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**LOYOLA UNIVERSITY CHICAGO**

**MORPHOGENESIS OF THE BACILLUS ANTHRACIS SPORE**

**A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE  
SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF  
PHILOSOPHY**

**PROGRAM IN MICROBIOLOGY & IMMUNOLOGY**

**BY MICHAEL MALLOZZI**

**CHICAGO, IL**

**DECEMBER 2010**

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## **CHAPTER 1**

### **INTRODUCTION**

One of the biggest challenges to life on earth is the ability to survive conditions of scarcity and adversity. From cacti which can survive for years without water (Nobel, 2003), to the myriad of microbes which thrive at temperatures exceeding 200°F (Kimura *et al.*, 2005); life, it seems, has an almost unbeatable capacity to adapt to extreme conditions. Perhaps the most tenacious of these adaptations is the ability of a group of bacteria, known as the Bacilli and Clostridia, to survive for years without food or water and to resist some of the most toxic and extreme environments known (Setlow, 2006), by forming a specialized cell-type called a spore. Far from being inert time capsules, spores have a variety of roles in the environment (Nicholson, 2002), but importantly, they are the cause of a significant threat to human health in the form of food-borne illnesses, hospital-acquired infections, and biological weapons (Nicholson, 2002).

There are a myriad of internal and external signals that govern the cell's decision to terminally differentiate into a spore (a process called sporulation) (Errington, 2003). However, starvation is probably the most-universal stimulus that induces this response (Kroos, 2007). Research in *Bacillus subtilis* (where sporulation has been best studied) has led to the discovery of a complex network of two-component signal transduction

systems, sigma factors, and transcription factors which act in concert to coordinate the production of a spore (Stephenson & Hoch, 2002, Kroos, 2007). My studies have focused on the assembly of the *B. anthracis* spore's outer structures which provide *B. anthracis* (and spores of many species) with many of their resistance properties (Bailey-Smith *et al.*, 2005, Boydston *et al.*, 2005, Chesnokova *et al.*, 2008, Cybulski *et al.*, 2009, Kim *et al.*, 2004, Nicholson *et al.*, 2000, Russell, 1999, Setlow, 2006).

Studies in the model organism *B. subtilis* have revealed that several distinct sets of protective structures that provide resistance to such diverse assaults as UV and ionizing radiation, heat, enzymatic degradation, toxic chemicals, and microbial predators (Carroll *et al.*, 2008, Klobutcher *et al.*, 2006, Setlow, 2006), allowing spores to survive almost any imaginable assault during their dormancy. The inner-most structure of the spore is the core, which contains the spore's chromosome. The chromosome is protected from heat and UV radiation by the action of small DNA-binding proteins known as SASPs (Fairhead *et al.*, 1993). Surrounding the core is a specialized peptidoglycan called the cortex that sits between the germ cell wall and the forespore membrane (Henriques *et al.*, 1998). The cortex provides resistance to heat, and helps to maintain its dry state (Atrih & Foster, 1999). The cortex is protected by a proteinaceous shell made up of more than 80 proteins collectively called the coat (Kim *et al.*, 2006). The coat acts as a molecular sieve that excludes large toxic molecules like lysozyme, it soaks up the toxic activities of anti-microbial chemicals like bleach, helps regulate the return to growth (germination), and likely has as of yet undiscovered functions (Ghosh *et al.*, 2008, Ferguson *et al.*, 2007,

Giorno *et al.*, 2007, Setlow, 2006, Moir, 1981, Moir, 2006). In some species, such as *Bacillus anthracis*, the spore is surrounded by an additional structure called the exosporium (Lewis, 1934, Hachisuka *et al.*, 1966). The purpose of the exosporium remains the topic of active investigation, but it has been shown to have roles in adherence, regulating germination, and facilitating pathogenesis (Chen *et al.*, Driks & Mallozzi, 2009).

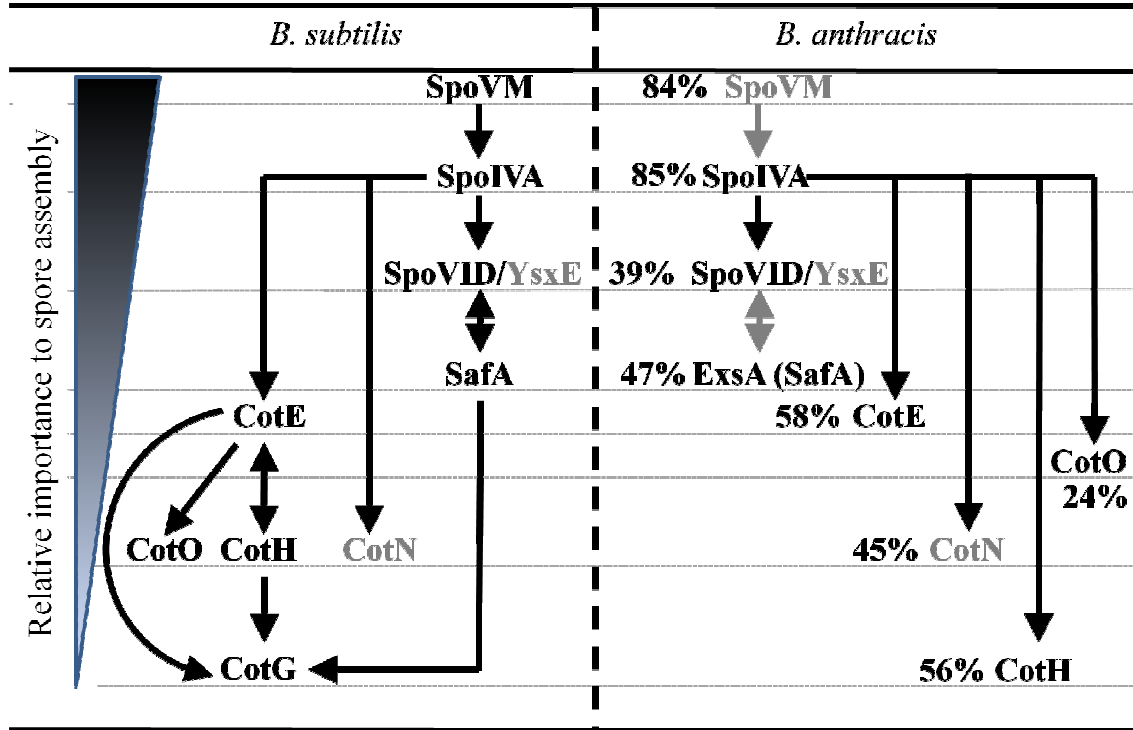
Our studies focused on the coat and exosporium of *B. anthracis* (Chada *et al.*, 2003, Giorno *et al.*, 2007, Giorno *et al.*, 2009, Lai *et al.*, 2003, Mallozzi *et al.*, 2008). There are about 80 proteins that have been identified in the coat and exosporium in *B. anthracis* (Driks, 2009). To organize these numerous proteins into a useful structure, spores rely on the action of just a handful of proteins. These so-called morphogenetic proteins are organized into a hierarchy of importance to spore assembly and act by controlling the assembly of many other proteins to the spore (Fig. 1) (Roels *et al.*, 1992, Levin *et al.*, 1993, Beall *et al.*, 1993, Takamatsu *et al.*, 1999, Zheng *et al.*, 1988, Naclerio *et al.*, 1996, Sacco *et al.*, 1995, McPherson *et al.*, 2005, Kim *et al.*, 2006). In the absence of a morphogenetic protein spores develop several morphological defects (as their name implies) visible by electron microscopy. While the organization and function of morphogenetic proteins has been extensively studied in *B. subtilis* little was known about how or if these proteins functioned similarly in other species. We wished to analyze the function of several orthologues of known morphogenetic proteins in *B. anthracis* to attempt to elucidate the program of assembly in another genetically tractable species.

This has led us to a deeper understanding of the way in which the program of assembly can vary from species to species and gives insights into the evolution of spore structure and function. In addition, we and others have identified a smaller number of proteins that constitute (and contribute towards) the proper assembly of the exosporium (Giorno *et al.*, 2009, Steichen *et al.*, 2003, Steichen *et al.*, 2005, Todd *et al.*, 2003, Sylvestre *et al.*, 2005, Sylvestre *et al.*, 2002, Thompson *et al.*, 2007, Waller *et al.*, 2005, Chesnokova *et al.*, 2008, Lai *et al.*, 2003, Redmond *et al.*, 2004, Severson *et al.*, 2009, Boydston *et al.*, 2006). Many of these proteins appear to be conserved solely in the *B. cereus* group of species (which includes *B. anthracis* and its close relatives) suggesting the formation of the exosporium is a clade specific adaptation.

To begin the analysis of spore coat and exosporium assembly in *B. anthracis* we generated mutations in six morphogenetic proteins (*spoIVA*, *spoVID*, *cotE*, *cotH*, and *cotO*) ((Giorno *et al.*, 2007) and results herein). All of these mutations had significant impact on the morphology of the resulting strains' spores suggesting that morphogenetic protein function is broadly-speaking conserved among species. Interestingly, however, the similarity in function and sequence between the protein orthologues in *B. subtilis* and *B. anthracis* varied more for morphogenetic proteins lower in the hierarchy than those present at the top positions in the hierarchy (Fig. 1). For example, the morphogenetic protein highest in the hierarchy (SpoIVA) is 85% identical to SpoIVA in *B. subtilis* and has a nearly identical function in *B. anthracis*. In contrast, CotO (a protein lower in the hierarchy) is only 24% identical to CotO in *B. subtilis* which controls the assembly of the

outer coat in *B. subtilis* (Fig. 1). However, CotO appears to be dispensable for coat assembly in *B. anthracis*, but is required for the assembly of the exosporium. Taken together, these findings suggest that proteins more fundamental to spore assembly (like SpoIVA) are highly conserved and are nearing the completion of their evolution, while those that are more auxiliary are still undergoing significant evolutionary flux.

As the sophistication of our understanding of the molecular biology of various processes in model organisms improves it has highlighted the deficiencies of our understanding of how these processes evolved. This study is intended to be a first effort towards an evolutionary understanding of spore morphogenesis. This study suggests that morphogenetic proteins can take on novel functions as new species evolve. Perhaps, evolution of morphogenetic proteins allow for spore producing bacteria to rapidly evolve novel spore structures, morphologies, and even functions.



**Figure 1. Schematic representing the model hierarchy of morphogenetic proteins.**

A model hierarchical structure of morphogenetic proteins in *B. subtilis* (left) and *B. anthracis* (right). Bolded proteins indicate the rank of the protein was empirically determined by its importance to spore assembly. Bolded arrows indicate one protein's control on another for assembly. Gray arrows and protein names indicate the presumed control for assembly and rank within the *B. anthracis* hierarchy based on the *B. subtilis* model. The role and rank of YsxE in the hierarchy has not yet been determined in either species, and its rank is hypothetically indicated in grey above. Percentages indicate the percent of amino acid identity that the *B. anthracis* homologues share with the *B. subtilis* proteins.

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **Spore-forming Bacteria**

Many if not all bacteria have evolved developmental pathways that allow them to adapt to constantly changing environmental conditions. These adaptations allow them to perform a variety of functions including the ability to: enter into pathogenic or symbiotic relationships with plants, animals, and insects (Moran, 2006), live in complex and diverse microbial communities (Torsvik & Øvreås, 2002), and even survive for eons without food or water (Cano & Borucki, 1995, Vreeland *et al.*, 2000). The (latter) developmental pathway that allows certain types of bacteria to form spores (called sporulation) will be the focus of this review.

Several genera of bacteria have evolved mechanisms for generating hardy cell types often called spores. Some bacteria in the genera *Myxococcus*, *Streptomyces*, *Methylosinus*, *Azotobacter*, *Bdellovibrio*, and *Cyanobacteria* can form a type of spore (sometimes called a cyst or exospore) (Titus *et al.*, 1982, Socolofsky & Wyss, 1962, Vela & Peterson, 1969, Mishustin & Nikitina, 1972, Kroos, 2007). While exospores are relatively tough



(compared to vegetative cells), they are formed by a process which involves the spore budding or growing out from one end of the cell. This type of sporulation is distinct from that used by the endospore-forming group of bacteria called the Firmicutes (Lopez *et al.*, 2009). Endospore-formation is generally non-reproductive, except in rare cases (Flint *et al.*, 2005), and overall endospores are considerably more resistant than exospores (Russell, 1999). Nonetheless, sporulation in all bacteria is controlled by complex signaling cascades and gene regulatory networks that involve the establishment and exploitation of cell polarity (Flardh & Buttner, 2009, Kroos, 2007, Shapiro *et al.*, 2002). The sporulation pathway of Firmicutes has been particularly well-studied in the model organism *Bacillus subtilis* and is considered in detail below.

## **Sporulation**

The production of spores by bacteria was first discovered more than 100 years ago by Koch and Cohn (Cohn, 1875, Koch, 1876). This bacterial developmental pathway has been the focus of research ever since. The major stimulus that instigates sporulation appears to be nutrient deprivation (Kroos, 2007). The sensing of nutrient poor-conditions, and the subsequent signaling events that lead to the commitment to sporulate will be discussed below.

### *Stages of Sporulation*

Sporulation can be divided into six phases, each with specific morphological characteristics (Errington, 2003). In the initial stage of development (stage 0) the cell's chromosome is duplicated. Next, the chromosomes in developing spore-forming cell's (called a sporangia) elongate, in stage I, as the mother and daughter chromosomes are separated and their origins of replication are taken to opposite ends of the cell. In stage II, just before or at nearly the same time, a polar septum begins to form which divides the cell into one-quarter and three-quarter sized compartments. In the last step of stage II, the daughter chromosome is completely translocated into the one-quarter compartment (the forespore) and the developing septum is then closed. Following this, in stage III, the smaller forespore is phagocytosed by the larger mother cell. The completion of this phagocytosis event marks the end of stage III and the beginning of stage IV. Stage IV is characterized by the dehydration of the forespore compartment, the formation of the cortex (between the inner and outer forespore membranes), and the condensation of the forespore chromosome by small acid soluble proteins (SASPs). In stage V the coat and other outer spore structures are assembled. In stage VI (also known as maturation) the spore's spore resistance properties are obtained and spore dormancy can be broken by germination at this stage. During the last stage of development (stage VII) the mother cell wall is lysed and the spore is released into the extracellular milieu.

### *Initiation of Sporulation*

The decision to sporulate is highly regulated to prevent this terminal developmental process from being inappropriately activated. Indeed, the process of sporulation is energetically taxing, non-reversible, and does not result in reproduction. It is therefore not surprising that the cell integrates an enormous amount of information from the environment in order to enter sporulation judiciously. So far, investigation has revealed that the cell monitors, as part of its decision making process, the presence of nitrogen, carbon, and phosphorus in the environment, the population density of its siblings in the environment, and the stage in the cell cycle (Mitrophanov & Groisman, 2008). If, and only if, all of these signals support the decision to sporulate, will the commitment to sporulation be made. To sample the extracellular environment and to relay information about the status of the environment across the cell wall and thus allow for adaption, bacterial cells use a variety of signaling and cognate effector molecules known as two component signal transduction systems. The simplest of these systems operates with just two proteins: 1) a sensor kinase that becomes auto-phosphorylated in the presence of its cognate signal and 2) a response regulator which accepts a phosphate from its corresponding sensor kinase and performs its effector function as a result (usually by binding to DNA and altering gene expression) (Koretke *et al.*, 2000). The way in which a sporulating cell manages to integrate information about the nutritional status, cell-cycle, and population density is simple (it uses only one protein), and elegant (since that protein is the master regulator of sporulation (SpoOA)) (Phillips & Strauch, 2002, Tjian & Losick, 1974). In this scheme, the level of phosphorylated SpoOA (SpoOA~P) is

determined by the rate of phosphorylation of SpoOA as a direct result of the internal and external state of the cell (Sun *et al.*, 1996). If the level of SpoOA~P remains low, then the cell may decide to enter another post-exponential developmental program (biofilm formation for example) (Hamon & Lazazzera, 2001, Hamon *et al.*, 2004, Stanley *et al.*, 2003). If however, the level of SpoOA~P reaches a critical threshold, it binds to the genetic element known as the OA box which results in the upregulation of sporulation-specific genes and the repression of vegetative cell genes (Trach *et al.*, 1991). The SpoOA regulon is comprised of approximately 500 genes, 121 of these genes are under direct control of SpoOA about 40 of which are upregulated and the rest are repressed (Molle *et al.*, 2003). The SpoOA regulon also includes several other transcription-regulating proteins including the sporulation sigma factors  $\sigma^E$  and  $\sigma^F$  (Molle *et al.*, 2003). The activation of  $\sigma^E$  and  $\sigma^F$  in the mother cell and forespore respectively commits the cell to sporulation (Dworkin & Losick, 2005).

