutrients into the spore, Ca-DPA out of the spore, or both. More work remains in order to fully elucidate the role these proteins are playing, but that knowledge will lead us to a more complete understanding of how *B. anthracis* regulates the complex process of germination.

Chapter V

Discussion and Future Directions

The overarching goal of this work was a better understanding of the earliest events of germination. Advancements in genetic tools for the manipulation of *B. anthracis* allowed significant strides toward fully understanding the germinants required to stimulate germination *in vitro* as well as in mouse models of infection. The creation of a panel of mutants allowed better dissection of germinant specificities and the mechanisms behind germination, providing insight into how these receptors contribute to infection and disease.

Germinants and their receptors

While a significant amount of work has been done by our laboratory, and others, to understand the process of spore germination, many details remain to be elucidated. One key step, which to this point has eluded understanding, is how germination is initiated. The germinants involved have been well studied, but the molecular mechanisms by which these molecules stimulate a rapid and well orchestrated cascade of events remains unclear. Learning how receptor complexes assemble and sense germinants will impart valuable clues into the initiation of germination. Determining how the three germinant receptor proteins insert themselves into the inner membrane of the spore, and how they interact with one another to form complete complexes, will be

crucial to fully understanding their behavior in response to germinants. Once we have a full appreciation of the structure of these germinant receptor complexes in dormant spores, it will be important to learn about any physical changes that occur during germination.

Chapter II focused on characterizing germinant profiles for each of the germinant receptors. One main theme that came out of these studies is the overlapping specificities of these receptors, as well as the range of germinants that each receptor can respond to. With the exception of GerX, all receptors are able to respond at least weakly to several different germinants, when expressed in the absence of all others. These broad germinant specificities raise questions about just how these receptors interact with their ligands. The most promiscuous germinant specificity is seen with GerH, as it is fully capable of inducing full germination of spores in response to inosine, when combined with any one of a wide variety of L-amino acids. Even this receptor, with its high degree of ligand flexibility, is not able to stimulate germination in response to any single purine or L-amino acid ligand species, however. It will be important to determine whether a single GerH receptor complex can interact with both L-amino acid and inosine germinant molecules, or whether two separate GerH receptors are required, with one binding inosine and the other binding the L-amino acid, in order to trigger germination. These questions can also be applied to the ligand/receptor interactions of the other four germinant receptors in order to provide insight into the mechanisms behind germinant interactions in a wild-type spore. Our quadruple mutants will be useful for investigating these interactions, by eliminating contributions of other germinant receptors.

Some purines, such as inosine, are potent germinants when paired with L-amino acids. Other purines, while still able to stimulate germination, are much less efficient at this process. This is the case of guanosine, as this purine is only able to stimulate about a third of the level of germination, when compared to the full germination response seen with inosine (Figure 2.3). The observation that these two purines may compete for interaction with germinant receptors provides insight into how these complexes may be functioning. If these small molecule germinants are able to compete with each other, this suggests that germinants physically interact with their receptor complexes. If a less efficient germinant, such as guanosine, binds first, the receptor is no longer able to contribute to a rapid germination response. Although these experiments suggest that germinants can compete for interaction with their receptor, binding has yet to be definitively shown. Determining whether binding actually occurs, perhaps by using labeled nutrient germinants, will be essential for fully characterizing early germination events.

If we assume that germinant molecules bind their receptors, it is possible that this binding event initiates a conformational change in the complex. As the process of germination is energy independent and all components necessary to mediate the process are prepackaged into the spore, I hypothesize that conformational changes are the key step in initiating Stage I germination events, including the opening of channels to release ions and Ca-DPA from the spore's core. These channels may involve additional spore components, such as the SpoVA proteins which have are important for DPA uptake, and release following exposure to chemical treatments, such as lysozyme or dodecylamine in *B. subtilis* (Tovar-Rojo *et al.*, 2002, Vepachedu and Setlow, 2007b). Additionally, the

SpoVA proteins have been shown to interact with the germinant receptor complexes of *B. subtilis* (Vepachedu and Setlow, 2007a). The channel could also include components of the germinant receptor complex itself. Determining exactly how germinant receptor complexes form and how they associate with other proteins in the spore will enable a more complete appreciation of how they respond to germinants. *In vitro* studies examining protein-protein interactions between spore components will help elucidate other factors in the processes of Stage I germination.

With two germinant molecules required for germination initiation, and three Ger proteins required to form the receptor complex, a variety of binding possibilities exist. Identifying whether one specific protein in the complex is responsible for binding germinants, or if the entire complex is required for ligand binding remains to be determined. Using tagged germinants and mutant strains lacking specific germinant receptor proteins will lead us closer to this goal. As all three receptor proteins are required for germination to occur, on hypothesis is that the complex as a whole is required for this binding event. Another is that one receptor protein binds to the germinant, while the other proteins stimulate a conformational shift and the opening of ion and water channels, either within the complex, or outside the complex with other spore proteins involved in channel formation. Additionally, the presence of three proteins per receptor complex, and 15 total proteins between the five germinant receptor complexes, raises the possibility that these proteins can form chimeric complexes that recognize additional germinants. Constructing chimeric operons and introducing these to the Δger_{null} strain could provide insight into the feasibility of these hybrid complexes in a wild-type spore.

In contrast to the relatively flexible specificities seen with the chromosomally-encoded germinant receptors, the pXO1-encoded GerX receptor was unable to respond to any germinants tested when expressed alone. This phenotype was identical to that seen with the Δ*ger*$_{null}$ strain lacking all germinant receptors, leaving its role in germination unclear. Several possible functions for GerX have been proposed, but additional work is needed to fully appreciate the role this receptor plays in germination. Small molecule screens may identify germinants able to stimulate this pXO1 encoded receptor, which may not be found otherwise. These germinants may be found in niches yet to be tested, such as in other mammalian hosts, organ sites, or the environment. GerX may also play a role in augmenting the germination responses of other receptors. By playing an additive role when present with other receptors, GerX may allow for a more rapid or efficient germination response. This response may be specific to a limited number of germinants, or it may be a global mediator of germination upon the sensing of nutrients, depending on whether GerX actually recognizes germinants or facilitates some other germination event. Testing germination responses in mutant backgrounds expressing GerX along with one or more other receptors will provide some insight into the role this receptor plays in germination. If a GerX mediated response is detected in these studies, it may provide additional evidence of cooperativity between the receptor complexes. Another possible role for GerX could be that it works somehow to *inhibit* germination. Perhaps this receptor helps to ensure that spores are in a hospitable environment before germination is initiated, by mediating when and how nutrients trigger germination via the other four receptors. Again, testing germination of mutant strains containing only GerX and one

other receptor and comparing these data to the quadruple mutant strains (containing only one receptor) could give a clearer picture of how GerX is functioning in the spore.

Germination in the host

A major goal of these studies was to identify the nutrient signals required for germination in the host. Using two routes of infection allowed comparison of germinant receptor requirements in different sites *in vivo.* Table 5.1 provides a summary of the various routes of infection described in these studies, and the host and spore components that may play a role in facilitating germination. The intratracheal infections of DBA/2 mice revealed a wide flexibility in the germinant receptors required to initiate disease, suggesting a nutrient rich germination environment (Figure 3.2). These data were supported by the experiments examining germinant receptor specificities in macrophages, which have been implicated in spore uptake and germination during an inhalation anthrax infection (Guidi-Rontani *et al.*, 1999b). These studies showed that GerH, GerK, GerL, and GerS were all able to stimulate at least a moderate level of germination (Figure 3.6). As the intratracheal inoculums were 100 times the calculated LD_{50} value of wild-type spores, even a 20% germination response in macrophages would correlate to a high infectious dose. These experiments should be repeated in additional cell types that may be involved in germination, such as dendritic cells and alveolar macrophages. As mentioned in Chapter III, increasing evidence suggests that dendritic cells are important for germination and trafficking during an inhalation anthrax infection, and it will be important to determine if the wide germinant receptor specificity trend occurs in these cells. (Brittingham *et al.*, 2005, Cleret *et al.*, 2007).

Table 5. 1. Potential methods of germination in different routes of B. *anthracis* infection

	Intratracheal	Subcutaneous	Injectional
Putative Germination sites	• Macrophages • Dendritic cells • Phagocytes	• Blood • Interstitial fluid • Lymph • Phagocytes	• Blood
Germinants	• Purines • L-amino acids	• Inosine • L-amino acids	• Inosine • L-amino acids
Germinant entry	• GerP • Unknown spore proteins	• GerP • Unknown spore proteins	• GerP • Unknown spore proteins
Germinant detection	• GerH • GerK • GerL • GerS	• GerH	• GerH

In a subcutaneous infection, germinant specificities were limited to those of the GerH receptor, as its presence was required for virulence (Figure 3.3A). Spores may come in contact with blood at this inoculation site, as was discussed in Chapter III. Inosine appears to be involved in germination in blood as well as cultured macrophages, as a purine analog specifically inhibited the inosine based germination response under these conditions (Figures 3.8 and 3.9). As mentioned above, macrophages may be involved in spore germination during inhalation anthrax. A new form of anthrax, termed "injectional anthrax" has emerged in intravenous drug users due to the injection of

contaminated heroin (Booth *et al.*, 2010). Blood based germination is a major concern in regards to these infections, so understanding the germinant signals involved is paramount. Although not directly tested in the mouse, Table 5.1 lists the spore and host components that may play roles in mediating this form of the disease.

Additional host components may be involved in germination, depending upon route of infection. Spores likely come into contact with other body fluids, including lymph or interstitial fluid in subcutaneous infections. These fluids should also be tested for germination using our panel of germinant receptor mutants to determine which receptors are required under each condition. This will provide additional information regarding potential germinants that spores may encounter in the host environment. Using the germination inhibitors described in Chapter III, as well as identifying additional inhibitors, will also aid in determining the germinants required in these potential germination sites. Together, this will increase our knowledge about the host environments that spores might encounter. Testing for germination in additional cell types, such as dendritic cells or primary alveolar macrophages, as described above, will also be important. Identifying all cell types involved in germination and trafficking of bacilli, as well as the germinants responsible for mediating these responses will provide additional targets for therapeutics, such as germination inhibitors, and mediate further development of prevention mechanisms.