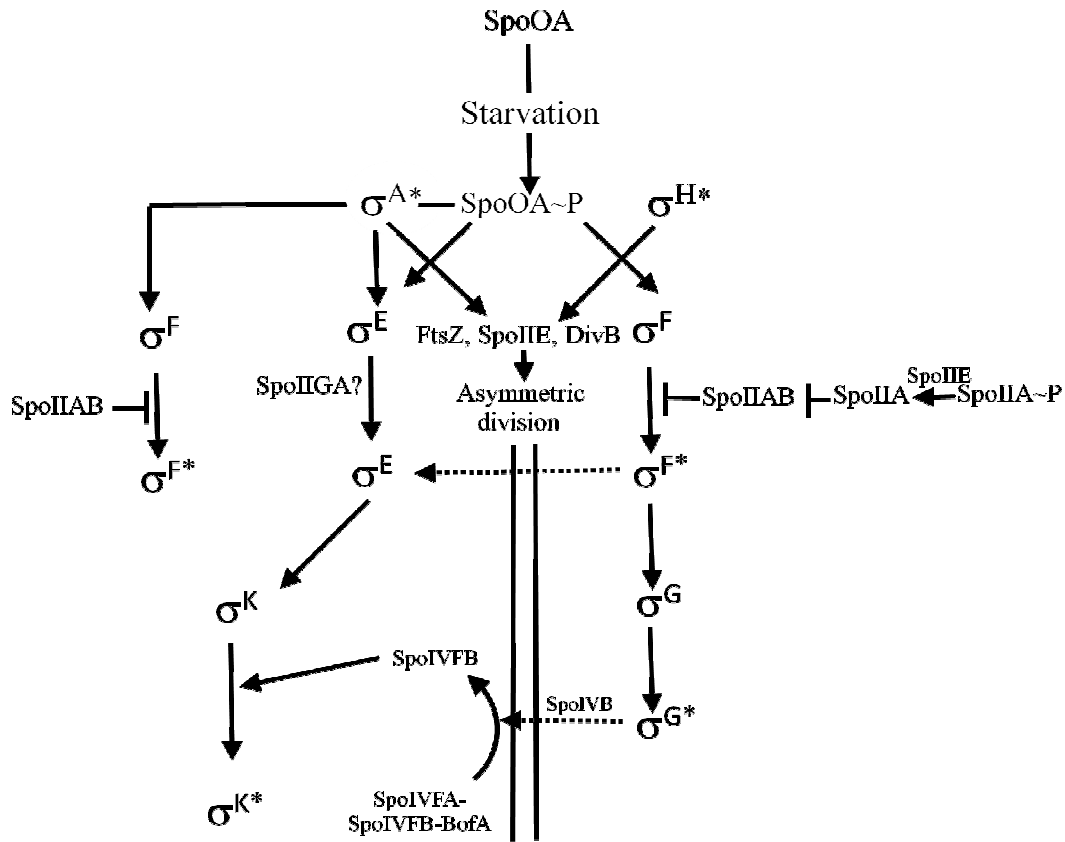
### *The sigma factor cascade*

The elucidation of the way in which the cell organizes the production of a spore has been the focus of research for over a century (Koch, 1876). The process, as it's now understood, requires the action of six sigma factors that control gene expression in the post-exponential vegetative cell, the forespore, and the mother cell respectively (see Fig. 2 for an overview). As mentioned above, SpoOA~P is responsible for activating the

expression of  $\sigma^E$  and  $\sigma^F$  which are held in an inactive state until they are activated specifically in the mother cell and forespore respectively (Dworkin & Losick, 2005). The production of  $\sigma^E$  also requires the house-keeping sigma factor ( $\sigma^A$ ), while the production of  $\sigma^F$  requires  $\sigma^H$  (a transition-state sigma present in post-exponential state cells) (Fig. 2). Together SpoOA~P,  $\sigma^H$ , and  $\sigma^A$  drive the expression FtsZ, SpoIIE, and DivIB: key factors that drive the formation of the polar septum (Fig. 2). The formation of the septum itself helps to drive the activation of  $\sigma^F$  in the forespore by way of SpoIIE. SpoIIE specifically activates an anti-sigma factor antagonist (SpoIIA) in the forespore by dephosphorylating it. Since SpoIIA is unstable and is, by definition, in less abundance in the forespore (as compared to the mother cell)  $\sigma^F$  is more likely to become activated in the fore spore. Additionally, since the gene encoding SpoIIAB is located in the mother cell side just after cell division (and is one of the last to be translocated into the forespore), the production of new SpoIIAB is confined to the mother cell and helps to keep  $\sigma^F$  inactive. These mechanisms lead to the specific activation  $\sigma^F$  in the forespore and leads to the activation and production of the other cell-specific sigma factors. The second cell-specific sigma factor to become active is  $\sigma^E$ , in the mother cell. Like  $\sigma^F$ ,  $\sigma^E$  is held in an inactive state until the formation of the forespore septum. However, unlike  $\sigma^F$ ,  $\sigma^E$  is synthesized as an inactive pro-protein which is cleaved by the action of an unknown protease (LaBell *et al.*, 1987, Stragier *et al.*, 1988). Nevertheless, it is likely that this protease is SpoIIGA because 1) it is required for the cleavage of pro- $\sigma^E$  (Kenney & Moran, 1987) and 2) because mutations in *spoIIGA* can suppress poorly cleaved alleles of

pro- $\sigma^E$  encoded by mutant alleles of *spoIIGB* (Stragier *et al.*, 1988). The  $\sigma^E$  regulon (discussed in more detail below) is made up of 253 genes (many of them coat protein genes) (Eichenberger *et al.*, 2003), and activation of  $\sigma^E$  is required for the subsequent activation of  $\sigma^G$  in the forespore. Until recently, the mechanism by which the  $\sigma^E$ -dependent activation of  $\sigma^G$  in the forespore was something of a black-box, with the only clues being mired in the interpretation of complicated genetic and biochemical experiments (Illing & Errington, 1991, Kellner *et al.*, 1996, Guillot & Moran, 2007, Londoño-Vallejo, 1997, Sun *et al.*, 2000). However, this year, two groups have developed similar models in which the activation of  $\sigma^G$  in the forespore relies on the formation of a channel connecting the cytoplasm of the forespore with that of the mother cell (Camp & Losick, 2009, Meisner *et al.*, 2008). In the model proposed by Camp and Losick the formation of the channel connecting the mother cell and forespore provides supportive care to the forespore via the introduction of small molecules (like nucleotides, amino acids, or ATP) required for the macromolecular synthesis necessary for forespore development, and indirectly, for the activation of  $\sigma^G$  (Camp & Losick, 2009). In contrast to this “feeding tube” model proposed by Camp and Losick, Meisner and colleagues propose that a specific regulator of  $\sigma^G$  crosses the channel and leads to  $\sigma^G$  activation and the subsequent development of the forespore (Meisner *et al.*, 2008). No matter which model proves to be correct, the identification of the role of the channel in this process is a fundamental step forward in the understanding of spore development.

The final sigma factor to be activated is the mother cell sigma,  $\sigma^K$ , an event which requires the  $\sigma^G$ -mediated expression of a single gene (*spoIVB*) in the forespore. The insertion of SpoIVB into the forespore triggers its auto-proteolysis and the release of proteolytic fragments into the intermembrane space (Wakeley *et al.*, 2000). These fragments act as signaling molecules which bind to a complex of three proteins (SpoIVFA-SpoIVFB-BofA) (Rudner & Losick, 2002), and triggers the release of the pro- $\sigma^K$  protease (SpoIVFB) (Wakeley *et al.*, 2000). Like  $\sigma^E$ ,  $\sigma^K$  also directs the expression of many of the proteins that make up spores outer layers (discussed below) (Eichenberger *et al.*, 2004).



**Figure 2. A schematic of sigma factor activation during sporulation (adapted from (Kroos, 2007)).**

Arrows indicate the activation ( $\rightarrow$ ) or inhibition ( $--|$ ) of one factor by another. Dashed arrows indicate signaling events that cross the polar septum. Asterisks indicate the active form of sigma factors. The ~P signifies that a protein is phosphorylated. The question mark after SpoIIIGA is meant to represent some ambiguity about the identity of the pro-sigma(E) protease which is likely to be SpoIIIGA (see text for details).



### *Expression of Outer-spore Proteins*

Approximately 80 proteins make up the *B. subtilis* coat (Kim *et al.*, 2006). It is similarly believed that the outer-structures of many species have equally complex compositions (Giorno *et al.*, 2007, Driks, 2009). In many cases (consider the flagellum, the viral particle, or the photosynthetic unit of Cyanobacteria), the cell's strategy for building complex macromolecular structures is to divide steps in assembly by controlling the timing of expression of genes important to the process. Likewise, the assembly of the coat in *B. subtilis* (where it's best studied), is activated by two master regulators ( $\sigma^E$  and  $\sigma^K$ ) and two transcription factors (SpoIID and GerE) (Eichenberger *et al.*, 2004). Therefore, the expression of outer-spore proteins occurs in four waves of gene expression under the control of  $\sigma^E$ , SpoIID,  $\sigma^K$ , and GerE respectively. Microarray studies in *B. anthracis* suggest that the organization of spore coat and exosporium genes is similarly organized, but the data from this organism are not as detailed (Bergman *et al.*, 2007, Bergman *et al.*, 2006, Passalacqua *et al.*, 2007).

About 13% (33 genes) of 253 genes that make up the  $\sigma^E$  regulon are coat proteins (Eichenberger *et al.*, 2004). It is noteworthy that all of the proteins critical to the development of the coat in *B. subtilis* (the so-called morphogenetic proteins – discussed in detail below) are expressed under the control of  $\sigma^E$  (Eichenberger *et al.*, 2004). Possibly, the expression of these morphogenetic proteins is required at this early stage of

coat assembly in order for the development to proceed normally. This conjecture is supported by the finding that dis-regulated late-expression of one of these proteins (CotE) causes defects in the morphology of spores (Costa *et al.*, 2007). In addition to activating the expression of important coat protein genes,  $\sigma^E$  (in cooperation with SpoIID) also activates the expression of  $\sigma^K$  which controls the expression of the remaining coat protein genes (Eichenberger *et al.*, 2004).

The  $\sigma^K$  regulon of *B. subtilis* though smaller than that of  $\sigma^E$  (comprised of 144 genes), has a larger proportion of the regulon dedicated to coat protein expression (21% vs. 13% respectively). Together  $\sigma^K$  and GerE are responsible for the expression of 30 coat proteins (Kim *et al.*, 2006, Eichenberger *et al.*, 2004). Many of these appear to be located in the outer coat (Kim *et al.*, 2006), suggesting that the expression of these proteins is timed to occur after inner coat assembly has completed. Intriguingly, seven genes (*cotE*, *cotH*, *cotB*, *cotG*, *cotX*, *cotY*, and *cotZ*) appear to be under the control of both  $\sigma^K$  and  $\sigma^E$  (Eichenberger *et al.*, 2004, Eichenberger *et al.*, 2003). This suggests that these coat proteins might be required at two separate stages of coat development (once early and once late), or that they might be present in two locations within the coat (in the inner and outer coats).

## Spore Structures

Prior to the advent of electron microscopy (EM), little was known about the detailed structure of bacterial spores. Indeed, without sub-light resolution, the imaging of spores could not be much improved from those images obtained by Koch more than 100 years ago (Koch, 1876). The first images of spores obtained by thin-section and scanning EM revealed a host of intricate structures which surround spores (Holt & Leadbetter, 1969, Gerhardt & Ribi, 1964, Knaysi *et al.*, 1947, Kawasaki *et al.*, 1969, Hodgkiss & Ordal, 1966, Hodgkiss *et al.*, 1967). More recently, cryo-EM, atomic force microscopy and freeze-etch microscopy have revealed ever more intricate detail of these structures first visualized by traditional TEM and SEM (Ball *et al.*, 2008, Chada *et al.*, 2003, Plomp & Malkin, 2009, Plomp *et al.*, 2005a, Plomp *et al.*, 2005b, Plomp *et al.*, 2005c, Mallozzi *et al.*, 2008, Giorno *et al.*, 2007).

### *The Core*

The interior-most of the spore's structures (called the core) contains the spore's chromosome. Detailed structure of the core interior remains an area of active investigation, but has been difficult to resolve using electron microscopy, probably due to its high electron density. We do know that the core is highly desiccated, and that the chromosome is wound into highly compacted chromatin (Pogliano *et al.*, 1995). The

chromosome is protected in this state by small acid soluble proteins (SASPs) that saturate the DNA and are responsible for protecting the DNA from damage due to UV radiation (Setlow, 2006, Setlow, 2007). Advances in cryo-EM techniques (and a new crystal structure) have revealed the spore chromatin is tightly packed into nucleoprotein helices, each packed into four-helix bundles, and which ultimately form the chromosome into a torus (Frenkiel-Krispin *et al.*, 2004, Lee *et al.*, 2008). In many cases this torus appears to occupy the periphery of the core leaving the remaining space packed with ribosomes, which can be visualized as darkly staining particles in the core's center (Frenkiel-Krispin *et al.*, 2004). Surrounding the core (and just outside of the germ-cell wall) is the spore cortex.

### *The Cortex*

The spore cortex is a specialized peptidoglycan which is synthesized between the inner and outer membranes of the forespore (Foster & Popham, 2001). The structure and make up of bacterial peptidoglycan is highly conserved in all bacteria, but is probably best-studied in *B. subtilis* (the gram-positive model organism) and is discussed in detail below. The cortex provides structural integrity to the spore (not unlike the peptidoglycan present in the cell wall), but also provides spores resistance to heat (Setlow, 2006, Nicholson *et al.*, 2000). Like cell wall peptidoglycan, present in the germ cell wall, the cortex peptidoglycan is a polymer of alternating N-acetylglucosamine and N-acetyl-muramic

acid residues connected with adjacent glycan strands via peptide bridges (Warth & Strominger, 1969). The characteristic feature of cortex peptidoglycan is the presence of muramic-d-lactam residues in place of about half of the N-acetyl-muramic acid ones (Popham, 2002). The peptides extending from the N-acetyl-muramic acid residues are anywhere from one L-alanine residue to a five amino acid peptide (Warth & Strominger, 1969). In *B. subtilis* the peptides are made from L-alanine, D-alanine, D-glutamic acid, and *meso*-diaminopimelic acid (Dpm) (Atrih *et al.*, 1996). These peptide bridges (between different glycan strands) are connected by trans-peptide bonds between D-ala and Dpm residues (Popham, 2002). Another characteristic feature of cortex peptidoglycan is a gradient of crosslinks (as opposed to uniform crosslinks in the germ cell wall) in which the inner-most layers of the cortex have more trans-peptide crosslinks than the outer layers do (Popham, 2002). Surrounding the cortex is a proteinaceous shell known as the coat, which protects the cortex from degradation by lytic enzymes like lysozyme (Moir, 1981).

### *The Coat*

In many species, including *B. subtilis* and *B. anthracis*, the coat is divided into a lightly-staining inner coat and a darkly-staining outer coat (Kim *et al.*, 2004, Beall *et al.*, 1993). The thickness of the *B. subtilis* coat allows for the visualization of coat features in greater detail. This resolution shows the inner coat has a more regular lamellar-like structure (in

cross-section) than the outer coat as visualized by thin-section EM (TEM) (Little & Driks, 2001). Recently, high-resolution imaging of the *B. subtilis* outer coat by atomic force microscopy has revealed that, the topological surface of the coat has a regular rodlet structure which surrounds the spore, giving it the appearance of weathered wood or impressionist brush-strokes (Chada *et al.*, 2003). Just beneath these arrays of rodlets is a honeycomb-like structure (Chada *et al.*, 2003). A distinguishing feature of the coat surface is a series of undulations or folds which extend along the long axis of the spore, which can be visualized by both SEM and AFM (Chada *et al.*, 2003). These folds appear as the spore shrinks during its dehydration (Freer & Levinson, 1967), and disappear as the spore germinates (Plomp *et al.*, 2007). In some species, such as *B. subtilis* the coat appears to be the outer-most structure surrounding spores (Driks, 2007). In others such as *B. anthracis* the coat is surrounded by an additional structure known as the exosporium (Lewis, 1934, Hachisuka *et al.*, 1966). The exosporium is separated from the coat by an electron-sparse structure known as the interspace.

### *The Interspace and Exosporium*

The exosporium as its name suggests is the outer-most structure of spores of many species. The exosporium is typically defined by its morphology (and not by its biochemical composition). Therefore, the definition has traditionally been a structure that fits loosely around the spore and is separated from the underlying structure by an

electron-sparse space known as the interspace. The composition of the interspace remains a mystery but, in *Bacillus anthracis*, careful preparation of spores visualized by deep freeze-etch electron microscopy reveal a reticular network of fibers that apparently connect the exosporium to the coat (Driks and Heuser unpublished data). The exosporium is known to be present in members of the *Bacillus cereus* group (including *Bacillus anthracis*, *Bacillus weihenstephanensis*, and *Bacillus megaterium*) (Ball *et al.*, 2008, Beaman *et al.*, 1972, Gerhardt *et al.*, 1976, Gerhardt & Ribi, 1964), but can also be found in the non-related species *Bacillus odyseyi* (La Duc *et al.*, 2004), *Brevibacillus laterosporus* (Fitz-James & Young, 1958), and several *Clostridium spp.* (Hodgkiss & Ordal, 1966). However, the exosporium has been best analyzed in members of the highly-related *B. cereus* group where much is known about its biochemical composition and its three dimensional structure (Ball *et al.*, 2008, Todd *et al.*, 2003, Lai *et al.*, 2003, Redmond *et al.*, 2004).

In the *B. cereus* group the exosporium appears to contain about 20 proteins (Giorno *et al.*, 2007, Giorno *et al.*, 2009, Todd *et al.*, 2003, Redmond *et al.*, 2004, Chen *et al.*, 2009, Driks & Mallozzi, 2009, Severson *et al.*, 2009, Steichen *et al.*, 2003, Steichen *et al.*, 2005, Steichen *et al.*, 2007). These proteins are organized into a paracrystalline-like array referred to as the basal “membrane” (it is not a lipid bilayer) which encircles the spore, and into hair-like projections that extend outward from the basal membrane, both of which can be visualized by TEM (Holt & Leadbetter, 1969, Beaman *et al.*, 1972).

When SEM is used, it becomes apparent that the exosporium is not uniformly distributed around the spore but rather extends off to one side giving it a balloon-like appearance (Gerhardt & Ribi, 1964). It was the high-resolution images of fragments and X-ray diffraction patterns of the exosporium which suggested the basal membrane is a paracrystalline array of hexagonal subunits (Gerhardt & Ribi, 1964). These findings were confirmed and extended by Ball and colleagues who showed that the basal membrane was made up of interlinked crown-like structures with three axes of symmetry (Ball *et al.*, 2008). The pores created by the crown structures were estimated to be 23-34Å, suggesting that the exosporium is relatively permeable to small molecules but is unlikely to allow for the passage of full-length proteins (Ball *et al.*, 2008). This porous array apparently serves as a scaffold upon which the hair-like projections are assembled. The hair-like fibers themselves are composed (at least in part) by the *Bacillus* collagen-like protein A (BclA) which forms collagen-like coiled-coils (Boydston *et al.*, 2005). Although the exosporium is the outer-most structure in many species, it is not always present, and thus could be considered an appendage. In fact, many other types of spore appendages have been seen in a diversity of species, which will be reviewed below.

### *Other Spore Appendages*

Although the exosporium has been the best-studied (and most commonly seen) spore appendage, there is a wealth of literature characterizing some elaborate structures present



on spores of Clostridia and Bacilli (Ball *et al.*, 2008, Beaman *et al.*, 1972, Granum & Stalheim, 2001, Hachisuka *et al.*, 1984, Iyer *et al.*, 2008, Krasil'nikov & Duda, 1966, Krasil'nikov *et al.*, 1968, Krasil'nikov *et al.*, 1973, Krasilnikov *et al.*, 1964, Krasil'nikov *et al.*, 1963, Mizuki *et al.*, 1998, Pope & Rode, 1969, Pope *et al.*, 1967, Rode, 1971, Samsonoff *et al.*, 1971, Walker *et al.*, 2007, Yolton *et al.*, 1972, Rubikas *et al.*, 1987, Yan *et al.*, 2007, Hannay, 1961, Fitz-James & Young, 1958, Fitz-James, 1962, Hodgkiss & Ordal, 1966, Hodgkiss *et al.*, 1967, Freer & Levinson, 1967, Short *et al.*, 1974, Youvan *et al.*, 1977, Duda & Makar'eva, 1977). One of the most striking examples of these structures is the ribbon-like appendages of some Clostridia species including *Clostridium* *sp. NI* and *Clostridium taeniosporum*. These species' spores have an elaborate tuft of ribbon-like protrusions that extend off of the mother-cell proximal surface of the coat and extend outwards into the mother cell cytoplasm (Krasil'nikov, Krasil'nikov & Duda, 1966, Krasilnikov *et al.*, 1964). These ribbons spread out from the base of the spore and spread out like the propellers of a helicopter, and have alternatively been described like the ribbons around a May pole (Driks, 2007). More recently, Walker *et al.*, measured the ribbon dimensions (4.5µm L X 0.5µm W X 30nm H), and showed that these ribbons are covered in hair-like fibers that extend off the surface of the ribbon-extensions (Walker *et al.*, 2007). Walker *et al.* also identified four proteins present in the ribbons. Two of these proteins are paralogues (P29a and P29b) and are 29kDa in size, one is a glycoprotein about 37kDa in size, (called GP85), and one an orthologue of the *B. subtilis* protein SpoVM (which is critical for the assembly of the coat) (Levin *et al.*, 1993, Walker *et al.*,

2007). The presence of a collagen-like region in GP85 is very reminiscent of BclA in *B. anthracis*, a collagen-like glycoprotein which makes up the hair-like fibers that extend off the surface of the exosporium (Walker *et al.*, 2007, Sylvestre *et al.*, 2002). It is tempting to speculate that GP85 may form the hair-like fibers present on the ribbon surface.

Finally, these ribbon-like appendages are not alone. In fact, a myriad of spore appendages have been described in the Bacilli and the Clostridia ranging from tubules, swords, feathers, to filaments (Ball *et al.*, 2008, Beaman *et al.*, 1972, Stalheim & Granum, 2001, Hachisuka *et al.*, 1984, Iyer *et al.*, 2008, Krasil'nikov, Krasil'nikov & Duda, 1966, Krasil'nikov *et al.*, 1968, Krasil'nikov *et al.*, 1973, Krasilnikov *et al.*, 1964, Krasil'nikov *et al.*, 1963, Mizuki *et al.*, 1998). The highly organized nature of these structures, the energy spent to make them, and the evolution that must have occurred to generate them make it highly-unlikely that these structures do not represent an adaptive advantage.

## **Spore Structure Assembly**

### *Morphogenetic Proteins*

In *B. subtilis* the coat is made up of about 80 proteins which are organized into layers in the coat. Interestingly, many coat proteins seem to be dispensable for the assembly of the

coat and do not appear to have any function that can be discerned in the laboratory. A handful of proteins, however, have dramatic effects on the assembly of this structure, and in their absence, severe aberrations in spore structure become apparent by electron microscopy. These proteins critical to assembly are known as morphogenetic proteins and they function by directing the assembly of other coat proteins to the developing coat. There are nine morphogenetic proteins that have been identified in *B. subtilis* (SpoIVA, SafA, SpoVID, SpoVM, CotE, CotO, CotH, CotG, and CotN) (Roels *et al.*, 1992, Levin *et al.*, 1993, Beall *et al.*, 1993, Takamatsu *et al.*, 1999, Zheng *et al.*, 1988, Naclerio *et al.*, 1996, Sacco *et al.*, 1995, McPherson *et al.*, 2005, Kim *et al.*, 2006), with YsxE being a possible tenth (Driks and Mallozzi (unpublished data) and (Carroll *et al.*, 2008)). These proteins are organized into a hierarchy in which the most interior of the proteins are most-important to spore development (Fig. 1).

SpoIVA and SpoVM are the first proteins to localize to the spore and are required for spore development (Roels *et al.*, 1992, Driks *et al.*, 1994, Levin *et al.*, 1993, van Ooij *et al.*, 2004). SpoIVA and SpoVM facilitate the development of the cortex and the attachment of the coat to the forespore surface (Roels *et al.*, 1992; Levin *et al.*, 1993) (Roels *et al.*, 1992, Levin *et al.*, 1993). SpoVM is a small protein (26 amino acids long) and is an amphipathic  $\alpha$ -helix (Prajapati *et al.*, 2000). The hydrophobic face of the helix binds to the forespore membrane so that the helix lies flat on surface (Ramamurthi & Losick, 2008). Next, SpoVM directs SpoIVA to the forespore surface (Price & Losick,

1999), which is most-likely mediated via an interaction with an N-terminal residue of SpoVM (Ramamurthi *et al.*, 2006). Once localized, SpoIVA polymerizes around the spore via an ATP-driven mechanism (Ramamurthi & Losick, 2008), forming a shell of protein that directs the assembly of all other coat proteins (Stevens *et al.*, 1992), including the next morphogenetic proteins in the hierarchy (SafA and SpoVID) (Costa *et al.*, 2006).

SpoVID, like SpoIVA, is required for the attachment of the coat to the spore, but *spoVID* mutant cells can still form spores with very thin coats and an ostensibly normal cortex (Beall *et al.*, 1993). As mentioned above, the YsxE might be a possible 10<sup>th</sup> morphogenetic protein that contributes to phenotypes previously associated solely with SpoVID. The *ysxE* gene sits just downstream of *spoVID* in the genomes of most *Bacillus spp.* and is predicted to be in an operon with *safA* in both *B. subtilis* and *B. anthracis*. Previous studies in *B. subtilis* (Beall *et al.*, 1993), have not clarified whether the phenotypes due to mutations in *spoVID* were in part due to polar effects on *ysxE*. In any case, SpoVID is known to interact with and helps to recruit another morphogenetic protein to the developing spore coat called SafA (Ozin *et al.*, 2000, Ozin *et al.*, 2001). The *safA* mutant phenotype is less severe than the *spoVID* mutant phenotype (Takamatsu *et al.*, 1999). *safA* mutant spores have a distinct inner and outer coat, although the outer coat is less electron-dense than in wild type spores and shards of abnormal coat appear to be attached to the coat surface (Takamatsu *et al.*, 1999). SpoVID directly interacts with

SpoIVA (Müllerová *et al.*, 2009), and SpoIVA is required for the assembly of the SpoVID (Costa *et al.*, 2006). Likewise, SpoIVA is required for SafA to be directed to the spore surface (Costa *et al.*, 2006) (possibly involving direct interaction SpoIVA (Müllerová *et al.*, 2009)). Once localized, SafA interacts directly with SpoVID (Costa *et al.*, 2006, Ozin *et al.*, 2001). Together these proteins help to stabilize the coat structure at the forespore surface (Takamatsu *et al.*, 1999, Driks *et al.*, 1994). The tri-molecular interaction between SpoIVA, SpoVID, and SafA (Müllerová *et al.*, 2009), may also help to stabilize the formation of the inner coat, providing a platform upon which the outer coat can be assembled. Specifically, SpoVID helps to stabilize CotE's localization at the coat surface, which is initially directed by SpoIVA (Driks *et al.*, 1994). In the current model of coat assembly SpoIVA, SpoVID, and SafA direct or stabilize the assembly of many if not all inner coat proteins including CotE, the next morphogenetic protein in the hierarchy (Driks *et al.*, 1994, Müllerová *et al.*, 2009, Kim *et al.*, 2006).

CotE is thought to localize to the interface between the inner and outer coat where it directs the assembly of the many if not all of the outer coat proteins and a handful of inner coat proteins (Zheng *et al.*, 1988, Kim *et al.*, 2006). In the absence of CotE the outer coat is absent but spores retain a normal looking inner coat (Zheng *et al.*, 1988). CotE has been shown to be required for the assembly of at least 24 proteins (CotA, CotI, CotR, CotW, Tgl, GerQ, CotX, CwlJ, YaaH, YknT, YlbD, YtxO, OxdD, CotO, CotS, CotH, CotSA, CotZ, , CotC, CotU, YusA, CotG, CotQ, CotB) (Kim *et al.*, 2006). Of

these, three (CotO, CotH, and CotG) have been shown to morphogenetic proteins (Kim *et al.*, 2006, McPherson *et al.*, 2005, Sacco *et al.*, 1995). Ultimately, the morphogenetic proteins provide the means to generate a robust spore structure that helps the spore to survive in the environment until it can break dormancy and return to vegetative growth.

### **Spore Functions**

Bacterial spores have a variety of roles in the environment (Nicholson, 2002). Spores have been shown to behave as pro-biotic commensal organisms in mammals (Rhee *et al.*, 2004, Rhee *et al.*, 2005, Rolfe, 2000), where they can promote the diversification of the immune repertoire and gastrointestinal health. The potential for spore-formers to undergo symbiosis appears to even be conserved in insects although the benefit to insects is less clear (Gilliam, 1978, Gilliam *et al.*, 1977), but at least one study has shown a selective advantage to insects bearing spore-forming bacteria closely related to *B. cereus* (Feinberg *et al.*, 1999). Spore have also been shown to have roles important to global processes since spore-specific enzymes play an important role in cycling manganese ions critical for ecological processes in the ocean (Tebo *et al.*, 2005). However, it seems that the primary role of spores is probably to resist environmental stresses. Spores are resistance to lysozyme, a variety of antimicrobial compounds, heat, ultraviolet and ionizing radiation (Nicholson, 2002, Nicholson & Galeano, 2003, Nicholson *et al.*, 2000,

Setlow, 2006, Setlow, 2007). Given the highly competitive and diverse environment of the soil, where many Bacilli occur, it's likely that at least some of these resistance properties evolved to resist killing by other microbial predators (Klobutcher *et al.*, 2006, Carroll *et al.*, 2008, Laaberki & Dworkin, 2008). These resistance properties may have allowed some species to survive with mammalian host and eventually cause disease. One possible explanation for the development of pathogenic spores comes from thorny plants, which could be protected from herbivores by promoting the growth and sporulation of pathogenic spore-forming species (Halpern *et al.*, 2007a, Halpern *et al.*, 2007b). This may also explain why *B. anthracis* infection can be endemic in domesticated herds (Hugh-Jones & Blackburn, 2009). Therefore, understanding the ecology, evolution, and mechanisms of resistance that *B. anthracis* spores have in the environment will likely be crucial for understanding the pathophysiology of the disease.

## **CHAPTER 3**

### **METHODS**

#### **Sporulation by Exhaustion**

Spores of *Bacillus anthracis* were generated by exhaustion sporulation in the following way. Strains to be sporulated were first struck out for single colonies on LB (Luria Broth) plates. Single colonies no older than 5 days were resuspended in 200ul of DSM (Difco Sporulation Media) broth and spread onto DSM plates then incubated overnight at room temperature or at 37°C for 6 hours. Lawns were then resuspended in 5ml of DSM broth and the OD<sub>600</sub> was measured. Resuspended cells were then used to inoculate 20-35ml of DSM broth at a final OD<sub>600</sub> of ~0.1 in 250ml Erlenmeyer flasks. Cells were then cultured at 37°C while shaking at 250rpm for 12-18hrs or until sporulation was complete as determined by microscopic observation.

#### **Extraction of Spore Proteins**

Extracts of spore proteins were prepared in the following way. 37ul of water washed dormant spores no older than 2 weeks at an OD<sub>600</sub> of 1.0 were pelleted in a 1.7ml microfuge tube and the supernatant was removed by aspiration. The spore pellet was the



resuspended in 27ul of 1X SDS-Loading Buffer (200mM Tris pH6.8, 8% SDS, 0.4% Bromophenol Blue, 40% glycerol) and 3ul of DTT by pipette and then vortexing for 1min. Spore suspensions were then alternately heated for 5min at 100°C and vortexed for 1min for a total of three cycles. Following this spores were further vortexed for 1min then were pelleted in the microfuge. 10ul of the spore extracts were loaded onto polyacrlyamide gels for SDS-PAGE.

### **Electrophoresis of Spore Proteins**

Spore extracts were resolved by SDS-PAGE using the following conditions. Spore proteins were resolved on reducing 6-12% (depending on the desired resolution) polyacrlyamide mini gels (0.75cm width) using the BioRad gel electrophoresis system. Gels were either run at constant voltage (at 100V with amperage not exceeding 20mA) or at constant amperage (at 15mA) until the loading dye had run out of the bottom of the gel.

### **Western Blots**

To analyze spore proteins by western blots spore protein extracts were electrophoresed as described above and then transferred to methanol activated PVDF membranes by electrotransferring at 100V for 2hrs or at 30V overnight in a water-cooled Owl

electrotransfer unit. Membranes were then blocked in a TBST with 5% milk. Next, membranes were probed in the 5% milk solution with the appropriate primary antibody at the concentrations described in (Giorno *et al.*, 2007, Severson *et al.*, 2009) for 1hr. Membranes were then washed for 3 times for 30min with fresh 5% milk solution and were then probed with either goat anti-mouse or goat anti-rabbit conjugated to horseradish peroxidase at a concentration of 1:20,000. Blots developed using chemiluminescent substrates as per the manufacturer's instructions (Sigma) were used to expose x-ray film for 30s to 10min until bands were easily visualized.

### **Lysozyme Resistance Assay**

To analyze spore resistance to lysozyme spores were suspended in PBS at an OD<sub>600</sub> of 1.0 in either 1cm (gap length) disposable cuvettes or in a 96 well microtiter plate. Spores were then exposed to lysozyme at a final concentration of 50ug/ml and spore optical density was measured at 600nm (in the spectrophotometer) or at 550nm in the microtiter plate reader every minute for a total of 21min or until the optical density of the spores had leveled.