The experiments using germinant analogs to identify the nutrient signals required *in vivo* pointed to L-amino acids and a purine nucleoside, inosine, as key germinants in blood and macrophages (Figures 3.7-3.9). Different hypotheses may account for why *B. anthracis* has evolved to require these specific germinants at the site of inoculation to

stimulate germination. One possibility is that these site-specific nutrients are important for outgrowth after germination until *B. anthracis* metabolism is fully functional. The small acid soluble proteins (SASPs) that protect DNA in the spore are used as nutrient sources upon germination, so it is reasonable to assume that L-amino acids in the environment could be used for the same purpose, assuming that they can be imported into the cell (Setlow, 1975a, Setlow, 1975b, Johnson and Tipper, 1981, Yuan *et al.*, 1981). Inosine catabolism may also provide an energy source to outgrowing bacilli, although the preference for this purine nucleoside over guanosine is unclear, if its sole purpose is to act as a nutrient source. It is possible that a high concentration of inosine at the site of germination may benefit the germinating bacilli in some other manner. In fact, inosine has been implicated as a regulator of the innate immune response, combating inflammation during injury (Hasko *et al.*, 2004). It may be advantageous for spores to germinate in an area that may provide some level of protection against the immediate innate immune response, until metabolism returns and bacilli are able to produce toxins and capsule to protect themselves from a hostile host environment.

In addition to the variability in virulence seen between the receptor mutant strains, we also observed differences in virulence between the mouse strains tested (Figure 3.4 and Figures A1.1 and A1.2). The differences observed between DBA/2 and A/J mice during subcutaneous or inhalation models of anthrax suggest that, in addition to complement components, other immune mechanisms are also essential for early defenses against disease in these strains. Evidence in the literature, as described in Appendix I, suggests that differences in IL-12 responses between these two mouse strains may be at least partially responsible for the differences seen in virulence and dissemination in these

two strains (Gieni *et al.*, 1996, Keane-Myers *et al.*, 1998, Mohan and Stevenson, 1998, Sam and Stevenson, 1999, Pina *et al.*, 2008). *In vitro* studies suggest that IL-12 is produced by phagocytic cells in response to infection with *B. anthracis* spores (Pickering and Merkel, 2004, Pickering *et al.*, 2004). The increased susceptibility and inability to control dissemination of bacilli in A/J mice could be due to a defect in IL-12 production, a defect in the ability to respond to IL-12, or a defect in another unknown immune component. Identification of this component could lead us to a greater understanding for host responses to anthrax infection. Fully understanding the inflammatory response stimulated by germinating bacilli may aid in the development of better therapeutics that harness the host's own immune response.

To better appreciate the differences between DBA/2 and A/J mice, additional studies are needed. Determining LD_{50} values for each route of infection would be an important first step in appreciating the full impact that the immune differences between these mouse strains has on their ability to combat anthrax infection. Once this is determined, we can better investigate the cause of the increased susceptibility in A/J mice. Detection of cytokines after infection in these two strains would allow us to directly compare the responses and determine which cytokines might be lacking in the immune response of A/J mice. Additionally, using knockout mice lacking IL-12, or other cytokines identified as candidates in the immune response, would allow for confirmation of the involvement of the suspected immune components. This could be performed in a normally resistant mouse background, such as C57/BL6. This would allow us to determine the extent of the role of IL-12, or the unknown cytokine or other immune

component, and whether the lack of this component is enough to impact resistance to *B. anthracis* spores.

The GerP proteins

The role of the GerP proteins in germination was also examined, as described in Chapter IV. Although these proteins are essential for nutrient-based germination in *B. subtilis* and *B. cereus,* their role in *B. anthracis* germination was less obvious (Figure 4.2). The mild germination defect in nutrient germinants did not result in any obvious virulence defect in *gerP* mutants *in vivo.* This suggests that *B. anthracis* may have additional spore components to facilitate germinant uptake or spore permeability. In contrast to the mild defect in nutrient germinants, these $\Delta gerP_{null}$ spores exhibited a severe germination defect when incubated in exogenous Ca-DPA, which can directly stimulate germination by activating the germination specific cortex lytic enzyme CwlJ1 *in vitro* (Paidhungat et al., 2001). It has yet to be tested whether *B. subtilis* or *B. cereus* have a similar defect when the GerP proteins are absent, but it is a likely possibility, considering the high degree of homology between the *gerP* operons of these strains.

It will be interesting to determine if *B. anthracis* has additional germinant uptake or permeability mechanisms in place, which renders the GerP proteins dispensable, at least in the case of nutrient germinant uptake. Additionally, it would be interesting to determine how germinant entry is facilitated in other spore formers, such as *Clostridial* species. Sequence alignments have suggested that a homologue does not exist outside the *Bacillus* genus, therefore an alternative mechanism must exist. Understanding the role GerP plays in Ca-DPA mediated germination, and whether this is physiologically relevant, will also be important. The release of endogenous Ca-DPA during Stage I germination stimulates the activation of CwlJ1 in *B. anthracis.* It is possible that the GerP proteins play a role in mediating this interaction between Ca-DPA and the

germination specific lytic enzyme it activates. Investigation into what spore components the GerP proteins interact with could provide great insight into how these coat proteins help facilitate rapid germination.

Implications for therapeutics

Gaining a better understanding of germinants themselves, and how the mammalian host responds to germinating spores, will expose some of the key nuances of anthrax infection that we do not sufficiently understand. Identifying germinants required to initiate disease may lead to better therapeutics in the case of a widespread attack. Current therapeutics are only effective against germinated spores and, therefore, are often required for long periods of time in order to ensure full clearance of the infection. Identifying potent *in vivo* germinants could lead to the development of a pro-germination therapeutic that forces germination of spores in the body, along with antibiotic therapy to clear the infection. This forced germination could eliminate the need for the long-term antibiotic therapy commonly used to protect against latent spore germination and disease. Additionally, forced germination could be used to decontaminate an area after release of spores into the environment, offering additional protection to potential victims, as vegetative bacilli are less infectious than spores (Dixon *et al.*, 1999, Fisher, 2006). Finding effective germination inhibitors could be useful as well. These therapeutics could be administered to victims at the location of spore release, until further treatment options or facilities become available. This would postpone germination and, therefore, disease establishment until the infected individual can be transported to a health care facility

where proper treatments can be administered, such as the germinant/antibiotic cocktail described above.

Together, the experiments described in this work have provided significant advancements in our understanding of germination and the signals required to stimulate this process. Although many questions were answered this study, an even larger number remain to be examined. Continued investigation into the composition and function of germinant receptors and their associated ligands will bring us even closer to a full understanding of the process of germination and how we can combat it in the event of human infection.

Appendix I

Comparison of A/J and DBA/2 mice, with respect to *B. anthracis* dissemination and virulence

BACKGROUND

Anthrax pathogenesis has been studied using a variety of animal models and infection methods. Two mouse strains have been classified as susceptible to infection with the *B. anthracis* Sterne strain, which lacks the pXO2 plasmid encoding the capsule (Sterne, 1939, Welkos *et al.*, 1986). Both the DBA/2 and A/J mouse strains have defects in the Hc locus which encodes complement factor 5 (C5), resulting in susceptibility to unencapsulated strains of *B. anthracis* (Welkos and Friedlander, 1988). This complement component is essential for resistance to these *B. anthracis* strains, as depletion of complement in the resistant mouse strain C57BL/6 via injection of cobra venom factor renders it susceptible to infection (Harvill *et al.*, 2005). Although complement deficiency is the primary means of susceptibility in these mice, we sought to determine whether additional, perhaps subtle, differences may exist between these two mouse strains, by means of exploiting our germinant receptor mutants as tools for comparison. We compared dissemination and survival in both aerosol and subcutaneous infections, using the *in vivo* imaging system described in Chapter III.

MATERIALS AND METHODS

Murine challenges for *in vivo* imaging

Aerosol challenges of 6 week old A/J mice (National Cancer Institute) were performed as previously described (Loving *et al.*, 2007). Briefly, groups of seven mice were exposed to spores of strains containing the bioluminescence cassette *luxCDABE*, as described in Chapter III, for 90 minutes using a nose-only exposure system. The spore inoculums contained 15 mL of $5x10^9$ spores/mL in distilled water with 0.01% Tween 80. Mice were exposed fresh air for 10 minutes before and after the challenge. After the challenge, 2 mice were euthanized and their lungs harvested, homogenized and plated in order to determine total inoculum. $5x10^6$ cfu/mouse were recovered from mice challenged with WT-*lux* spores, while $2.5x10^4$ cfu/mouse were recovered from Δger_{null}-*lux* spores.

Subcutaneous challenges were performed as described in Chapter III. Briefly, groups of A/J or DBA/2 (National Cancer Institute) mice were injected with $4x10^4$ or $4x10^5$ spores in the scruff of the neck. Mice were monitored for a period of 10 days. All mice for these studies were housed and maintained at the Center for Biologics Evaluation and Research animal facility at the Food and Drug Administration under the approval of the Institutional Animal Care and Use Committee, in collaboration with Tod Merkel and Scott Stibitz.

In vivo imaging of infected animals was performed as described in Chapter III. Briefly, bioluminescent strains of my germinant receptor mutants were constructed by Scott Stibitz, and imaging was performed using an IVIS 100 *in vivo* imaging system (Xenogen). Mice were imaged at various time points after infection, as noted in the text.

RESULTS AND DISCUSSION

Comparison of subcutaneous infections in DBA/2 and A/J mice

DBA/2 and A/J mice were inoculated subcutaneously with WT-*lux* spores. Mice were infected with $5x10^5$ spores/mouse, and imaged hourly during the first 12 hours post infection (Figure A1.1).