### **CP51ts Mediated Transduction**

To generate a working stock of CP51ts phage, plaques were made by infecting the *Bacillus cereus* 569 indicator strain with infected spores of the same species using the soft-agar overlay technique. To do this 5mL of molten soft-agar was inoculated with 100ul of an overnight culture of *B. cereus* 569 (grown in Luria Broth) and 100ul of water-washed infected spores (containing approximately 100 plaque forming units (PFUs) of CP51). The molten agar was then mixed vigorously and laid over phage assay (PA) plates (Thorne, 1968) and allowed to solidify. Plates were then inverted and incubated overnight at 30°C and plaques too numerous to count (TNTC) were harvested from the plate by scraping up the soft agar with a sterile Pasteur pipette in 5ml of PA broth. The resuspended phage solution was vortexed rigorously and the agar was cleared from the solution by centrifugation. The supernatant was then filtered once through a 0.45µl pore size filter and subsequently filtered thru a 0.22µm pore size filter to create the working solution of phage. Next, 100µl of the donor strain (also grown overnight in LB) was infected a 1:10 dilution series of the working solution of phage in 5ml of molten soft-agar and plated onto PA plates and incubated at 30°C as before. Plates with plaques TNTC were scraped up as before with 5ml of PA broth and 100µl of the working solution of the phage prepared from the donor strain was used to infect the recipient strain grown up overnight in LB. To do this tubes containing 1ml of the recipient strain were infected with 100ul of phage solutions containing undiluted phage or a 1:10 serial dilution of

phage from the donor strain. The infections were then allowed to proceed for 30min on the bench followed by 1hr 30min in a rotisserie at 25°C. The bacteria were then pelleted in a microfuge and resuspended in 200µl of PA broth then grown overnight at 37°C on LB plates containing the appropriate antibiotic corresponding to the antibiotic resistance marker present in the donor strain.

### **Germination Assay by Loss of Optical Density**

Germination of spores was measured by the loss of optical density at 600nm in the spectrophotometer or in a plate reader at 550nm. Water washed spores were heat activated at 65°C for 15min, then resuspended in 50mM Tris-HCl (pH 7.5) at an OD<sub>600</sub> of 1.0. To initiate germination spores were exposed to a final concentration of 10% v/v BHI and the optical density was measured every minute for 30-40mins. The percent loss of optical density was calculated as follows:  $[1 - (\text{OD}_{600} \text{ at time } t / \text{OD}_{600} \text{ at time } 0)] \times 100$ .

### **Germination Assay by Tetrazolium Metabolism**

Germination of *B. anthracis* as measured by tetrazolium metabolism was measured as described in *Molecular biological methods for Bacillus* (Cutting, S. M., and P. B. Vander Horn. 1990 eds), with the following modifications. The germination media was

supplemented with L-alanine (1.7 g/liter instead of 1 g/liter for *B. subtilis*), Casamino Acids (0.4g/liter), and adenosine (1.7 g/liter).

### **Fluorescence and Immunofluorescence Microscopy of Spores and Sporangia**

To prepare slides for fluorescence and immunofluorescence microscopy, wells of a multi-well microscope slide (MP Biomedicals) were treated with a solution of 0.01% (wt/vol) poly-L-lysine (Sigma) and allowed to dry. Next, 10 $\mu$ l of purified spores or whole sporulation culture was pipette into a single well. Wells were then washed with 10 $\mu$ l of phosphate-buffered saline (PBS) and then treated with a blocking solution containing 2% (wt/vol) bovine serum albumin (in PBS) prior to the addition of primary antibody. We used the following primary antibodies: mouse immunoglobulin IgG1 control antibody (0.5 mg/ml; Pharmingen) at a 1:2,000 dilution, mouse monoclonal anti-BclA BA-Mab5 antibody (1.0 mg/ml; Critical Reagents Program) at a 1:5,000 dilution, or mouse anti-ExsK anti-sera (Knight Lab) at a 1:200 dilution. Wells were then washed 10 times with 20 $\mu$ l of PBS and then 10 $\mu$ l of a goat anti-mouse antibody conjugated to Alexa Fluor 568 (Molecular Probes) was applied at a 1:300 dilution. Wells were again washed 10 times with 20 $\mu$ l of PBS and either treated with the DNA-specific dye Hoechst 33342 (Sigma) (where appropriate), and/or treated with the anti-fade mounting media ProLong Gold (Invitrogen). Images were collected using a Leica DM IRB fluorescence microscope

equipped with a MagnaFire cryo-cooled charge-coupled-device camera and processed with Adobe Photoshop 7.0 software.

### **Thin Section Electron Microscopy**

To visualize spores and sporangia we collected 10-15ml of culture and pelleted the cells by centrifugation in a swing bucket table top centrifuge at  $\geq 15,000g$  for 10min. Pelleted cells were resuspended in 840 $\mu$ l of 10mM sodium phosphate buffer, pH7.5.

Resuspended cells were then fixed by the addition of 160 $\mu$ l of glutaraldehyde for a final concentration of 4%. Cell suspensions were then mixed and incubated overnight at 4°C.

The following day fixed cells were pelleted as before and washed twice with a 0.5M solution of  $\text{NH}_4\text{Cl}$ . After washing cells were re-pelleted and suspended in 300 $\mu$ l in the 10mM sodium phosphate buffer containing 1%  $\text{OsO}_4$ , and incubated overnight at 4°C.

After osmification the samples were washed twice with the  $\text{NH}_4\text{Cl}$  solution as before, and then embedded in 3% agarose. Next, the agarose was washed for 15min to 1hr in each of a 30%, 50%, and 70% aqueous ethanol solutions followed by three washes in 100%

ethanol. Following the ethanol dehydration the plug was washed three times (as before) with 100% propylene oxide, and then incubated in a rocker overnight in a 1:1 solution of propylene oxide and Spurr's resin (consisting of 5g of ERL 4221, 3g of DER 736, 13g of NSA, and 0.15g of DMAE). The following day the 1:1 solution was replaced with a 1:2 of propylene oxide to resin and incubated for 8hrs. Then, the plug was placed into a

solution of 100% resin and incubated, while rocking, overnight at room temp. Lastly, the resin-infiltrated sample was placed into a mold, baked overnight at 65°C, then sectioned and placed on grids for visualization in the electron microscope.

### **Flow Cytometry of Spores**

Spores prepared for flow cytometry were stained with either the mouse anti-ExsK serum or the mouse BA-Mab5 monoclonal anti-BclA antibody (at a 1:200 dilution) followed by Dylight™ 649-goat Fab anti-mouse IgG (Jackson ImmunoResearch) (at a 1:200 dilution). Samples were then analyzed using FACSCanto™ flow cytometer (Becton Dickinson) at the Loyola University Medical Center FACS Core Facility. Data analysis was conducted using the FlowJo software program (©Tree Star, Inc).

### **Renographin Purification of Spores**

Water washed spores were purified by density gradient centrifugation using Renographin-60 (a 60% Renographin solution from Bracco Diagnostics). Spores were first pelleted and then resuspended in 400µl of 20% Renographin and layered over a solution of 50% Renographin in a 1.7mL microfuge tube. The spores were then centrifuged for 10min at  $\geq 13,500$ rpm and the purified spore pellet was washed three times in de-ionized water.

## CHAPTER 4

### RESULTS

#### Spore Assembly, Maturation, and Function

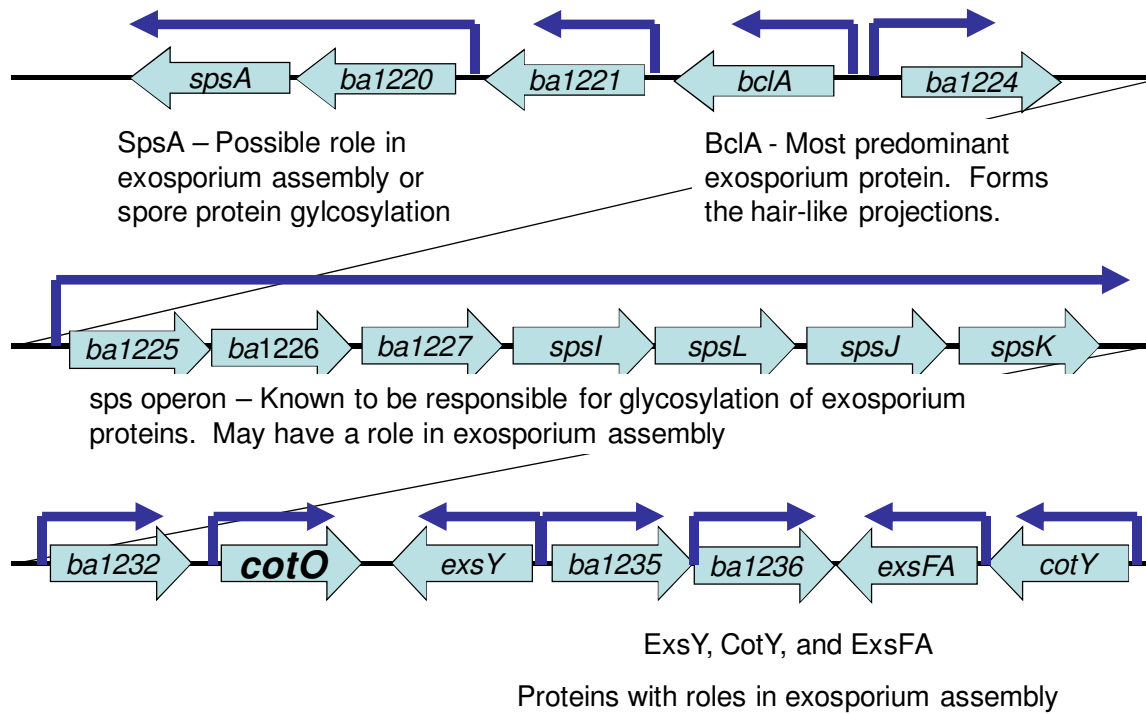
##### *Defects of Mutant Spores Lacking the Exosporium*

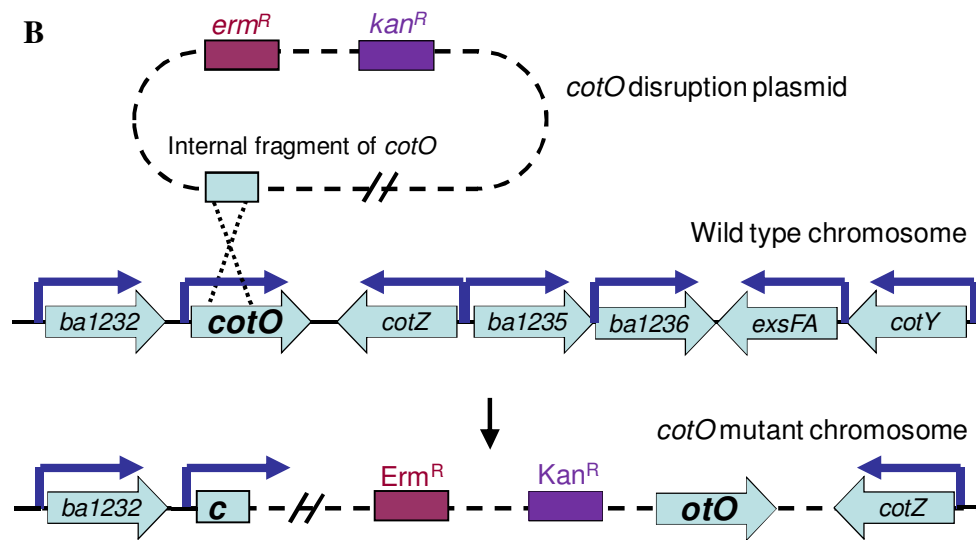
Some of the most basic questions in spore biology ask: why is there such diversity in spore-surface structures, what is their purpose, and how are they formed? The *B. anthracis* exosporium has been the focus of these questions in recent years. Of the proteins involved in exosporium morphogenesis, three (CotE, ExsY, and CotY) are orthologues of *B. subtilis* outer coat proteins (Giorno *et al.*, 2007, Boydston *et al.*, 2006, Steichen *et al.*, 2007). We wondered whether other coat protein orthologues known to be involved in spore coat morphogenesis might also be important for the assembly of the exosporium. I chose to investigate CotO, which is important for the assembly and organization of the *B. subtilis* outer coat (McPherson *et al.*, 2005), and whose *B. anthracis* orthologue sits in a cluster of genes previously shown to be important for exosporium assembly and maturation (Fig. 3A) (Boydston *et al.*, 2006, Bozue *et al.*, 2005, Johnson *et al.*, 2006, Sylvestre *et al.*, 2005, Sylvestre *et al.*, 2002, Steichen *et al.*, 2005). Therefore, Dr. Joel Bozue (USAMRIID) and I inactivated *cotO* in the Ames and



Sterne 34F2 strains of *B. anthracis* respectively, via the Campbell-type integration of plasmid pMGM3 (Fig 3B). We then analyzed the resulting strains' (MGM76 and JABAmescotO) spores by thin-section electron microscopy (TEM) (Fig. 4). We found that, unlike wild type, many *cotO* mutant spores lacked an exosporium, although all spores had an intact coat that was indistinguishable from wild types spores (Fig. 4A). To quantify the percentage of spores that lacked exosporia we stained spores with an antibody against the prominent exosporium protein BclA and visualized spores with and without exosporia using fluorescence microscopy and flow cytometry (Fig. 5). We found that about 70% of spores lacked exosporia (Fig. 5). This morphological defect is specific to the disruption of the *cotO* gene and the mutation is unlikely to be polar since: 1) the downstream open reading frame is oriented in the opposite direction (Fig. 3B), and 2) multiple independent disruptions in two different strains of *B. anthracis* gave morphological phenotypes that were indistinguishable (Fig. 4).

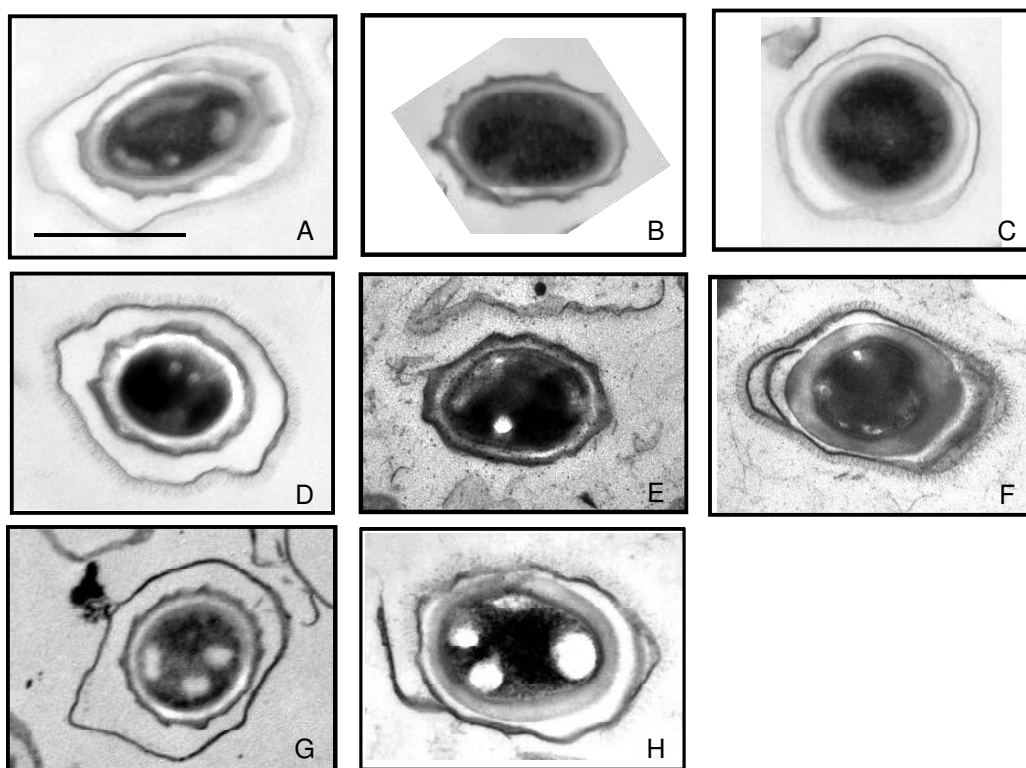
A

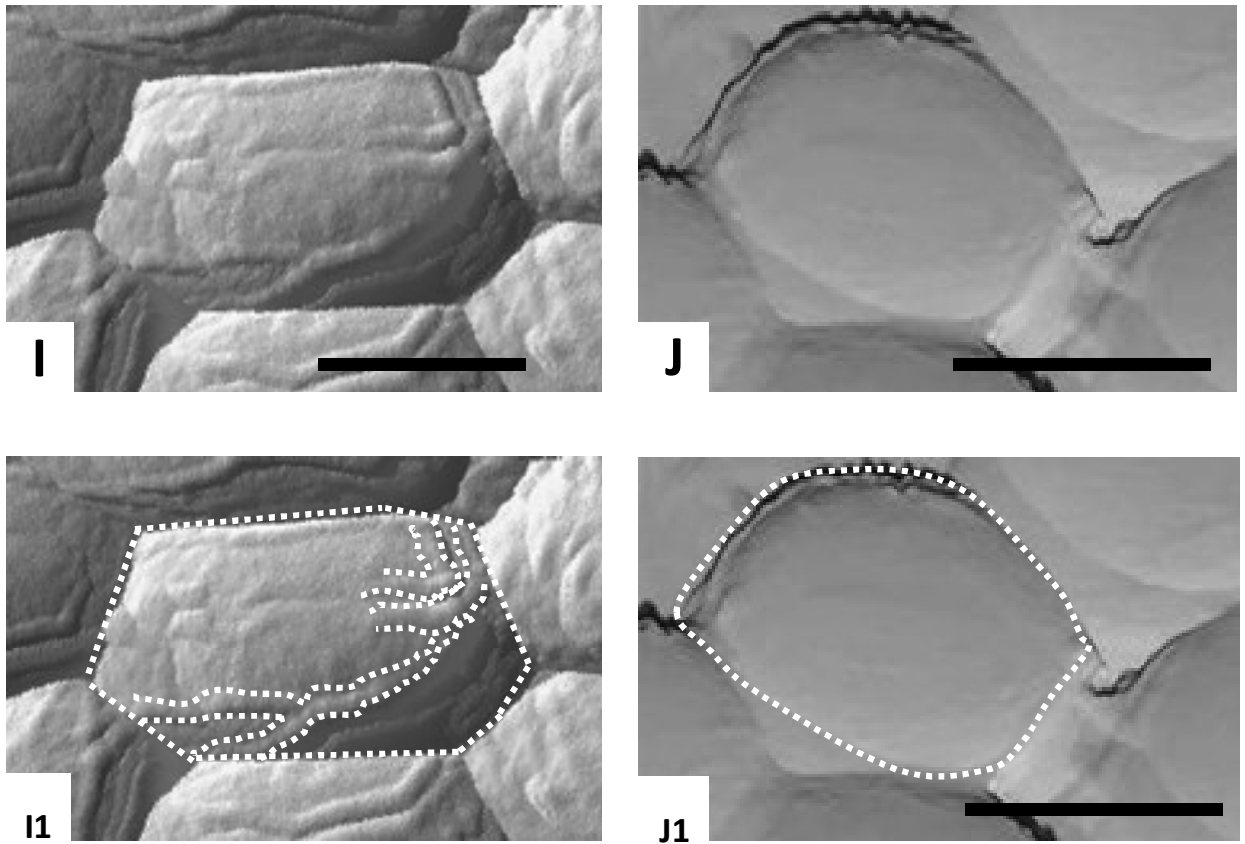




**Figure 3. The exosporium gene cluster and the disruption of *cotO*.**

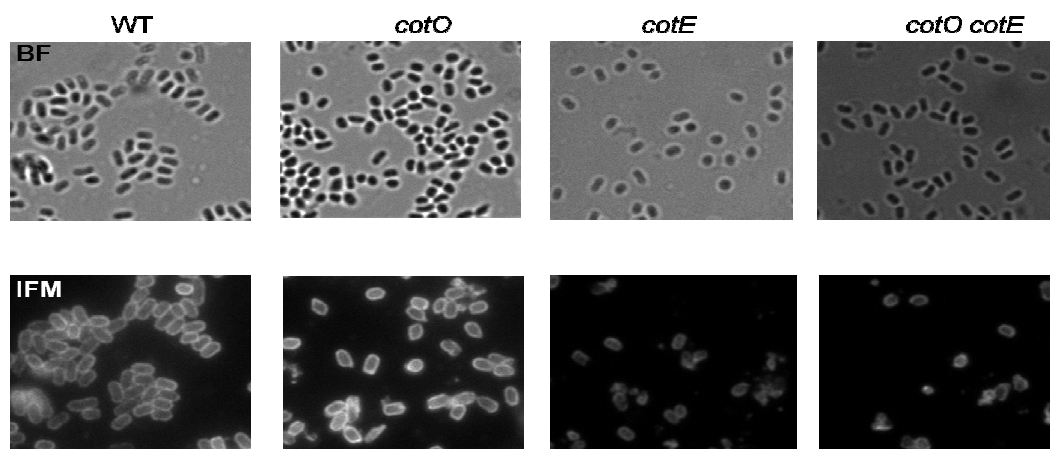
A) The function, orientation, and structure of the genes comprising the exosporium gene cluster in *B. anthracis*. B) The disruption of the *cotO* gene in strain MGM76 via the integration of plasmid pMGM3.



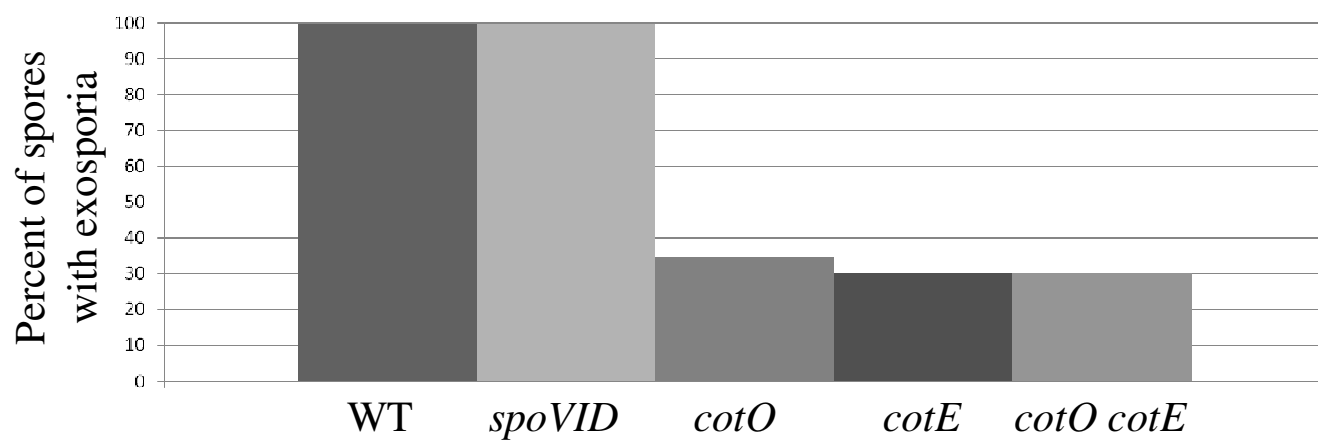


**Figure 4. Thin-section electron and atomic force micrographs of wild type and mutant *B. anthracis* spores.** Thin-section electron micrographs (A – H) and atomic force micrographs (I and J) of wild type (A and D) and mutant (B, C, E, F, G, H) Ames (A – C and G) and Sterne (D – F, and H-J) strain spores. *cotO* mutant spores (B and E) are pictured lacking the exosporium. *spoVID ysxE* mutant spores (C, F, I) are pictured lacking the coat. Bars (A, I and J) represent 100nm in all micrographs. White dotted lines in I1 and J1 outline the spore and spore ridges pictured in I and J respectively.

A



B



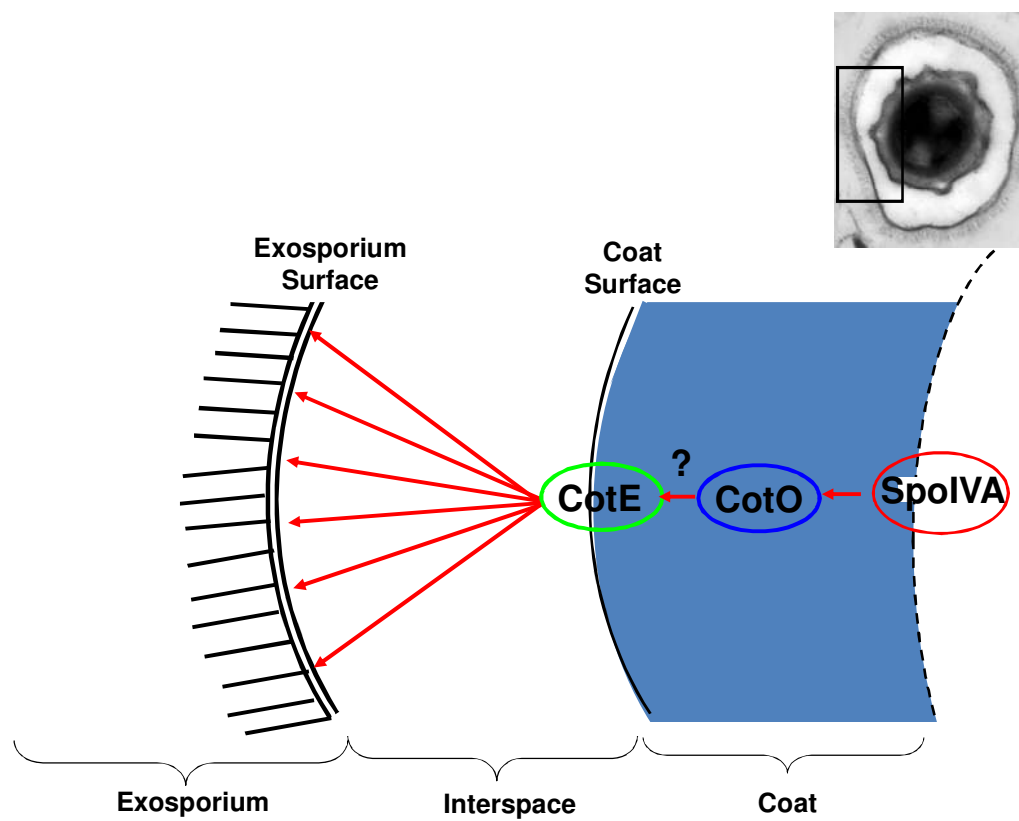
**Figure 5. Analysis of exosporium assembly in wild type and mutant spores.**

A) Corresponding bright field (BF) and immunofluorescence (IFM) micrographs of wild type (WT), *spoVID*, *cotO*, *cotE*, *cotO cotE* mutant spores stained with an anti-exosporium antibody directed against BclA. B) Combined averages of spores bearing exosporia as determined by immunofluorescence microscopy, as shown in (A) and by flow cytometry (data not shown).

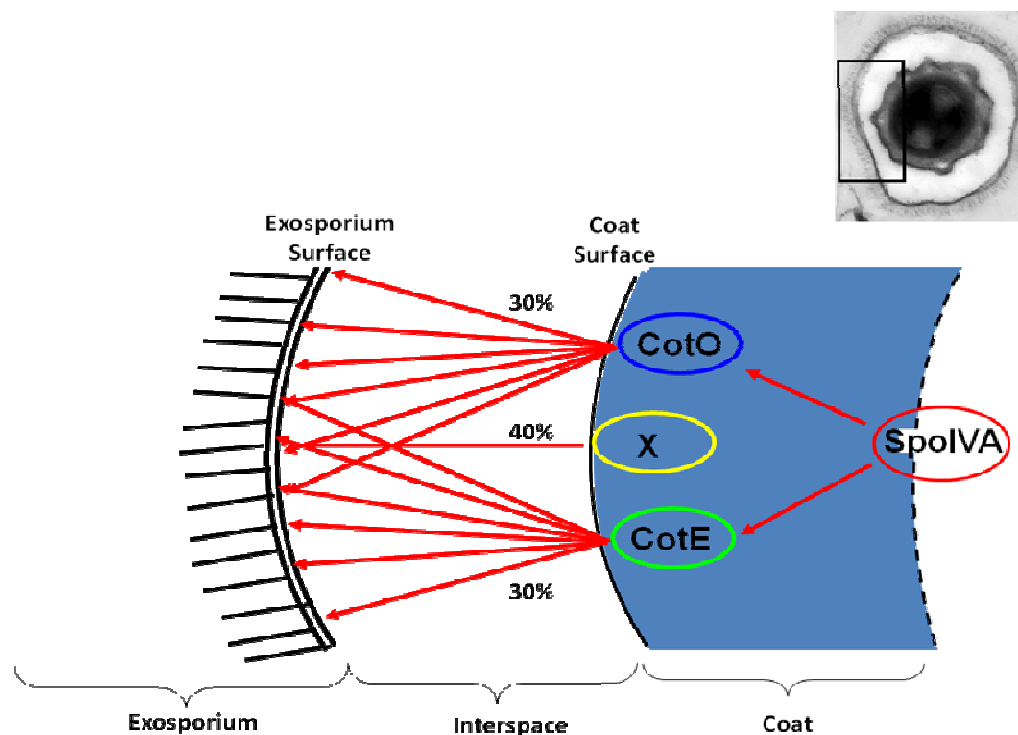
The morphological defects we observed for the *cotO* mutant spores were reminiscent of those that we previously observed in *cotE* mutant spores (Giorno *et al.*, 2007) (Fig. 4H). *cotE* mutant spores also lack exosporia, and although they have an intact coat it appears to be unfastened from the underlying spore structure (Fig. 4H) (Giorno *et al.*, 2007). These results, along with those of Boydston *et al.* and Johnson *et al.* (as mentioned above) suggested that multiple genes were involved in the assembly of the exosporium (Boydston *et al.*, 2006, Johnson *et al.*, 2006). Given this, and the striking similarity between the *cotO* and *cotE* mutant phenotypes, I hypothesized that CotO may control the assembly of CotE which, in turn, controls the assembly of the exosporium (Fig 6.). If this hypothesis were true then *cotO* mutant spores should have diminished levels or lack entirely the CotE protein. Therefore, to test this hypothesis I extracted spore proteins from wild type and *cotO* mutant spores and analyzed the extracts for the presence of the CotE protein using anti-CotE anti-sera (Fig. 7). I found that CotE was present in the *cotO* mutant spores suggesting that CotO does not control the assembly of CotE, and therefore that CotO plays a role in exosporium attachment that is independent of CotE (Fig 6B). However, the blot showed that, of the two species of CotE detected (approximately 20kDa and 23kDa); the larger species was more abundant in the mutant spores. Possibly, CotO is required for the efficient assembly of this 23kDa CotE species.



A



B



**Figure 6. Model of the role that CotE and CotO play in exosporium assembly.**

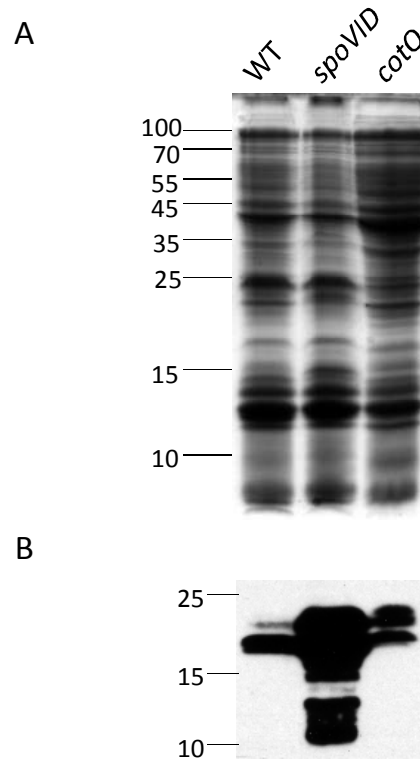
A) The diagram depicts the section of the spore indicated by a box over the spore pictured in the upper right hand corner. In this hypothetical arrangement of morphogenetic proteins SpoIVA controls the assembly of CotO, which in turn controls the assembly of CotE. Finally, CotE is required for the attachment of the exosporium. The question mark indicates the hypothesis under consideration (that CotO controls CotE's assembly to the spore). B) In this revised assembly model CotE and CotO each independently direct approximately 30% of exosporium attachment, while a third unknown factor (X) directs the remainder of exosporium attachment.

Further, the 23kDa species is required for exosporium attachment. If true, then specific species of CotE have specific functions within the spore, and their assembly is controlled by separate proteins as well. Given these results and interpretations, I wondered if I could clarify whether CotO acted in concert with, or independently from CotE, to control exosporium attachment. To test this hypothesis I generated a *cotO cotE* mutant strain by introducing the *cotO* mutation into the *cotE* mutant (RG56) via generalized transduction using the CP51ts transducing phage (Thorne, 1968), and confirmed the presence of both mutations using PCR. Next, I analyzed the extent of exosporium assembly in *cotO*, *cotE*, and *cotO cotE* mutant spores by staining the exosporium with the anti-BclA antibodies and quantifying the number of spores with and without intact exosporia using immunofluorescence microscopy (Fig. 5). I found that the percentage of spores with intact exosporia were similar in all of the mutant spore populations suggesting that CotO and CotE do not act in concert to control exosporium attachment since the absence of either or both proteins does not change the amount of spores that form without exosporia (Fig. 5). Nevertheless, these results left open the question of whether or not CotE is required for the assembly of CotO. However, this is unlikely to be the case because proteins profiles generated from *cotO* mutant spores lack a band which is consistent with the predicted molecular weight of CotO ((21kDa) see Fig. 7), and *cotE* mutant spores do not, suggesting that CotO is still present in *cotE* mutant spores. Taken together, these data suggest that although both CotO and CotE are required for robust exosporium assembly they are part of separate assembly pathways (Fig. 6). These data further

suggest that factor(s) which remain to be identified also participate in exosporium attachment since 30% of spores still have an exosporium in the absence of CotO and CotE (Fig. 6). The identification of the gene(s) involved in this third pathway of exosporium attachment will be an important step forward in the creation of a complete model of exosporium assembly.