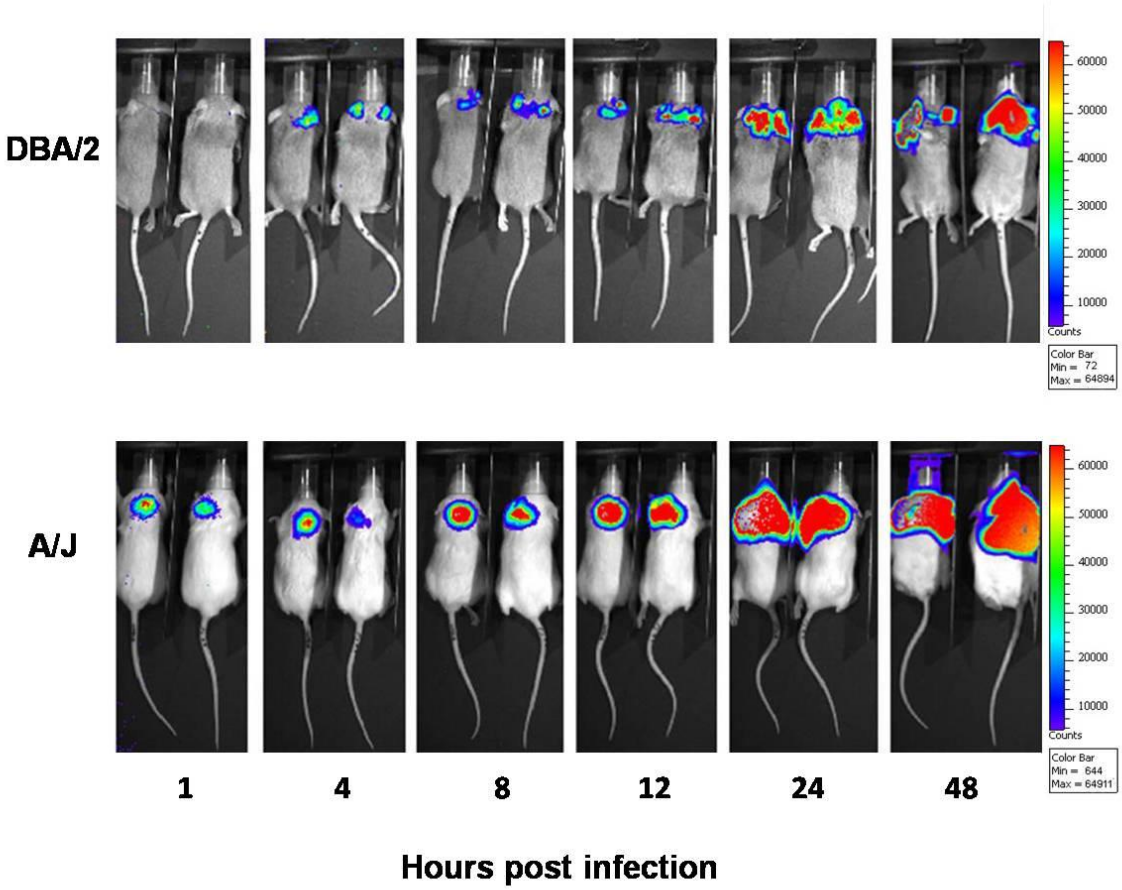

Figure A1. 1. Time course of subcutaneous inoculations into DBA/2 and A/J mice. $5x10^5$ spores/mouse were injected into the scruff of the neck of each animal. Animals were imaged every four hours for the first twelve hours, then once per day afterward. Each panel shows disease progression of two individual animals. The 48 hour panel shows animals rotated onto their backs, as bacteria had disseminated throughout the body. Images stop at 48 hours, as these animals succumbed to infection at this point.

Based on the images obtained during this time course, it appears that *B. anthracis* exhibits very different patterns of dissemination in these two susceptible strains of mice. Luminescence was easily detectable within one hour post infection in the A/J mice, while no luminescence was seen in the DBA/2 mice until four hours. Additionally, bacilli appeared to be trafficked away from the injection site after germination in DBA/2 mice, while the signal from A/J mice did not traffic the entire course of infection. Instead, the luminescence signal in these A/J mice was concentrated at the site of injection, with the signal radiating outward from this site as time elapsed. As infection progressed in DBA/2 mice, the luminescence was sequestered into punctate areas of light suggesting that, perhaps, these mice had additional mechanisms to control infection that the A/J mice did not. The disparity between these two strains of mice became even more apparent at a lower inoculum (Figure A1.2). When infected with $4x10^4$ spores/mouse, luminescence could still be detected very early in A/J mice, while only a weak signal was seen in DBA/2 mice until 48 hours post infection. It should be noted that median time to death was not significantly different between mouse strains, when infected with wild-type spores at these doses. Based upon these data, however, it is reasonable to predict that at a lower inoculum virulence may diverge between these two mouse strains, leading to differing LD_{50} values.

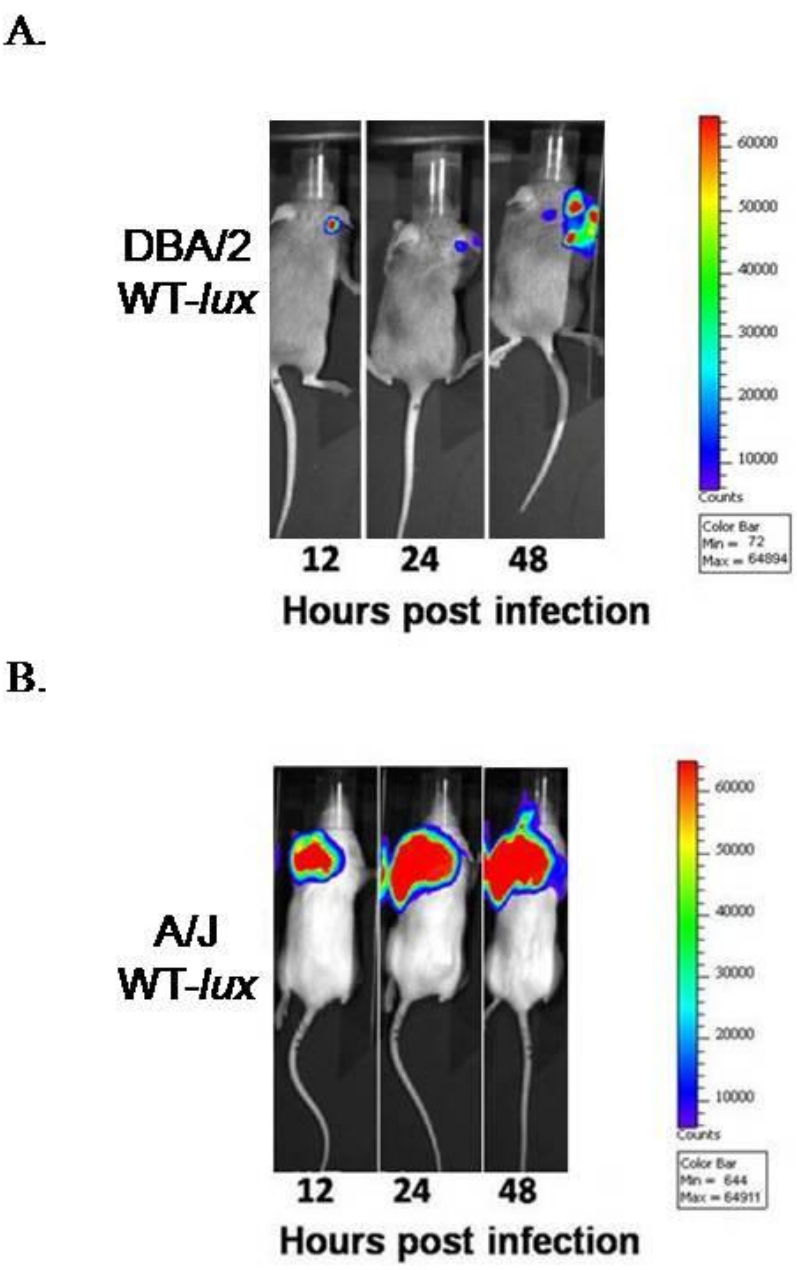

Figure A1. 2. Inoculation of 5x10^4 WT-*lux* spores into DBA/2 and A/J mice.
Spores were injected into the scruff of the neck of either (A) DBA/2 or (B) A/J mice. Animals were imaged at the time points indicated. Each panel shows disease progression of one representative animal. Images stop at 48 hours, as these animals succumbed to infection at this point.

A/J mice were also challenged subcutaneously with Δ*ger*$_{null}$-*lux* spores. When DBA/2 mice were inoculated with the non-luminescent version of these spores, lacking all functional germinant receptors, no significant virulence was seen over the course of infection (Figure 3.3B). In contrast to the high degree of attenuation in DBA/2 mice, A/J mice appeared to be much more susceptible to infection with these spores. The appearance of luminescence in these mice was delayed by several days, when compared to wild-type spores, but once luminescence appeared, disease progression was rapid, resulting in death of two out of the three mice challenged (Figure A1.3). As described in detail in Chapter II, Δ*ger*$_{null}$ spores have a severe defect in colony forming efficiency, when compared to wild-type (Figure 2.2). This deficiency could be correlated with the attenuation seen in infections of DBA/2 mice, as a 1,000 fold defect in colony forming efficiency correlated with a similar attenuation *in vivo* (Table 3.2). The loss of all germinant receptors in these spores does not result in a complete lack of germination, as a 0.1% spores are still able to germinate on rich media, and a low level of virulence was seen in DBA/2 mice (Figures 3.2 and 3.3). It appears that although this inefficient level of germination was severely attenuating in DBA/2 mice, this germination defect was not enough to cause the same level of attenuation in A/J mice.

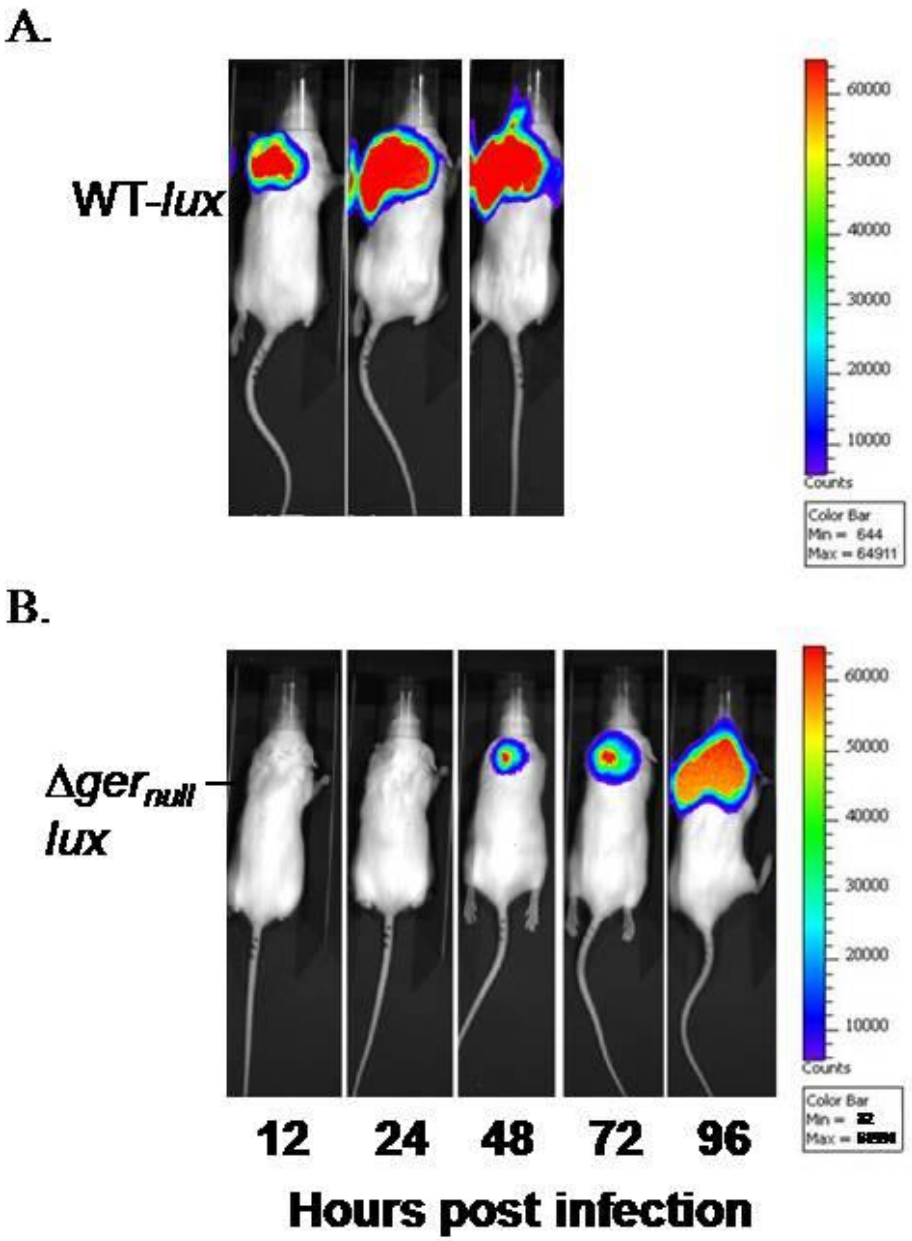

Figure A1. 3. Dissemination of Δ*ger*$_{null}$-*lux* in subcutaneously challenged A/J mice. WT-lux or Δgernull-lux spores were injected into the scruff of the neck of each animal at a dose of 5x104 spores/mouse. Animals were imaged at the first 12 hours, then once per day afterward.

Aerosol challenges of A/J mice

Dissemination during an inhalational anthrax infection was also examined. 10 A/J mice were inoculated with $5x10^6$ spores/mouse of either WT-*lux* or Δ*ger*$_{null}$-*lux* spores via an aerosol system and monitored for 10 days (Figure A1.4). DBA/2 mice were not tested due to a limited number of spore stocks available. A/J mice were infected using a forced breathing aerosol system, then imaged and monitored for disease for a period of 10 days, or until death. After inoculation, two mice were sacrificed, and lungs were homogenized and plated on rich media to determine the spore inoculum. $5x10^6$ cfu/mouse were recovered from mice challenged with wild-type spores, while only $2.5x10^4$ cfu/mouse were recovered from Δ*ger*$_{null}$-*lux* spores. As described above, this difference may be explain w++96+9/8ned by the defect in colony forming efficiency seen with this spore strain (Figure 2.2). Since similar initial inoculums led to fewer cfu/mouse when inoculated with the Δ*ger*$_{null}$-*lux* strain, this suggests that these spores were less able to germinate, essentially leading to a lower infectious dose than in the WT-*lux* challenge.

Loving *et al.* previously categorized bacterial dissemination during an aerosol challenge into three stages (Loving *et al.*, 2009). Stage I consisted of bacteria detected in the nasal associated lymphoid tissues (NALT). Stage II was defined as the detection of luminescence in the central lymph nodes (cLN). Stage III was defined as dissemination past the central lymph nodes, when the infection became systemic (Loving *et al.*, 2009). When A/J mice were infected with WT-*lux* spores, luminescence could be detected within 12 hours in the NALT (Figure A1.4A). Within 48 hours, bacteria had spread to the cLN, with systemic infection being detected at day 3. Stage III is considered to be a

fatal systemic infection and, indeed, none of the mice that reached this stage survived the infection.

A/J mice were also challenged with Δ*ger*$_{null}$-*lux* spores in the aerosol model. In an intratracheal infection of DBA/2 mice, these spores, lacking all functional germinant receptors, caused no significant virulence over the course of infection (Figure 3.2). In contrast to the high degree of attenuation in DBA/2 mice, A/J mice appeared to be much more susceptible to infection with these spores. Similar to the subcutaneous infections with these mutant spores, the appearance of luminescence in these mice was delayed by several days, but disease progression was rapid following the detection of luminescence, resulting in 70% mortality (Figure A1.4B). It appears that although this inefficient germination was severely attenuating in DBA/2 mice, this germination defect was not enough to cause the same level of attenuation in A/J mice.

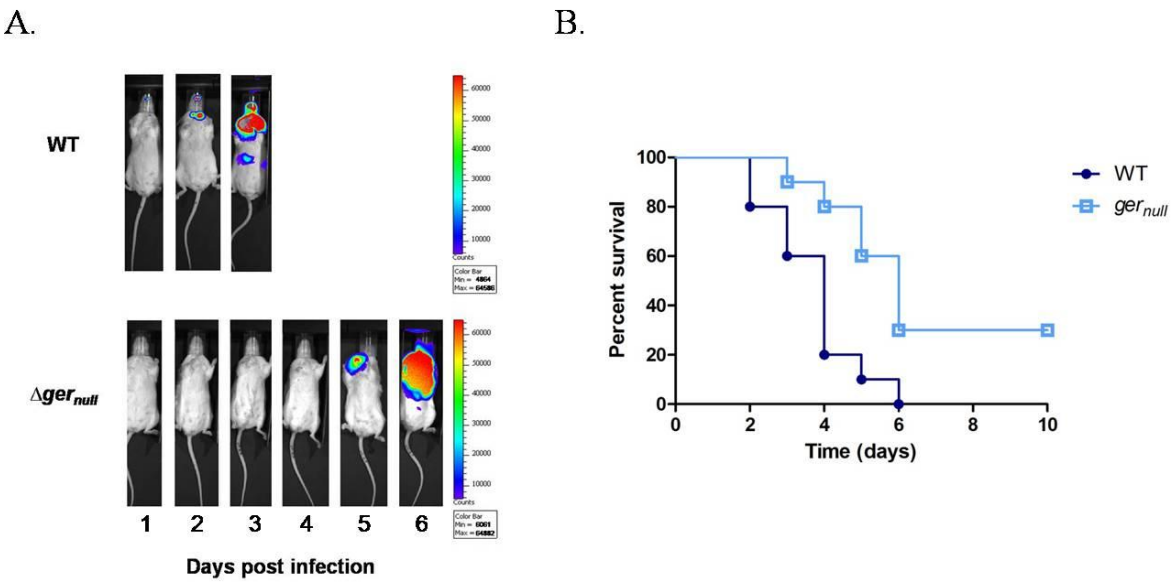

Figure A1. 4. Survival and dissemination following aerosol challenge.
Groups of A/J mice were exposed to either wild-type or Δ*ger*$_{null}$-*lux* spores using an aerosol challenge model. (A) Bacterial dissemination via luminescence detection and (B) survival curve are shown. Luminescence data shown are from representative mice showing typical bacterial dissemination patterns.

Although a direct comparison cannot be made between the A/J and DBA/2 challenges due to the different methods of infection (aerosol *vs.* intratracheal, respectively), we can at least make some observations as both challenges deliver spores to the lungs, mimicking inhalation anthrax. The striking variability between A/J and DBA/2 mice challenged with Δ*ger*$_{null}$ spores suggests that there may be some differences in their immune responses to anthrax infection (Figure 3.2 and Figure A1.4B). Although both strains are considered to be susceptible to anthrax due to their complement deficiency, DBA/2 mice appear to have some additional resistance to spores lacking in A/J mice that allow for resistance to these germination-defective spores.