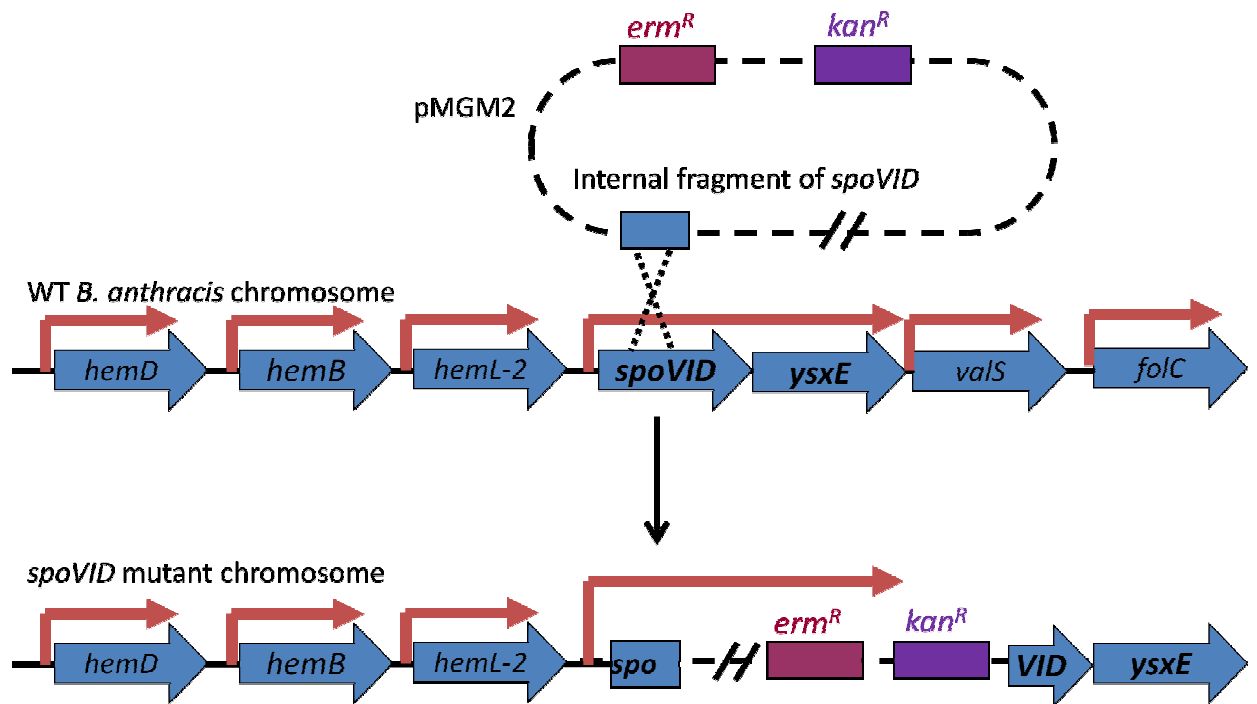
#### *Defects of Mutant Spores Lacking the Coat*

Bioinformatic analysis of the *B. anthracis* reveals that about 80% of spore coat proteins are conserved between *B. anthracis* and *B. subtilis* (Kim *et al.*, 2006, Henriques & Moran, 2007, Driks, 2009), Mallozzi and Driks (unpublished data)). Despite this high degree of similarity in composition, there are striking morphological differences between the spores of these two species. We have sought to understand how both novel spore proteins and well-conserved morphogenetic proteins contribute to these



**Figure 7. Analyses of spore protein extracts from wild type and mutant spores.**

A) Spore protein extracts of wild type, *spoVID* mutant (*spoVID*), and *cotO* mutant spores (*cotO*) separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Molecular weight markers are indicated in kilodaltons. B) Species of CotE detected by western blot from the spore extracts corresponding to the gel depicted in (A).



**Figure 8. Construction of a *spoVID* mutant.**

Disruption of *spoVID* via the Campbell-type integration of pMGM2 into the *B. anthracis* chromosome. The insertion of pMGM2 into the *spoVID* locus disrupts the operon predicted to contain *spoVID* and *ysxE*.

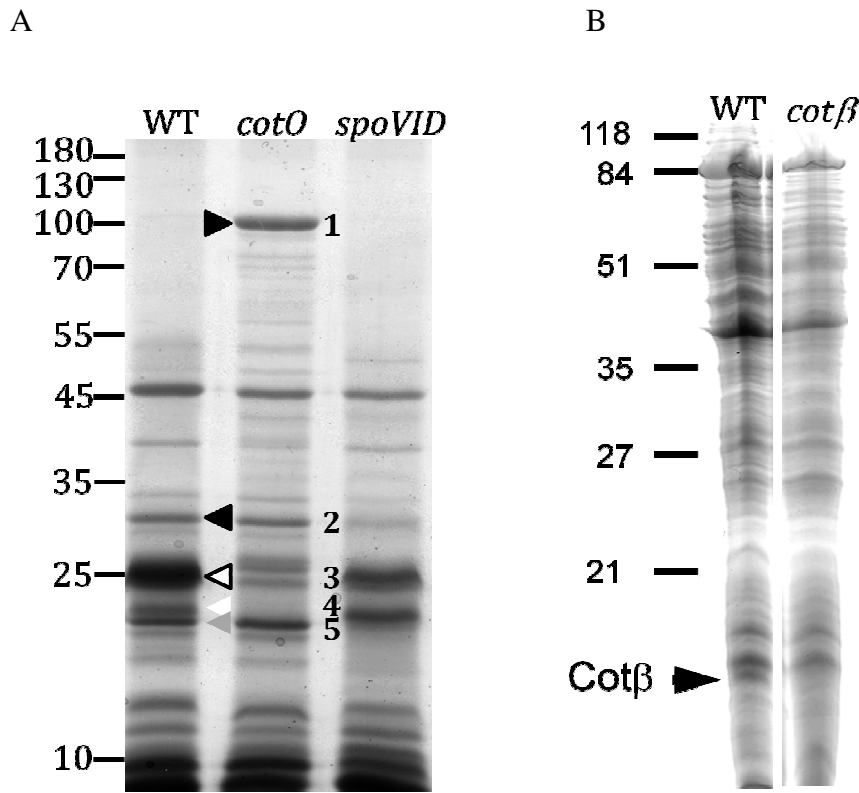
observed differences (Mallozzi *et al.*, 2008, Giorno *et al.*, 2007, Giorno *et al.*, 2009, Lai *et al.*, 2003, Severson *et al.*, 2009). To further our goal of understanding of how well-conserved morphogenetic proteins function in a variety of species we chose to investigate the role that *spoVID* has in *B. anthracis* spore assembly. To do this we generated a plasmid (pMGM2) that integrates into the chromosome of the Sterne 34F2 and Ames strains of *B. anthracis* and insertionally-inactivates *spoVID* generating strains MGM84 and JABAmess*spoVID**ysxE* respectively (Fig. 8). It should be noted that this mutation is likely to be polar on the downstream open reading frame which is an orthologue of the *B. subtilis* spore protein YsxE. To examine the effect of this mutation on the appearance of spore structure Dr. Bozue and I examined the Ames and Sterne mutant spores (respectively) by thin section electron and atomic force microscopy (Fig. 4). TEM of spores from both mutant strains (MGM84 and JABAmess*spoVID**ysxE*) revealed that the spores did not have an identifiable coat, and the cortex appeared thicker than usual (Figs. 4C and F). Despite this severe morphological defect all Sterne mutant spores had exosporia (as determined by TEM and IFM (Figs. 4C and 5). The presence of exosporia was unexpected since at least one coat protein (CotE) is known to be important for the attachment of the exosporium to the coat (Giorno *et al.*, 2007). We therefore wanted to determine if CotE was present in *spoVID* mutant spores by analyzing mutant spore extracts (from MGM84) using an anti-CotE antibody via western blot (Fig 7B). We found that *spoVID* mutant spores contained an abundance of CotE protein although it migrated as multiple species ranging from 10-23kDa in contrast to the two species

previously detected in wild type spores ranging in size from 20-23kDa (Fig 7B). This finding suggested that although the coat structure was not visible by TEM at least some coat proteins were present in the spore. Furthermore, it suggested CotE is not post-translationally modified in *spoVID* mutant spores as it is in wild type spores. It's possible that this lack of CotE modification contributes to *spoVID* phenotypes. The potential roles of the post-translational modification of CotE has in spore assembly will be discussed Chapter 5. To analyze the *spoVID* mutant morphological phenotype in more detail we imaged mutant spores from strain MGM84 by atomic force microscopy (AFM) (Fig 4J). AFM produces topological relief maps of the spore surface (Chada *et al.*, 2003, Plomp *et al.*, 2005a, Plomp *et al.*, 2005b, Plomp *et al.*, 2005c, Wang *et al.*, 2007). We found that the *spoVID* mutant spores have a smooth appearance (Fig. 4J), in contrast to the wild type spores which have a characteristic pattern of ridges that extend the length of the spore (a feature of the coat) (Fig 4I and (Wang *et al.*, 2007, Plomp *et al.*, 2005a, Chada *et al.*, 2003)). These results suggest that although coat proteins are present in *spoVID* mutant spores they are not assembled into an organized structure. This interpretation is supported by the finding that *B. subtilis* spores which have an exposed cortex due to the loss of most of the coat layers (as a result of mutations in *cotE* and *gerE*) also appear smooth by atomic force microscopy (Ghosh *et al.*, 2008). Taken together, these data suggest that the *spoVID* mutation leads to the loss of coat structural integrity in *B. anthracis* and the exposure of the underlying cortex.



*Identification and Assembly of Novel Spore Proteins*

To gain a better understanding of the molecular basis for the morphological and functional defects that we observed for *spoVID* and *cotO* mutant spores, we wished to identify changes in spore-protein composition that occurred as a result of the mutations. Identifying the proteins that depend on CotO and SpoVID for their assembly to the spore is the first step towards a complete model of spore assembly, the role of the spore structures in germination and disease, and will be the focus of future work. To analyze spore-protein composition we extracted proteins from wild type Sterne, *cotO* mutant (MGM76), and *spoVID* mutant (MGM84) spores by boiling them in an SDS-containing buffer as previously described (Little & Driks, 2001), and separated the proteins by 1D SDS-PAGE followed by staining with Coomassie Brilliant Blue (Fig. 9). This analysis identified two protein-bands that were missing or were dramatically reduced in *cotO* mutant spores (bands 3 and 4, Fig. 9), and one novel band (band 1, Fig. 9). *spoVID* mutant spores also showed a reduction in the proteins present in bands 2 and 3, and lacked or had undetectable levels of protein present in band 5 (Fig. 9). To determine which proteins are present in the aforementioned bands, we excised the bands present in the wild type extract that corresponded to the band that was missing or reduced in the mutant spore extracts (and the novel band present in the *cotO* mutant spores) and used MALDI-TOF mass spectrometry to identify the proteins. The results of this analysis are summarized in Table 1.



**Figure 9. Spore extracts analyzed by mass spectrometry.**

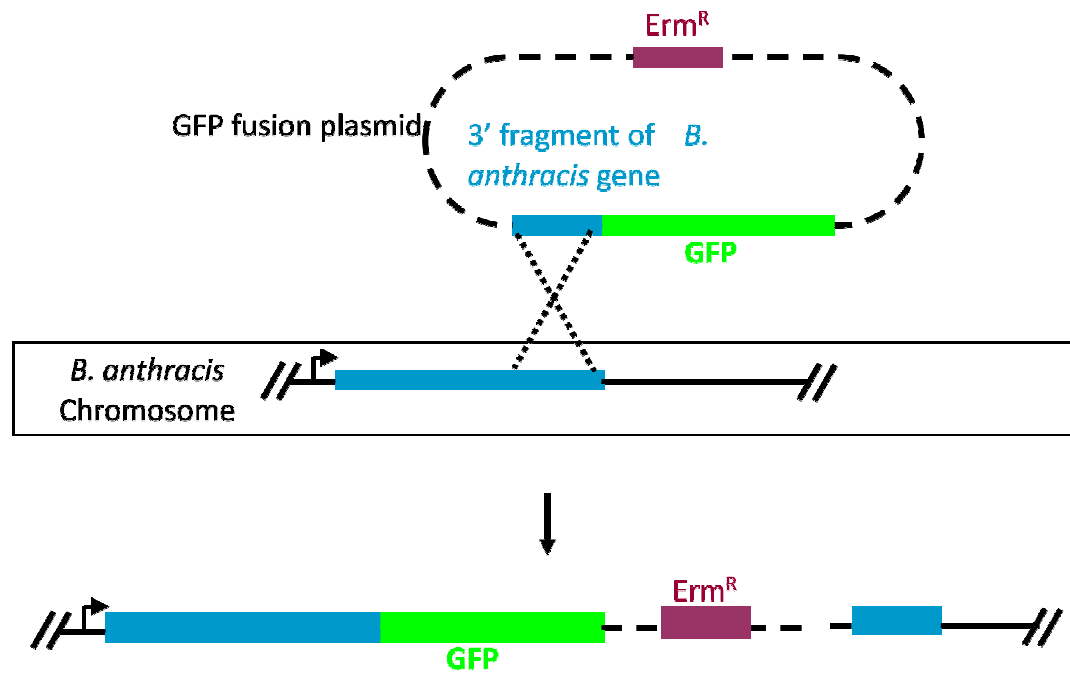
A) Protein extracts from wild type (WT), *cotO* mutant (*cotO*), and *spoVID* mutant (*spoVID*) spores were prepared and separated as described in chapter 3. The bands indicated by triangles and numbers were analyzed at the Vincent Coates Foundation Mass Spectrometry Laboratory (Stanford University Mass Spectrometry), and the protein identities are indicated in the text. B) Protein extracts from wild type and *cotβ* mutant (*cotβ*) spores separated by SDS-PAGE. The Cotβ band (indicated) was identified by Dr. Janine Maddock (University of Michigan) using mass-spectrometry and was reduced and/or missing from the *cotβ* mutant strain.

**Table 1 Proteins Identified by Mass Spectrometry**

<b>Band</b>	<b>Accession</b>	<b>Protein Name</b>	<b>Known or (Putative) Function</b>	<b>Known or (Putative) Subcellular Location</b>
1	BA0887	EA1	S-layer protein	Vegetative spore surface
2	BA1021	BA1021	Unknown function	Unknown location in spore
2	BA3606	IunH2	(Inosine-uridine preferring nucleoside hydrolase)	Unknown location in spore
2	BA4658	YrbB	Unknown function	(Between the coat and cortex)
3	BA1477	ExsJ	Unknown function	Exosporium
3	BA2554	ExsK	Unknown function/ inhibition of germination	Exosporium
3	BA4173	Tgl	(Transglutaminase)	(Spore coat)
3	BA5030	YisY	(Spore coat protein of unknown function)	(Spore coat)
3	BA5149	BA5149	Unknown Function	Unknown
4	BA0252	Alr	Alanine racemase /inhibition of germination	Exosporium
4	BA4710	BA4710	Unknown function	Unknown
4	BA4772	BA4772	(ABC transporter)	Unknown
5	BA0803	CotJC	Unknown	(Spore coat)
5	BA1489	SodA	Superoxide dismutase (protein crosslinking)	Unknown
5	BA4266	Cot $\alpha$	Chemical resistance	Spore coat
Cot $\beta$	BAS2377	Cot $\beta$	Controls spore surface morphology	Spore coat

The novel band present in *cotO* mutant spores was identified as the vegetative cell surface layer protein EA1, and is a common contaminant of *B. anthracis* spore protein extracts (Williams & Turnbough, 2004). The proteins present in band 2 are BA1021 (a protein of unknown function previously found to be associated with spores (Lai *et al.*, 2003, Redmond *et al.*, 2004, Todd *et al.*, 2003)), IunH a protein similar to other inosine-uridine preferring nucleoside hydrolases and which has a role in regulating germination in *B. thuringiensis* (Liang *et al.*, 2008), and an orthologue of YrbB (also called CoxA (a *B. subtilis* protein localized to the cortex-coat interface (Takamatsu *et al.*, 1998))). Band 3 contained a protein of unknown function previously shown to be associated with the exosporium (ExsK) (Severson *et al.*, 2009, Redmond *et al.*, 2004, Giorno *et al.*, 2007), an orthologue of a *B. subtilis* coat protein of unknown function (YisY) (Kim *et al.*, 2006), Tgl an orthologue of a *B. subtilis* coat protein and transglutaminase shown to be involved in the crosslinking of spore coat proteins (Ragkousi & Setlow, 2004), BA5149 a hypothetical protein of unknown function, and ExsJ a protein previously found to be associated with the exosporium (Todd *et al.*, 2003, Redmond *et al.*, 2004). Band 4 contained BA4710 a protein of unknown function, BA4722, a hypothetical protein similar to other ABC transporters, and alanine racemase (Alr) a protein previously shown to be localized to the exosporium and to regulate germination (Chesnokova *et al.*, 2008). Lastly, band 5 contained SodA a superoxide dismutase and an orthologue of a *B. subtilis* spore coat protein known to be involved in coat protein crosslinking (Passalacqua *et al.*,

2006), Cota a *B. anthracis* spore coat protein involved in the assembly of the outer coat and in resistance to antimicrobial chemicals (Kim *et al.*, 2004), and CotJC an orthologue of the *B. subtilis* spore coat protein of unknown function (Seyler *et al.*, 1997, Henriques *et al.*, 1995). In addition to the proteins identified in this study, we identified another protein (BAS1956) in a previous analysis of spore protein extracts which we renamed Cot $\beta$  (Fig. 9B) (Lai *et al.*, 2003, Mallozzi *et al.*, 2008). We wished to determine if the proteins we identified by mass spectrometry were bone fide spore proteins. We and others have previously used GFP-fusions of sporulation proteins (Severson *et al.*, 2009, Giorno *et al.*, 2009, Kim *et al.*, 2006, Mallozzi *et al.*, 2008, Webb *et al.*, 1995, van Ooij *et al.*, 2004, King *et al.*, 1999, Fujita & Losick, 2002, Eichenberger *et al.*, 2003), to determine if the timing of expression and localization of the protein within the sporangia is consistent with that of known spore and sporangia proteins. We were able to generate C-terminal *gfp*-fusion alleles of six of the genes/proteins (two from bands missing in *cotO* mutant spores, two from bands missing in *spoVID* mutant spores, and a protein (Cot $\beta$ ) we previously identified in the course of other work (Lai *et al.*, 2003)). To generate the *gfp*-fusion alleles we generated plasmid constructs which target the endogenous copy of the gene encoding these proteins and replaces them with the *gfp*-fusion allele via a Campbell-type recombination event (Fig. 10).