By inoculating DBA/2 and A/J mice side by side in the subcutaneous infections, we could directly compare susceptibility and dissemination patterns that have not previously been examined (Figure A1.1 and A1.2). Indeed, when these mouse strains were inoculated with wild-type bioluminescent spores, we saw stark differences between the dissemination patterns, as DBA/2 mice appeared much more capable of controlling the infection at early time points, when compared to A/J mice. This inability to combat early dissemination resulted in greater virulence when infected with the Δ*ger*$_{null}$ strain (Figures 3.3B and A1.3).

The cytokine interleukin-12 (IL-12) may be responsible for at least some of the difference seen between these mouse strains. Studies examining cytokine production in response to *B. anthracis* infection have detected IL-12 in cell culture models, including BALB/c derived mouse macrophages (J774A.1), human dendritic cells, and mouse splenocytes (Pickering and Merkel, 2004, Pickering *et al.*, 2004, Glomski *et al.*, 2007). Several studies have suggested that IL-12 is important in the defense against intracellular pathogens, and that A/J mice have a defect in its production (Keane-Myers *et al.*, 1998, Mohan and Stevenson, 1998, Sam and Stevenson, 1999, Pina *et al.*, 2008). Additionally, DBA/2 mice are considered to have a robust IL-12 response in regards to some pathogens (Gieni *et al.*, 1996). IL-12 is a heterodimeric pro-inflammatory cytokine produced by professional antigen presenting cells, including macrophages, dendritic cells, and neutrophils. IL-12 production stimulates the production of IFN-γ, a key activator of macrophages, and also promotes the differentiation of T helper 1 (T_H1) cells (Trinchieri, 2003).

If IL-12 is, indeed, vital for the defense against vegetative *B. anthracis* then A/J mice may lack the ability to combat early moments of infection. In an IL-12 competent strain, such as DBA/2, mice may be able to mount an early response, as seen with the sequestration of luminescence near the lymph nodes (Figure A1.1). This early response may prevent the rapid dissemination that was seen in the A/J mice. Further, a Δger_{null} strain, which has a severe defect in colony forming efficiency *in vitro* and is highly attenuated in DBA/2 mice when injected intratracheally, showed a radically different level of virulence in A/J mice when introduced in an aerosol model of infection (Figures 3.2 and A1.4B). DBA/2 mice, which may have better IL-12 responses, were able to combat infection with a strain of *B. anthracis* that has a severe germination defect. In an A/J model, however, the inability to mount the same early response resulted in a much higher level of virulence. Although the complement component C5 has been shown to be essential for resistance, the data in this study suggest that IL-12, or some other immune component, may also play a role in the early defense against *B. anthracis* infection *(*Harvill *et al.,* 2005*).* Future studies will aim to further our understanding of these early immune responses, by definitively identifying the immune components necessary to mount an effective response against this pathogen.

References

Abramova, F., L. Grinberg, O. Yampolskaya & D. Walker, (1993) Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979. *Proc Nat Acad Sci USA* 90: 2291-2294.

Akoachere, M., R. C. Squires, A. M. Nour, L. Angelov, J. Brojatsch & E. Abel-Santos, (2007) Identification of an in vivo Inhibitor of *Bacillus anthracis* Spore Germination. *J Biol Chem* 282: 12112-12118.

Albrink, W. S., (1961) Pathogenesis of Inhalation Anthrax. *Microbiol Mol Biol Rev* 25: 268-273.

Andersson, A., U. Rönner & P. E. Granum, (1995) What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *International Journal of Food Microbiology* 28: 145-155.

Arnesen, L., A. Fagerlund & P. E. Granum, (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS microbiol Rev* 32: 579-606.

Ash, C., J. A. E. Farrow, M. Dorsch, E. Stackebrandt & M. D. Collins, (1991) Comparative Analysis of *Bacillus anthracis, Bacillus cereus*, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S rRNA. *Int J Syst Bacteriol* 41: 343-346.

Atluri, S., K. Ragkousi, D. E. Cortezzo & P. Setlow, (2006) Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. *J Bacteriol* 188: 28-36.

Atrih, A. & S. J. Foster, (2001a) Analysis of the role of bacterial endospore cortex structure in resistance properties and demonstration of its conservation amongst species. *J Appl Microbiol* 91: 364-372.

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

Atrih, A., P. Zollner, G. Allmaier & S. J. Foster, (1996) Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. *J Bacteriol* 178: 6173-6183.

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

Barth, H., K. Aktories, M. R. Popoff & B. G. Stiles, (2004) Binary Bacterial Toxins: Biochemistry, Biology, and Applications of Common *Clostridium* and *Bacillus* Proteins. *Microbiol Mol Biol Rev* 68: 373-402.

Bartkus, J. M. & S. H. Leppla, (1989) Transcriptional regulation of the protective antigen gene of *Bacillus anthracis*. *Infect. Immun.* 57: 2295-2300.

Beall, B., A. Driks, R. Losick & C. P. Moran, Jr., (1993) Cloning and characterization of a gene required for assembly of the *Bacillus subtilis* spore coat. *J. Bacteriol.* 175: 1705-1716.

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

Behravan, J., H. Chirakkal, A. Masson & A. Moir, (2000) Mutations in the *gerP* locus of *Bacillus subtilis* and *Bacillus cereus* affect access of germinants to their targets in spores. *J Bacteriol* 182: 1987-1994.

Bergman, N. H., E. C. Anderson, E. E. Swenson, M. M. Niemeyer, A. D. Miyoshi & P. C. Hanna, (2006) Transcriptional profiling of the *Bacillus anthracis* life cycle in vitro and an implied model for regulation of spore formation. *J Bacteriol* 188: 6092-6100.

Bergman, N. H., K. D. Passalacqua, P. C. Hanna & Z. S. Qin, (2007) Operon Prediction for Sequenced Bacterial Genomes without Experimental Information. *Appl Environ Microbiol* 73: 846-854.

Betty, J. M. & W. G. Murrell, (1970) Symposium on Bacterial Spores : Paper IX. Biophysical Analysis of the Spore. *J Appl Microbiol* 33: 103-129.

Blaustein, R. O., T. M. Koehler, R. J. Collier & A. Finkelstein, (1989) Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. *Proc Nat Acad Sci USA* 86: 2209-2213.

Boland, F. M., A. Atrih, H. Chirakkal, S. J. Foster & A. Moir, (2000) Complete spore-cortex hydrolysis during germination of *Bacillus subtilis* 168 requires SleB and YpeB. *Microbiology* 146 (Pt 1): 57-64.

Booth, M. G., J. Hood, T. J. Brooks & A. Hart, (2010) Anthrax infection in drug users. *Lancet* 375: 1345-1346.

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

Bradley, K. A., J. Mogridge, M. Mourez, R. J. Collier & J. A. T. Young, (2001) Identification of the cellular receptor for anthrax toxin. *Nature* 414: 225-229.

Brahmbhatt, T. N., B. K. Janes, E. S. Stibitz, S. C. Darnell, P. Sanz, S. B. Rasmussen & A. D. O'Brien, (2007) *Bacillus anthracis* Exosporium Protein BclA Affects Spore Germination, Interaction with Extracellular Matrix Proteins, and Hydrophobicity. *Infect Immun* 75: 5233-5239.

Brittingham, K. C., G. Ruthel, R. G. Panchal, C. L. Fuller, W. J. Ribot, T. A. Hoover, *et al.*, (2005) Dendritic Cells Endocytose Bacillus anthracis Spores: Implications for Anthrax Pathogenesis. *J Immunol* 174: 5545-5552.

Brown, W. C., D. Vellom, I. Ho, N. Mitchell & P. McVay, (1982) Interaction between a *Bacillus cereus* spore hexosaminidase and specific germinants. *J Bacteriol* 149: 969-976.

Burbulys, D., K. A. Trach & J. A. Hoch, (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell* 64: 545-552.

Candela, T., M. Mock & A. Fouet, (2005) CapE, a 47-Amino-Acid Peptide, Is Necessary for *Bacillus anthracis* Polyglutamate Capsule Synthesis. *J Bacteriol* 187: 7765-7772.

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

Carlson, P. E., Jr., K. A. Carr, B. K. Janes, E. C. Anderson & P. C. Hanna, (2009) Transcriptional Profiling of *Bacillus anthracis* Sterne (34F2) during Iron Starvation. *PLoS ONE* 4: e6988.

Chakrabarty, K., W. Wu, J. L. Booth, E. S. Duggan, K. M. Coggeshall & J. P. Metcalf, (2006) Bacillus anthracis Spores Stimulate Cytokine and Chemokine Innate Immune Responses in Human Alveolar Macrophages through Multiple Mitogen-Activated Protein Kinase Pathways. *Infect. Immun.* 74: 4430-4438.

Chesnokova, O. N., S. A. McPherson, C. T. Steichen & C. L. Turnbough, Jr., (2009) The Spore-Specific Alanine Racemase of *Bacillus anthracis* and Its Role in Suppressing Germination during Spore Development. *J Bacteriol* 191: 1303-1310.

Chirakkal, H., M. O'Rourke, A. Atrih, S. J. Foster & A. Moir, (2002) Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. *Microbiology* 148: 2383-2392.

Cleret, A., A. Quesnel-Hellmann, A. Vallon-Eberhard, B. Verrier, S. Jung, D. Vidal, *et al.*, (2007) Lung Dendritic Cells Rapidly Mediate Anthrax Spore Entry through the Pulmonary Route. *J Immunol* 178: 7994-8001.

Collier, R. J. & J. A. T. Young, (2003) Anthrax Toxin. *Ann Rev Cell Dev Biol* 19: 45-70.

Corfe, B. M., A. Moir, D. Popham & P. Setlow, (1994a) Analysis of the expression and regulation of the *gerB* spore germination operon of *Bacillus subtilis* 168. *Microbiology* 140: 3079-3083.

Corfe, B. M., R. L. Sammons, D. A. Smith & C. Mauel, (1994b) The *gerB* region of the *Bacillus subtilis* 168 chromosome encodes a homologue of the *gerA* spore germination operon. *Microbiology* 140: 471-478.

Cutting, S., A. Driks, R. Schmidt, B. Kunkel & R. Losick, (1991) Forespore-specific transcription of a gene in the signal transduction pathway that governs Pro-sigma K processing in *Bacillus subtilis*. *Genes Dev* 5: 456-466.

Dai, Z. & T. M. Koehler, (1997) Regulation of anthrax toxin activator gene (*atxA*) expression in *Bacillus anthracis*: temperature, not CO2/bicarbonate, affects AtxA synthesis. *Infect Immun* 65: 2576-2582.

Daniel, J. R., J. J. Mark & Y. G. Michael, (2003) An extracellular calcium-binding domain in bacteria with a distant relationship to EF-hands. *FEMS Microbiol Lett* 221: 103-110.

Dixon, T. C., M. Meselson, J. Guillemin & P. C. Hanna, (1999) Anthrax. *N Engl J Med* 341: 815-826.

Driks, A., (2002) Maximum shields: the assembly and function of the bacterial spore coat. *Trends Microbiol* 10: 251-254.

Druett, H. A., D. W. Henderson, L. Packman & S. Peacock, (1953) Studies on respiratory infection I. The influence of particle size on respiratory infection with anthrax spores. *J Hyg* 51: 359-371.

Drysdale, M., A. Bourgogne, S. G. Hilsenbeck & T. M. Koehler, (2004) *atxA* Controls *Bacillus anthracis* Capsule Synthesis via *acpA* and a Newly Discovered Regulator, *acpB*. *J Bacteriol* 186: 307-315.

Drysdale, M., A. Bourgogne & T. M. Koehler, (2005a) Transcriptional Analysis of the *Bacillus anthracis* Capsule Regulators. *J. Bacteriol.* 187: 5108-5114.

Drysdale, M., S. Heninger, J. Hutt, Y. Chen, C. R. Lyons & T. M. Koehler, (2005b) Capsule synthesis by *Bacillus anthracis* is required for dissemination in murine inhalation anthrax. *EMBO J* 24: 221-227.

Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel, T. D. Copeland, *et al.*, (1998) Proteolytic Inactivation of MAP-Kinase-Kinase by Anthrax Lethal Factor. *Science* 280: 734-737.

Elliott, J. L., J. Mogridge & R. J. Collier, (2000) A Quantitative Study of the Interactions of *Bacillus anthracis* Edema Factor and Lethal Factor with Activated Protective Antigen. *Biochemistry* 39: 6706-6713.

Errington, J., (1993) *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol Mol Biol Rev* 57: 1-33.

Feavers, I. M., J. Foulkes, B. Setlow, D. Sun, W. Nicholson, P. Setlow & A. Moir, (1990) The regulation of transcription of the *gerA* spore germination operon of *Bacillus subtilis*. *Mol Microbiol* 4: 275-282.

Fey, G., G. W. Gould & A. D. Hitchins, (1964) Identification of d-Alanine as the Auto-Inhibitor of Germination of *Bacillus globigii* Spores. *J Gen Microbiol* 35: 229-236.

Finlay, W. J., N. A. Logan & A. D. Sutherland, (2002) *Bacillus cereus* emetic toxin production in cooked rice. *Food Microbiology* 19: 431-439.

Fisher, N., (2006) On the Role of *Bacillus anthracis* Cellular Differentiation in the Establishment of Anthrax. In: Microbiology and Immunology. Ann Arbor: University of Michigan, pp.

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

Fox, K. & B. D. Eder, (1969) Comparison of Survivor Curves of *Bacillus subtilis* Spores Subjected to Wet and Dry Heat. *J Food Sci* 34: 518-521.

Friedlander, A. M., (1986) Macrophages are sensitive to anthrax lethal toxin through an acid- dependent process. *J Biol Chem* 261: 7123-7126.

Friedlander, A. M., (1999) Anthrax. In: Vaccines (3rd edn). S. Plotkin & W. Orenstein (eds). Philadelphia: WB Saunders Company, pp. 629-637.

Giebel, J. D., K. A. Carr, E. C. Anderson & P. C. Hanna, (2009) Germination-Specific Lytic Enzymes SleB, CwlJ1, and CwlJ2 each contribute to *Bacillus anthracis* Spore Germination and Virulence. *J Bacteriol*: JB.00408-00409.

Gieni, R. S., Y. Fang, G. Trinchieri, D. T. Umetsu & R. H. DeKruyff, (1996) Differential production of IL-12 in BALB/c and DBA/2 mice controls IL-4 versus IFN-{gamma} synthesis in primed CD4 lymphocytes. *Int. Immunol.* 8: 1511-1520.

Glomski, I. J., J. H. Fritz, S. J. Keppler, V. Balloy, M. Chignard, M. Mock & P. L. Goossens, (2007) Murine splenocytes produce inflammatory cytokines in a MyD88-dependent response to *Bacillus anthracis* spores. *Cell Microbiol* 9: 502-513.

Gonzalez, J. M., B. J. Brown & B. C. Carlton, (1982) Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc Nat Acad Sci USA* 79: 6951-6955.

Gordon, V. M., S. H. Leppla & E. L. Hewlett, (1988) Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. *Infect Immun* 56: 1066-1069.

Gould, G. W., (1977) Recent Advances in the Understanding of Resistance and Dormancy in Bacterial Spores. *J Appl Microbiol* 42: 297-309.

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

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

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

Haldenwang, W. G., (1995) The sigma factors of *Bacillus subtilis*. *Microbiol Rev* 59: 1-30.

Hammond, S. E. & P. C. Hanna, (1998) Lethal Factor Active-Site Mutations Affect Catalytic Activity In Vitro. *Infect Immun* 66: 2374-2378.

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

Harvill, E. T., G. Lee, V. K. Grippe & T. J. Merkel, (2005) Complement Depletion Renders C57BL/6 Mice Sensitive to the *Bacillus anthracis* Sterne Strain. *Infect Immun* 73: 4420-4422.

Hasko, G., M. Sitkovsky & C. Szabo, (2004) Immunomodulatory and neuroprotective effects of inosine. *Trends Pharm Sci* 25: 152-157.

Heffernan, B. J., B. Thomason, A. Herring-Palmer, L. Shaughnessy, R. McDonald, N. Fisher, *et al.*, (2006) *Bacillus anthracis* phospholipases C facilitate macrophage-associated growth and contribute to virulence in a murine model of inhalation anthrax. *Infect Immun* 74: 3756-3764.

Heffron, J. D., B. Orsburn & D. L. Popham, (2009) Roles of Germination-Specific Lytic Enzymes CwlJ and SleB in *Bacillus anthracis*. *J Bacteriol* 191: 2237-2247.

Helgason, E., O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, *et al.*, (2000) *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*---One Species on the Basis of Genetic Evidence. *Appl. Environ. Microbiol.* 66: 2627-2630.

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

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

Hoffmaster, A. R. & T. M. Koehler, (1999) Autogenous Regulation of the *Bacillus anthracis pag* Operon. *J. Bacteriol.* 181: 4485-4492.

Hofte, H. & H. R. Whiteley, (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Mol Biol Rev* 53: 242-255.

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

Hudson, K. D., B. M. Corfe, E. H. Kemp, I. M. Feavers, P. J. Coote & A. Moir, (2001) Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. *J Bacteriol* 183: 4317-4322.

Igarashi, T. & P. Setlow, (2005) Interaction between individual protein components of the GerA and GerB nutrient receptors that trigger germination of *Bacillus subtilis* spores. *J Bacteriol* 187: 2513-2518.

Illing, N. & J. Errington, (1991) Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of sigma E and sigma F in prespore engulfment. *J Bacteriol* 173: 3159-3169.

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

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

Janes, B. K. & S. Stibitz, (2006) Routine Markerless Gene Replacement in *Bacillus anthracis*. *Infect Immun* 74: 1949-1953.

Jenkinson, H. F., D. Kay & J. Mandelstam, (1980) Temporal dissociation of late events in *Bacillus subtilis* sporulation from expression of genes that determine them. *J Bacteriol* 141: 793-805.

Jernigan, J., D. Stephens, D. Ashford, C. Omenaca, M. Topiel, M. Galbraith, *et al.*, (2001) Bioterrorism-Related Inhalational Anthrax: The First 10 Cases Reported in the United States. *Emerg Infect Dis* 7: 933-944.

Johnson, W. C. & D. J. Tipper, (1981) Acid-soluble spore proteins of *Bacillus subtilis*. *J. Bacteriol.* 146: 972-982.

Kang, T. J., M. J. Fenton, M. A. Weiner, S. Hibbs, S. Basu, L. Baillie & A. S. Cross, (2005) Murine Macrophages Kill the Vegetative Form of *Bacillus anthracis*. *Infect Immun* 73: 7495-7501.

Keane-Myers, A., M. Wysocka, G. Trinchieri & M. Wills-Karp, (1998) Resistance to Antigen-Induced Airway Hyperresponsiveness Requires Endogenous Production of IL-12. *J Immunol* 161: 919-926.

Kennedy, M. J., S. L. Reader & L. M. Swierczynski, (1994) Preservation records of micro-organisms: evidence of the tenacity of life. *Microbiology* 140: 2513-2529.

Kim, H. & J. Goepfert, (1974) A sporulation medium for *Bacillus anthracis*. *J Appl Bact* 37: 265-267.

Kim, S. O., Q. Jing, K. Hoebe, B. Beutler, N. S. Duesbery & J. Han, (2003) Sensitizing Anthrax Lethal Toxin-resistant Macrophages to Lethal Toxin-induced Killing by Tumor Necrosis Factor-alpha. *J Biol Chem* 278: 7413-7421.

Klimpel, K. R., S. S. Molloy, T. G & S. H. Leppla, (1992) Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc Nat Acad Sci USA* 89: 10277-10281.

Klobutcher, L. A., K. Ragkousi & P. Setlow, (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: 165-170.