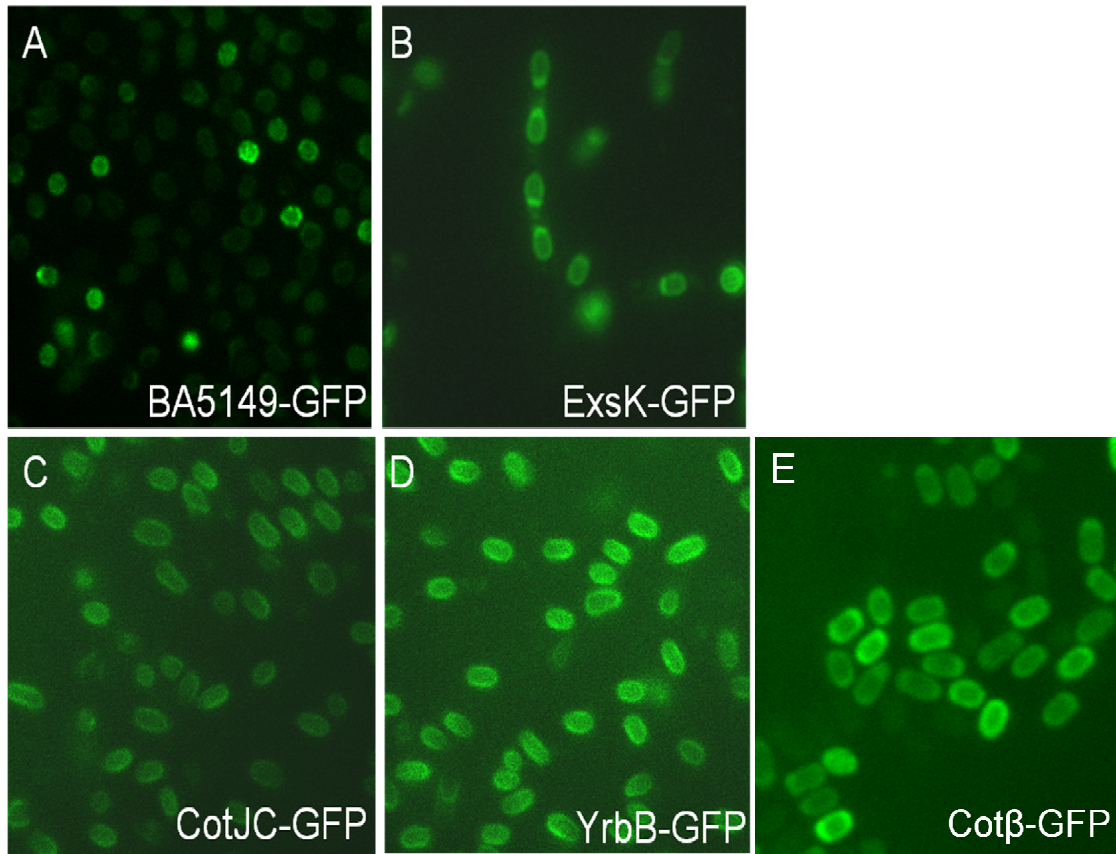
**Figure 10. Construction of a *gfp*-fusion allele.**

Integration of a plasmid bearing a 3' fragment of a gene translationally fused to *gfp* generates a full-length fusion allele at the endogenous locus and a promoter-less fragment of the gene downstream of the integration site (not shown).

We found all of the GFP-fusion proteins were expressed specifically during sporulation (data not shown), and that five of the fusions localized to spores forming rings of fluorescence that surrounded the spores (Fig. 11). The GFP-fusion of BA1021, although expressed specifically during sporulation, was not assembled to the spore, and instead filled the mother cell cytoplasm during sporulation (data not shown). The ring-pattern of fluorescence is characteristic of a coat or exosporium protein (Giorno *et al.*, 2009, Mallozzi *et al.*, 2008, Kim *et al.*, 2006, Severson *et al.*, 2009). To better understand the where some of these proteins were localized within the spore Kari Severson and I further analyzed the location of ExsK and Cot $\beta$  using an approach that detects surface exposed protein before and after the exosporium has been physically or genetically removed from the spores. This allowed us to determine whether ExsK or Cot $\beta$  is present on the exosporium surface, the exosporium basal layer, or beneath the exosporium (either in the interspace or on the coat surface) using antibodies directed against GFP (for the case of Cot $\beta$ -GFP) or against ExsK itself (using anti-ExsK anti-sera). Using this approach I was able to determine that Cot $\beta$ -GFP is present on the coat surface, since it could not be detected on the surface of spores in which the exosporium was present (in *cot $\beta$ -gfp* spores), and was exposed when the exosporium was absent due the absence of CotE (in a *cotE cot $\beta$ -gfp* mutant strain) Fig. 12A (Mallozzi *et al.*, 2008). Perhaps, more interestingly is the localization of ExsK-GFP. ExsK was first identified in an analysis of proteins isolated from an exosporium-rich fraction of spore proteins (Redmond *et al.*, 2004). Given this, it was perhaps not surprising that Kari Severson was able to find that ExsK

surrounds spores and is displayed on the exosporium surface as detect using an anti-ExsK antibody Fig 12B (Severson *et al.*, 2009). This analysis revealed also revealed that ExsK surrounded spores but was most concentrated at one pole (data not shown). We hypothesized that if ExsK was solely located in the exosporium then sonicated spores (which often lack exosporia) should not stain with the anti-ExsK antibody. Surprisingly, Kari found that some sonicated spores still stained with the anti-ExsK anti-sera. To determine if these ExsK-positive spores had intact exosporia she stained them with the anti-exosporium antibody directed against BclA (Fig. 12B). She found that spores with and without exosporia (as determined by exosporium staining) stained positive for the presence of ExsK (Fig. 12B). These results suggest that ExsK is present in two locations: one at the exosporium surface and one underneath the exosporium (on the interspace or coat surface). This is the first published evidence of a *B. anthracis* spore protein being located in multiple locations in the spore, and may represent an important assembly strategy that cells use to accomplish the task of spores assembly (see Discussion).

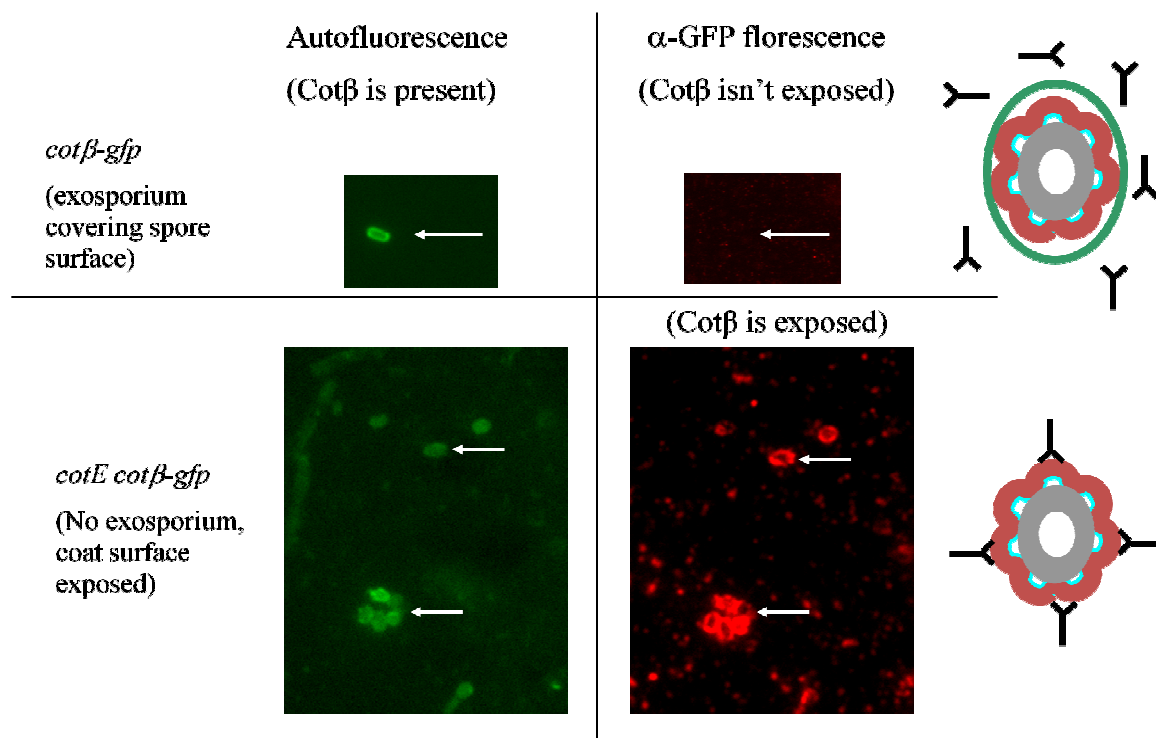


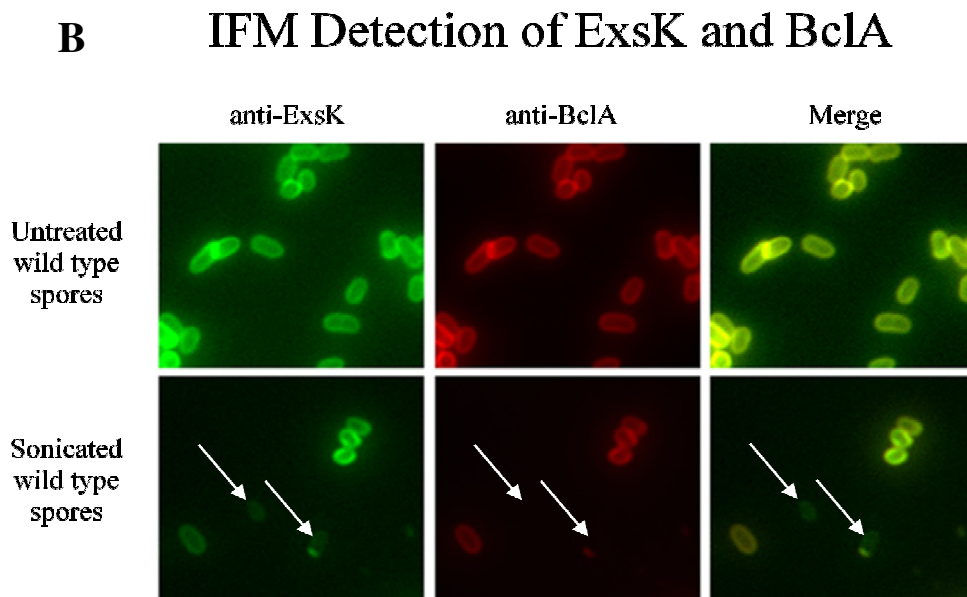


**Figure 11. Spores bearing GFP-fusions to candidate coat or exosporium proteins.**

Fluorescence micrographs of spores bearing GFP-fusions to BA5149 (A), ExsK-GFP (B), CotJC-GFP (C), YrbB-GFP (D), or Cotβ-GFP (E). A ring of fluorescence is a two-dimensional cross-section of a sphere of fluorescent protein that surrounds the spore.

A

IFM Detection of Cot $\beta$ -GFP



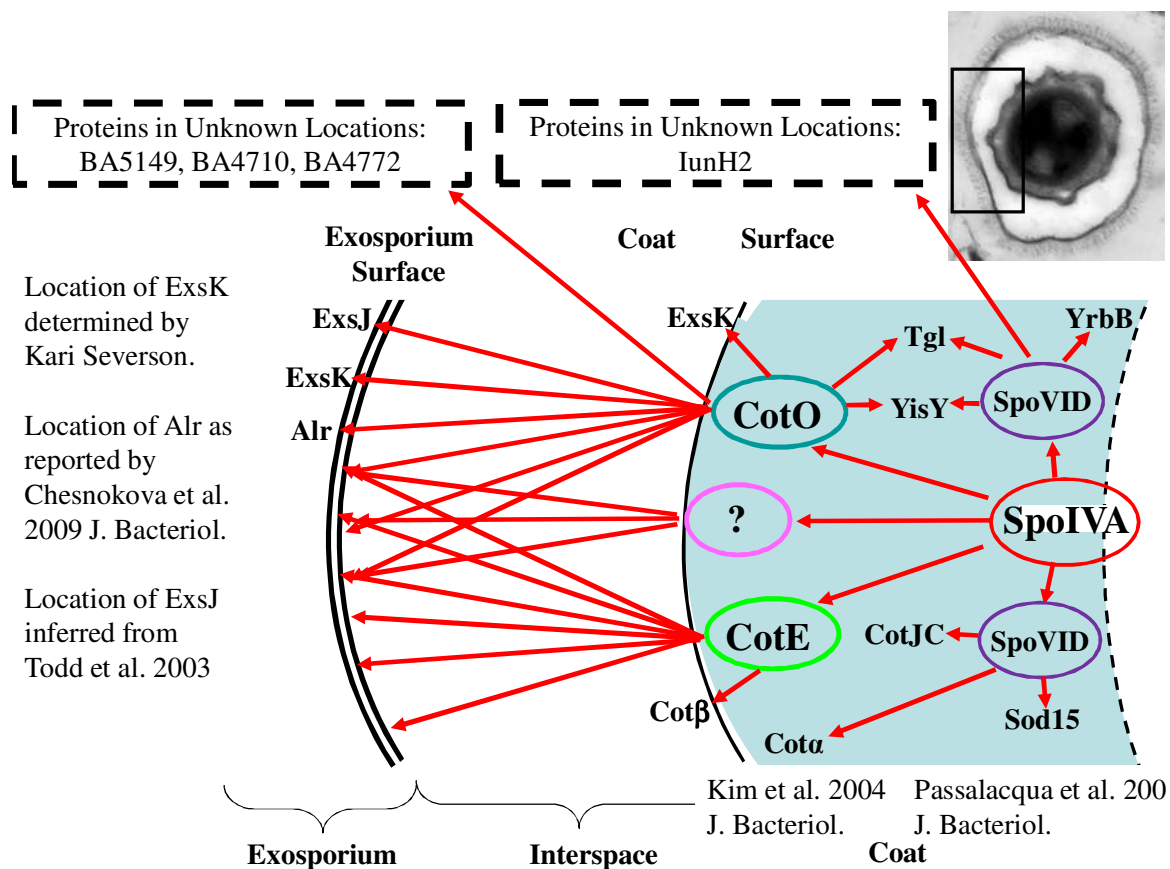
**Figure 12. Localization of spore proteins using immunofluorescence microscopy.**

Fluorescence and immunofluorescence micrographs of either wild type spores or spores bearing a Cot $\beta$ -GFP fusion. A) Left panel: autofluorescence of spores bearing Cot $\beta$ -GFP. Top right panel: autofluorescence of spores bearing Cot $\beta$ -GFP. Bottom right panel: immunofluorescence of spores stained with anti-GFP and a corresponding secondary antibody conjugated to a red fluorophore. B) Top panels immunofluorescence of untreated wild type spores; bottom panels immunofluorescence of sonicated wild type spores. Left panels: immunofluorescence of spores stained with anti-ExsK anti-sera and a corresponding secondary antibody conjugated to a green fluorophore. Middle panels: immunofluorescence of spores stained with anti-BclA monoclonal antibodies and a corresponding secondary antibody conjugated to a red fluorophore. Right panels: Merged images.

*A Model of Spore Assembly in Bacillus anthracis.*

Given the number of proteins putatively under the control of CotO and SpoVID I wished to generate a model of the spore assembly pathway in *B. anthracis* (Fig. 13). Such a model allows for the development and testing of various hypotheses about the program of spore assembly. As discussed previously, it had become clear that CotO and CotE are both involved in exosporium attachment. This analysis also revealed that other unknown factor(s) are involved in exosporium attachment as well. To generate a more complete model of spore assembly I integrated information about the assembly and localization of spore proteins from three sources into this model: First, the data we obtained from the analyses of the *cotO*, *cotE*, *exsK*, and *spoVID* mutants. Second, the microscopic localization of ExsK, Cot $\beta$ , CotJC, YrbB, and BA5149. And lastly, the previously reported locations of spore proteins in the literature (Kim *et al.*, 2004, Passalacqua *et al.*, 2006, Todd *et al.*, 2003, Chesnokova *et al.*, 2008, Severson *et al.*, 2009, Giorno *et al.*, 2007). In this model SpoIVA sits highest in the hierarchy of morphogenetic proteins and controls the assembly of all other outer-spore proteins, consistent with its role in *B. subtilis* (Roels *et al.*, 1992, Giorno *et al.*, 2007). SpoVID, then next most important morphogenetic protein controls the assembly the coat proteins YrbB, Sod15, CotJC, Tgl, YisY, and Cota (present on the coat surface (Kim *et al.*, 2004)). Additionally, SpoVID controls the assembly of IunH2, an outer-spore protein whose location has not yet been

determined. CotO and CotE, two morphogenetic proteins are responsible for the attachment of the exosporium and are themselves controlled by SpoIVA. As of yet unknown protein(s) (indicated by a question mark in Fig. 13) also participate in exosporium attachment. CotE partially controls the assembly of Cot $\beta$  which is present on the coat surface. CotO controls the assembly of three coat proteins including YisY and Tgl and ExsK (also located in the exosporium). CotO additionally controls the assembly of three other proteins (BA5149, BA4710, and BA4772) whose location has not yet been elucidated. Lastly, exosporium-localized ExsK, ExsJ, and Alr are present on the exosporium surface and are controlled by CotO (Todd *et al.*, 2003, Chesnokova *et al.*, 2008, Severson *et al.*, 2009).