Knott, A. G., A. D. Russell & B. N. Dancer, (1995) Development of resistance to biocides during sporulation of *Bacillus subtilis*. *J Appl Microbiol* 79: 492-498.

Koch, R., (1876) The etiology of anthrax, based on the life history of *Bacillus anthracis Beitr. Biol. Pflanz* 2: 277-310.

Koehler, T. M. & R. J. Collier, (1991) Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. *Mol Microbiol* 5: 1501-1506.

Koike, J., T. Oshima, K. A. Koike, H. Taguchi, R. Tanaka, K. Nishimura & M. Miyaji, (1992) Survival rates of some terrestrial microorganisms under simulated space conditions. *Adv Space Res* 12: 271-274.

Krantz, B. A., A. D. Trivedi, K. Cunningham, K. A. Christensen & R. J. Collier, (2004) Acid-induced Unfolding of the Amino-terminal Domains of the Lethal and Edema Factors of Anthrax Toxin. *J Mol Bio* 344: 739-756.

Kroos, L. & Y.-T. N. Yu, (2000) Regulation of sigma factor activity during *Bacillus subtilis* development. *Current Opinion in Microbiology* 3: 553-560.

Kumar, P., N. Ahuja & R. Bhatnagar, (2002) Anthrax Edema Toxin Requires Influx of Calcium for Inducing Cyclic AMP Toxicity in Target Cells. *Infect. Immun.* 70: 4997-5007.

Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, *et al.*, (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* 390: 249-256.

Laaberki, M.-H. & J. Dworkin, (2008) Role of Spore Coat Proteins in the Resistance of *Bacillus subtilis* Spores to *Caenorhabditis elegans* Predation. *J Bacteriol* 190: 6197-6203.

Lee, J. Y., B. K. Janes, K. D. Passalacqua, B. F. Pfleger, N. H. Bergman, H. Liu, *et al.*, (2007) Biosynthetic analysis of the petrobactin siderophore pathway from *Bacillus anthracis*. *J Bacteriol* 189: 1698-1710.

Lee, K. S., D. Bumbaca, J. Kosman, P. Setlow & M. J. Jedrzejas, (2008) Structure of a protein-DNA complex essential for DNA protection in spores of *Bacillus* species. *Proc Nat Acad Sci USA* 105: 2806-2811.

Leonard, C. G. & C. B. Thorne, (1961) Studies on the Nonspecific Precipitation of Basic Serum Proteins with gamma-Glutamyl Polypeptides. *J Immunol* 87: 175-188.

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

Levin, P. A. & R. Losick, (1996) Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in *Bacillus subtilis*. *Genes Dev* 10: 478-488.

Liu, H., N. H. Bergman, B. Thomason, S. Shallom, A. Hazen, J. Crossno, *et al.*, (2004) Formation and composition of the *Bacillus anthracis* endospore. *J Bacteriol* 186: 164-178.

Loving, C. L., M. Kennett, G. M. Lee, V. K. Grippe & T. J. Merkel, (2007) Murine Aerosol Challenge Model of Anthrax. *Infect Immun* 75: 2689-2698.

Loving, C. L., T. Khurana, M. Osorio, G. M. Lee, V. K. Kelly, S. Stibitz & T. J. Merkel, (2009) Role of Anthrax Toxins in Dissemination, Disease Progression, and Induction of Protective Adaptive Immunity in the Mouse Aerosol Challenge Model. *Infect. Immun.* 77: 255-265.

Lu, S., R. Halberg & L. Kroos, (1990) Processing of the mother-cell sigma factor, sigma K, may depend on events occurring in the forespore during *Bacillus subtilis* development. *Proc Nat Acad Sci USA* 87: 9722-9726.

Lu, S. & L. Kroos, (1994) Overproducing the *Bacillus subtilis* mother cell sigma factor precursor, Pro-sigma K, uncouples sigma K-dependent gene expression from dependence on intercompartmental communication. *J Bacteriol* 176: 3936-3943.

Makino, S., C. Sasakawa, I. Uchida, N. Terakado & M. Yoshikawa, (1988) Cloning and CO2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. *Mol Microbiol* 2: 371-376.

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

Mandelstam, J. & S. A. Higgs, (1974) Induction of Sporulation During Synchronized Chromosome Replication in *Bacillus subtilis*. *J Bacteriol* 120: 38-42.

McCann, K. P., C. Robinson, R. L. Sammons, D. A. Smith & B. M. Corfe, (1996) Alanine germination receptors of *Bacillus subtilis*. *Letters in Applied Microbiology* 23: 290-294.

McCormick, N. G., (1965) Kinetics of Spore Germination. *J Bacteriol* 89: 1180-1185.

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

Mesnage, S., E. Tosi-Couture, M. Mock & A. Fouet, (1999) The S-layer homology domain as a means for anchoring heterologous proteins on the cell surface of *Bacillus anthracis*. *J Appl Microbiol* 87: 256-260.

Metcalfe, N., (2004) The history of woolsorters' disease: a Yorkshire beginning with an international future? *Occup Med (Lond)* 54: 489-493.

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

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

Mock, M. & A. Ullmann, (1993) Calmodulin-activated bacterial adenylate cyclases as virulence factors. *Trends Microbiol* 1: 187-192.

Mohan, K. & M. Stevenson, (1998) Interleukin-12 corrects severe anemia during blood-stage Plasmodium chabaudi AS in susceptible A/J mice. *Exp Hematol* 26: 45-52.

Moir, A., (2003) Bacterial spore germination and protein mobility. *Trends Microbiol* 11: 452-454.

Moir, A., B. M. Corfe & J. Behravan, (2002) Spore Germination. *Cell Mol Life Sci*: 403-409.

Moir, A., E. Lafferty & D. A. Smith, (1979) Genetics analysis of spore germination mutants of *Bacillus subtilis* 168: the correlation of phenotype with map location. *J Gen Microbiol* 111: 165-180.

Moriyama, R., S. Kudoh, S. Miyata, S. Nonobe, A. Hattori & S. Makino, (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: 5330-5332.

Nicholson, W., B. Setlow & P. Setlow, (1991) Ultraviolet irradiation of DNA complexed with alpha/beta-type small, acid-soluble proteins from spores of *Bacillus* or *Clostridium* species makes spore photoproduct but not thymine dimers. *Proc Natl Acad Sci U S A* 88: 8288-8292.

Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh & P. Setlow, (2000) Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiol Mol Biol Rev* 64: 548-572.

OIE, (1997) OIE Animal health and disease control report 1997. In. Paris, France: Office International des Epizooties, pp.

Okinaka, R. T., K. Cloud, O. Hampton, A. R. Hoffmaster, K. K. Hill, P. Keim, *et al.*, (1999) Sequence and Organization of pXO1, the Large *Bacillus anthracis* Plasmid Harboring the Anthrax Toxin Genes. *J Bacteriol* 181: 6509-6515.

Paidhungat, M., K. Ragkousi & P. Setlow, (2001) Genetic Requirements for Induction of Germination of Spores of *Bacillus subtilis* by Ca2+-Dipicolinate. *J Bacteriol* 183: 4886-4893.

Paidhungat, M., B. Setlow, W. B. Daniels, D. Hoover, E. Papafragkou & P. Setlow, (2002) Mechanisms of Induction of Germination of *Bacillus subtilis* Spores by High Pressure. *Appl. Environ. Microbiol.* 68: 3172-3175.

Paidhungat, M. & P. Setlow, (2000) Role of Ger Proteins in Nutrient and Nonnutrient Triggering of Spore Germination in *Bacillus subtilis*. *J Bacteriol* 182: 2513-2519.

Paidhungat, M. & P. Setlow, (2001) Localization of a Germinant Receptor Protein (GerBA) to the Inner Membrane of *Bacillus subtilis* Spores. *J Bacteriol* 183: 3982-3990.

Paidhungat, M. & P. Setlow, (2002) Spore germination and outgrowth. In: *Bacillus subtilis* and its Closest Relatives: from Genes to Cells. A. L. Sonenshein, J. Hoch & R. Losick (eds). Washington DC: American Society for Microbiology, pp. 537-548.

Pannucci, J., R. Okinaka, E. Williams, R. Sabin, L. Ticknor & C. Kuske, (2002) DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria. *BMC Genomics* 3: 34.

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

Pasteur, L., (1881) De I'attenuation des virus et de leur retour a la virulence.
. *C.R. Acad. Sci. Agric. Bulg.* 92: 429-435.

Pickering, A. K. & T. J. Merkel, (2004) Macrophages Release Tumor Necrosis Factor Alpha and Interleukin-12 in Response to Intracellular *Bacillus anthracis* Spores. *Infect Immun* 72: 3069-3072.

Pickering, A. K., M. Osorio, G. M. Lee, V. K. Grippe, M. Bray & T. J. Merkel, (2004) Cytokine Response to Infection with *Bacillus anthracis* Spores. *Infect Immun* 72: 6382-6389.

Piggot, P. J. & D. W. Hilbert, (2004) Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* 7: 579-586.

Pile, J. C., J. D. Malone, E. M. Eitzen & A. M. Friedlander, (1998) Anthrax as a Potential Biological Warfare Agent. *Arch Intern Med* 158: 429-434.

Pimental, R.-A. L., K. A. Christensen, B. A. Krantz & R. J. Collier, (2004) Anthrax toxin complexes: heptameric protective antigen can bind lethal factor and edema factor simultaneously. *Biochem Biophys Res Commun* 322: 258-262.

Pina, A., S. Bernardino & V. L. G. Calich, (2008) Alveolar macrophages from susceptible mice are more competent than those of resistant mice to control initial *Paracoccidioides brasiliensis* infection. *J Leukoc Biol* 83: 1088-1099.

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

Popham, D. L., J. Helin, C. E. Costello & P. Setlow, (1996) Muramic Lactam in Peptidoglycan of *Bacillus subtilis* Spores is Required for Spore Outgrowth but not for Spore Dehydration or Heat Resistance. *Proc Natl Acad Sci U S A* 93: 15405-15410.

Rasko, D. A., J. Ravel, O. A. Okstad, E. Helgason, R. Z. Cer, L. Jiang, *et al.*, (2004) The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucl. Acids Res.* 32: 977-988.

Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, *et al.*, (2003) The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* 423: 81-86.

Reed, L. J. & H. Muench, (1938) A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 27: 493-497.

Rhie, G.-E., M. H. Roehrl, M. Mourez, R. J. Collier, J. J. Mekalanos & J. Y. Wang, (2003) A dually active anthrax vaccine that confers protection against both bacilli and toxins. *Proc Nat Acad Sci USA* 100: 10925-10930.

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

Sam, H. & M. M. Stevenson, (1999) In Vivo IL-12 Production and IL-12 Receptors {beta}1 and {beta}2 mRNA Expression in the Spleen Are Differentially Up-Regulated in Resistant B6 and Susceptible A/J Mice During Early Blood-Stage *Plasmodium chabaudi* AS Malaria. *J Immunol* 162: 1582-1589.

Sammons, R. L., A. Moir & D. A. Smith, (1981) Isolation and Properties of Spore Germination Mutants of *Bacillus subtilis* 168 Deficient in the Initiation of Germination. *J Gen Microbiol* 124: 229-241.

Schneerson, R., J. Kubler-Kielb, T.-Y. Liu, Z.-D. Dai, S. H. Leppla, A. Yergey, *et al.*, (2003) Poly(gamma-d-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of *Bacillus anthracis*: A potential addition to the anthrax vaccine. *Proc Nat Acad Sci USA* 100: 8945-8950.

Scobie, H. M., G. J. A. Rainey, K. A. Bradley & J. A. T. Young, (2003) Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc Nat Acad Sci USA* 100: 5170-5174.

Scorpio, A., T. Blank, W. Day & D. Chabot, (2006) Anthrax vaccines: Pasteur to the present. *Cell Mol Life Sci* 63: 2237-2248.

Setlow, B., S. Atluri, R. Kitchel, K. Koziol-Dube & P. Setlow, (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: 3740-3747.

Setlow, B., A. E. Cowan & P. Setlow, (2003) Germination of spores of *Bacillus subtilis* with dodecylamine. *J Appl Microbiol* 95: 637-648.

Setlow, B., E. Melly & P. Setlow, (2001) Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. *J Bacteriol* 183: 4894-4899.

Setlow, B. & P. Setlow, (1993) Dipicolinic Acid Greatly Enhances Production of Spore Photoproduct in Bacterial Spores upon UV Irradiation. *Appl Environ Microbiol* 59: 640-643.

Setlow, P., (1975a) Identification and localization of the major proteins degraded during germination of *Bacillus megaterium* spores. *J Biol Chem* 250: 8159-8167.

Setlow, P., (1975b) Protein metabolism during germination of *Bacillus megaterium* spores. II. Degradation of pre-existing and newly synthesized protein. *J Biol Chem* 250: 631-637.

Setlow, P., (1992) DNA in dormant spores of *Bacillus* species is in an A-like conformation. *Mol Microbiol* 6: 563-567.

Setlow, P., (1995) Mechanisms for the Prevention of Damage to DNA in Spores of *Bacillus* Species. *Annu Rev Microbiol* 49: 29-54.

Setlow, P., (2001) Resistance of spores of *Bacillus* species to ultraviolet light. *Environ Mol Mutagen* 38: 97-104.

Setlow, P., (2003) Spore germination. *Curr Opin Microbiol* 6: 550-556.

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

Shah, I. M., M.-H. Laaberki, D. L. Popham & J. Dworkin, (2008) A Eukaryotic-like Ser/Thr Kinase Signals Bacteria to Exit Dormancy in Response to Peptidoglycan Fragments. *Cell* 135: 486-496.

Singh, Y., K. R. Klimpel, C. P. Quinn, V. K. Chaudhary & S. H. Leppla, (1991) The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. *J Biol Chem* 266: 15493-15497.

Sirard, J. C., M. Mock & A. Fouet, (1994) The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. *J Bacteriol* 176: 5188-5192.

Sirisanthana, T., N. Navachareon, P. Tharavichitkul, V. Sirisanthana & A. E. Brown, (1984) Outbreak of Oral-Oropharyngeal Anthrax: an Unusual Manifestation of Human Infection with *Bacillus anthracis*. *Am J Trop Med Hyg* 33: 144-150.

Sirisanthana, T., K. E. Nelson, J. W. Ezzell & T. G. Abshire, (1988) Serological Studies of Patients with Cutaneous and Oral-Oropharyngeal Anthrax from Northern Thailand. *Am J Trop Med Hyg* 39: 575-581.

Smego, R., B. Gebrian & G. Desmangels, (1998) Cutaneous manifestations of anthrax in rural Haiti.

. *Clin Infect Dis* 26: 97-102.

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

Stanley, J. L. & H. Smith, (1963) The Three Factors of Anthrax Toxin: their Immunogenicity and Lack of Demonstrable Enzymic Activity. *J Gen Microbiol* 31: 329-337.

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

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

Stewart, G. S., K. Johnstone, E. Hagelberg & D. J. Ellar, (1981) Commitment of bacterail spores to germinate: A measure of the trigger reaction. *Biochem J* 198: 101-106.

Stragier, P. & R. Losick, (1996) Molecular genetics of sporulation in *Bacillus subtilis*. *Ann Rev Gen* 30: 297-341.

Swerdlow, B. M., B. Setlow & P. Setlow, (1981) Levels of H+ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. *J Bacteriol* 148: 20-29.

Taia, T. W., F. F. Patricia, J. L. Terrance & H. L. Alexander, (2004) Induction of opsonic antibodies to the gamma-d-glutamic acid capsule of *Bacillus anthracis* by immunization with a synthetic peptide-carrier protein conjugate. *FEMS Immunol Med Mic* 40: 231-237.

Thomas, C. & 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: 717-726.

Todd, S. J., A. J. G. Moir, M. J. Johnson & A. Moir, (2003) Genes of *Bacillus cereus* and *Bacillus anthracis* Encoding Proteins of the Exosporium. *J Bacteriol* 185: 3373-3378.

Tovar-Rojo, F., M. Chander, B. Setlow & P. Setlow, (2002) The Products of the *spoVA* Operon Are Involved in Dipicolinic Acid Uptake into Developing Spores of *Bacillus subtilis*. *J Bacteriol* 184: 584-587.

Trinchieri, G., (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3: 133-146.

Turnbull, P., R. Bohm, O. Cosivi, M. Doganay, M. Hugh-Jones, D. Joshi, *et al.*, (1999) Guidelines for the Surveillance and Control of Anthrax in Humans and Animals. 3rd edition. In. D. o. C. D. S. a. Response (ed). pp. 1-18.

Turnbull, P. C. B., (1991) Anthrax vaccines: past, present and future. *Vaccine* 9: 533-539.

Uchida, I., J. M. Hornung, C. B. Thorne, K. R. Klimpel & S. H. Leppla, (1993) Cloning and characterization of a gene whose product is a trans-activator of anthrax toxin synthesis. *J. Bacteriol.* 175: 5329-5338.

Uchida, I., T. Sekizaki, K. Hashimoto & N. Terakado, (1985) Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. *J Gen Microbiol* 131: 363-367.

Vepachedu, V. R. & P. Setlow, (2007a) Analysis of interactions between nutrient germinant receptors and SpoVA proteins of *Bacillus subtilis* spores. *FEMS Microbiol Lett* 274: 42-47.

Vepachedu, V. R. & P. Setlow, (2007b) Role of SpoVA Proteins in Release of Dipicolinic Acid during Germination of *Bacillus subtilis* Spores Triggered by Dodecylamine or Lysozyme. *J Bacteriol* 189: 1565-1572.

Warth, A. D. & J. L. Strominger, (1969) Structure of the peptidoglycan of bacterial spores: Occurence of the lactam of muramic acid. *Proceedings of the National Academy of Sciences of the United States of America* 64: 528-535.

Warth, A. D. & J. L. Strominger, (1972) Structure of the peptidoglycan from spores of *Bacillus subtilis*. *Biochemistry* 11: 1389-1396.

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

Weiner, M. A., T. D. Read & P. C. Hanna, (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. & A. M. Friedlander, (1988) Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. *Microb Path* 4: 53-69.

Welkos, S. L., T. J. Keener & P. H. Gibbs, (1986) Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infect Immun* 51: 795-800.

WHO, (1994) Anthrax control and research, with special reference to national programme development in Africa: memorandum from a WHO meeting. *Bull World Health Organ* 72.

Wilcks, A., N. Jayaswal, D. Lereclus & L. Andrup, (1998) Characterization of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. *Microbiology* 144: 1263-1270.

Wu, W., H. Mehta, K. Chakrabarty, J. L. Booth, E. S. Duggan, K. B. Patel, *et al.*, (2009) Resistance of Human Alveolar Macrophages to *Bacillus anthracis* Lethal Toxin. *J Immunol* 183: 5799-5806.

Wuytack, E. Y., S. Boven & C. W. Michiels, (1998) Comparative Study of Pressure-Induced Germination of *Bacillus subtilis* Spores at Low and High Pressures. *Appl. Environ. Microbiol.* 64: 3220-3224.

Yi, X. & P. Setlow, (2010) Studies of the Commitment Step in the Germination of Spores of *Bacillus* Species. *J Bacteriol*: JB.00326-00310.

Yuan, K., W. C. Johnson, D. J. Tipper & P. Setlow, (1981) Comparison of various properties of low-molecular-weight proteins from dormant spores of several *Bacillus* species. *J. Bacteriol.* 146: 965-971.

Zuberi, A., A. Moir & I. M. Feavers, (1987) The nucleotide sequence and gene organization of the *gerA* spore germination operon of *Bacillus subtilis* 168. *Gene* 51: 1-11.