**Figure 13. A model of spore assembly in *Bacillus anthracis*.**

The diagram depicts an arc of the spore indicated by the box over the spore pictured in the upper-right hand corner. Morphogenetic proteins are circled and red arrows extending from the morphogenetic proteins represent control for assembly. The localization of Sod15, Cot $\alpha$ , ExsJ, and Alr are from published observations as indicated.

### *Evidence of Spore Protein Crosslinking*

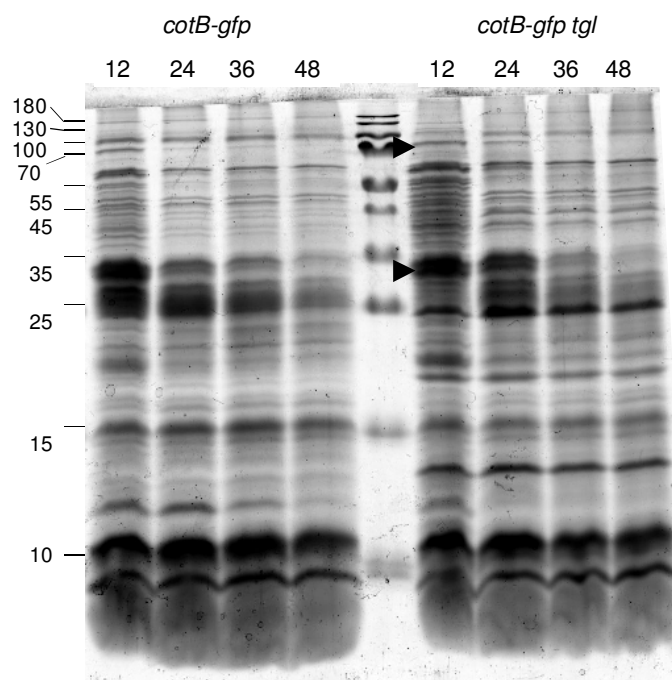
Up to this point spore assembly has been considered in terms of how proteins are targeted to and organized within the coat and exosporium during sporulation. Recently, it was discovered that the spore assembly process in *B. subtilis* continues even after mother cell lysis (Ragkousi & Setlow, 2004). Ragkousi *et al.* discovered that *B. subtilis* spore proteins continue to be modified even after their assembly to the spore is complete; finding that GerQ, a coat protein, becomes covalently crosslinked into high molecular weight protein-complexes (Ragkousi & Setlow, 2004). Furthermore, Ragkousi *et al.* were able to show that Tgl (a *B. subtilis* transglutaminase that catalyzes the formation of  $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide bridges between lysine and glutamine residues present within or between proteins) was responsible for the formation of these complexes. We wished to determine if Tgl might also crosslink other coat proteins into insoluble complexes in *B. subtilis*. The first indication that other proteins (besides GerQ) might becoming crosslinked in *B. subtilis* spores came from our observation that individual bands were becoming reduced in intensity the longer they remained in culture (Fig. 14A). These bands correspond to the coat proteins CotB, and CotG respectively. For the case of CotB we confirmed that the loss of band intensity corresponded to a decrease in the amount of detectable CotB-GFP as determined by western blot (Fig. 14B). We suspect that the loss of the bands is due to Tgl-mediated crosslinking of CotB and CotG into complexes that do not run into the gel, given that the rate of decrease in band intensity is

at least partially dependent on spores having a functional copy of *tgl* (Fig. 14B).

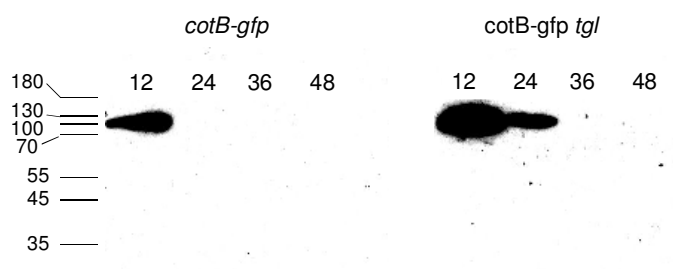
Nevertheless, these data were not conclusive evidence of protein crosslinking since the loss of the CotB and CotG bands could be due to degradation or proteins being lost to the culture media. To address this question we wished to analyze both the insoluble (gel excluded) and soluble fractions of one of these proteins (CotB). To do this we monitored the fluorescence intensity of CotB-GFP bearing spores over 48hrs. We found that spores bearing CotB-GFP or CotG-GFP at 12hrs after the initiation of sporulation had their highest level of fluorescence intensity (Fig. 14C). After 48hrs the fluorescence intensity of both sets of spores had dropped by at least one log (Fig. 14C). In contrast, spores bearing either of the fusions and a mutation in *tgl* retained a population of spores that fluoresced at maximal intensity after

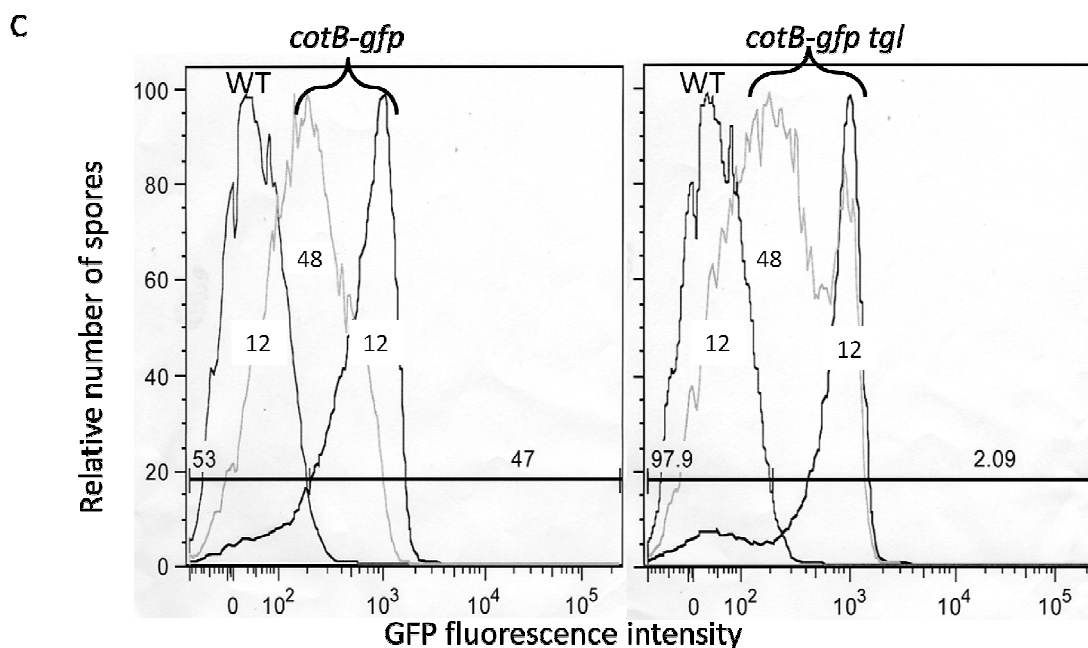


A



B





**Figure 14. The fate of CotB-GFP in wild type and *tgl* mutant spores.**

A) Spores isolated from cultures of wild type *B. subtilis* (WT), cells bearing *cotB-gfp* (*cotB-gfp*), or *tgl* mutant cells bearing a copy of *cotB-gfp* (*cotB-gfp tgl*) at 12, 24, 36, or 48hrs after the onset of sporulation (as indicated) were isolated, and crosslinking activity was stopped by centrifugation of the spores thru a Renographin density gradient (described in Chapter 3). A) Spore protein profiles of spores isolated at each time point. The ~100kDa CotB band and ~35kDa band are indicated by closed triangles. B) Detection of CotB-GFP by western blot using an anti-GFP antibody (described in Chapter 3). C) Fluorescence intensity of GFP-bearing spores over time as determined by flow cytometry.

48hrs. Taken together, these data suggest that Tgl is partly responsible for the loss of detectable and CotB-GFP in this assay. However, it is not clear what the cause of the decrease in fluorescence was. Possibly, spore protein crosslinking is reduced in the *tgl* mutant backgrounds preventing the decrease in fluorescence intensity. Alternatively, it's possible that crosslinking inactivates GFP-fluorescence, and in the absence of Tgl fluorescence-inactivation is reduced; more experiments will be needed to address this question.

## **Interaction of Spores with the Host**

### *Role of spore structures in disease*

While several studies have investigated and elucidated several functions of spore structures and proteins in interacting with the host immune system (Driks, 2009), it is still unclear what properties of the spore are essential for the ability to cause disease animals or people. We have had a long standing interest in identifying roles for spore proteins, structures, and functions in disease (Giorno *et al.*, 2007, Giorno *et al.*, 2009, Mallozzi *et al.*, 2008, Severson *et al.*, 2009). Therefore, we wished to determine if the morphological and functional defects caused by the *cotO* and *spoVID* might also have an impact on disease in an animal model. To do this we tested the ability of *cotO* and *spoVID* mutant spores to cause disease in the Guinea pig intramuscular disease model via collaboration

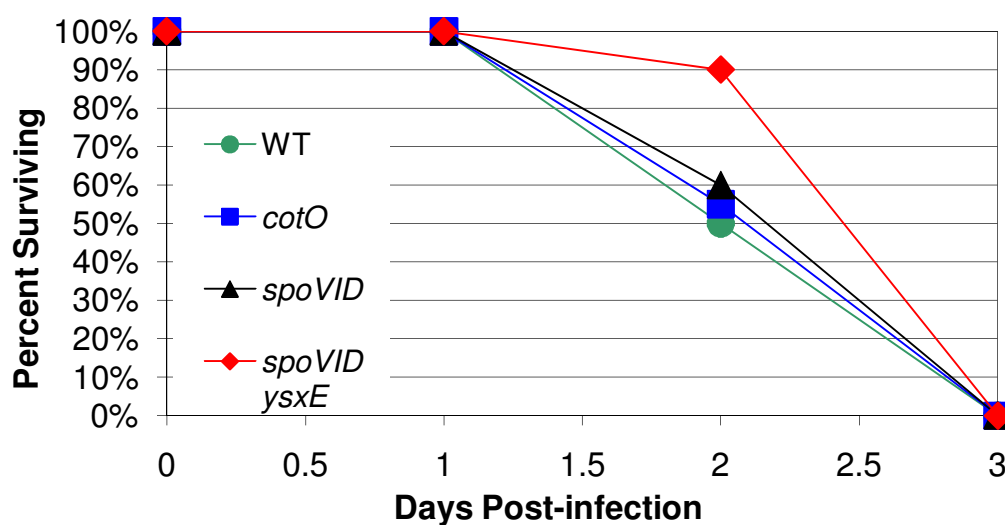
with Dr. Joel Bozue (USAMRIID). First, Dr. Bozue infected 10 female Hartley Guinea pigs injected intramuscularly with approximately 2,000 spores of wild type, *spoVID* mutant, or *cotO* mutant strains, representing the equivalent of 20 Ames 50% lethal doses (LD50s). The guinea pigs were monitored several times each day, and mortality rates were recorded. We have previously shown that the exosporium is dispensable for disease (Giorno *et al.*, 2007). Consistent with these findings *cotO* mutant spores were indistinguishable from wild type spores in this model and all animals had succumbed to the disease by day three post-infection (Fig. 15). In contrast, *spoVID* mutant spores had a one day delay in the onset of the disease as 90% of the animals had survived at two days post-infection (compared to 50% of animals infected with wild type or *cotO* mutant spores) (Fig. 15). Interestingly, this delay did not lead to an increased survival rate as all of the animals succumbed at day three post-infection.

#### *Role of Spore Proteins in Phagocytosis by Host-cells*

Phagocytosis by a macrophage or dendritic cell is thought to be one of the first steps in the initiation of anthrax (Frankel *et al.*, 2009). However, the factors that influence the phagocytosis of *B. anthracis* spores are still poorly understood. Nevertheless, it is known that opsonization of spores by the complement protein C3 can enhance phagocytosis of spores by human macrophages (Premanandan *et al.*, 2009). This may be related to the presence of a binding site on spores for gC1q-R/p33 (a mammalian cell surface receptor

that binds to the globular heads of the complement protein C1q) (Ghebrehiwet *et al.*, 1994, Ghebrehiwet *et al.*, 2001, Ghebrehiwet *et al.*, 2007). Interestingly, the C-terminus of BclA contains a globular domain with high structural identity to C1q (Réty *et al.*, 2005). BclA has also been shown to bind to Mac-1 (CR3 (a macrophage cell-surface integrin)) which may promote phagocytosis (Oliva *et al.*, 2009, Oliva *et al.*, 2008). Furthermore, spores appear to possess another protein (SoaA) that influences the ability of anti-spore antibodies to opsonize spores, and thus FcR mediated phagocytosis (Cote *et al.*, 2008). Given these examples of spore-specific factors that influence phagocytosis, we wished to determine if the changes to the spore structures that resulted from the *spoVID* and *cotO* mutations might reveal a role for those structures (or even specific spore proteins) in regulating spore phagocytosis. To determine this we analyzed the phagocytosis of wild type Sterne strain spores, along with the derived *cotO*, *cotE*, and *spoVID* mutant strains via a collaboration with Drs. Steve Blanke and Angela Prouty at the University of Illinois Urbana-Champaign. To do this Dr. Prouty infected either the RAW 267.4 macrophage-like cell line or primary human alveolar macrophages with wild type or mutant spores at a multiplicity of infection of 250. Next, she fixed samples for electron microscopy at 5 minute intervals for 120 minutes total and quantified spore uptake by counting the number of intracellular spores at each time point. Surprisingly, Dr. Prouty found that *spoVID* mutant spores were largely ignored by both the macrophage-like cell line RAW 267.4 and by primary human macrophages isolated from the lung. In fact, she found that less than 1% of *spoVID* mutant spores were

phagocytosed by macrophages after 120 min as compared to 75% of wild type spores under the same conditions. These data suggest that *spoVID* mutant spores lack a critical pro-phagocytic factor that activates macrophages and promotes spore phagocytosis. In contrast, the efficiency of phagocytosis of *cotO* and *cotE* mutant spores was indistinguishable from that of wild type spores, but TEM analysis revealed that mutant spores that lacked exosporia (*cotO* and *cotE* mutant spores) were often observed with other spores in the same phagocytic vesicle as compared to wild type spores which were always strictly observed to be in a 1:1 ratio of spore to phagocytic vesicle (data not shown). However, this may be due to the tendency of *cotO* and *cotE* mutant spores to clump together (data not shown), in which case they might be phagocytosed in pairs or more groups of three. Further analysis will be needed to resolve this question.



**Figure 15. Pathogenesis of wild type and mutant spores.**

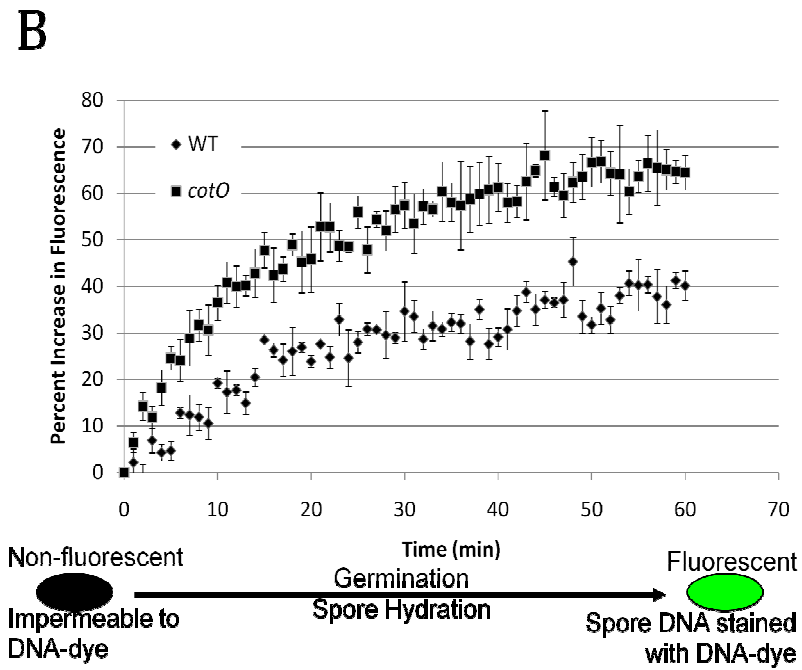
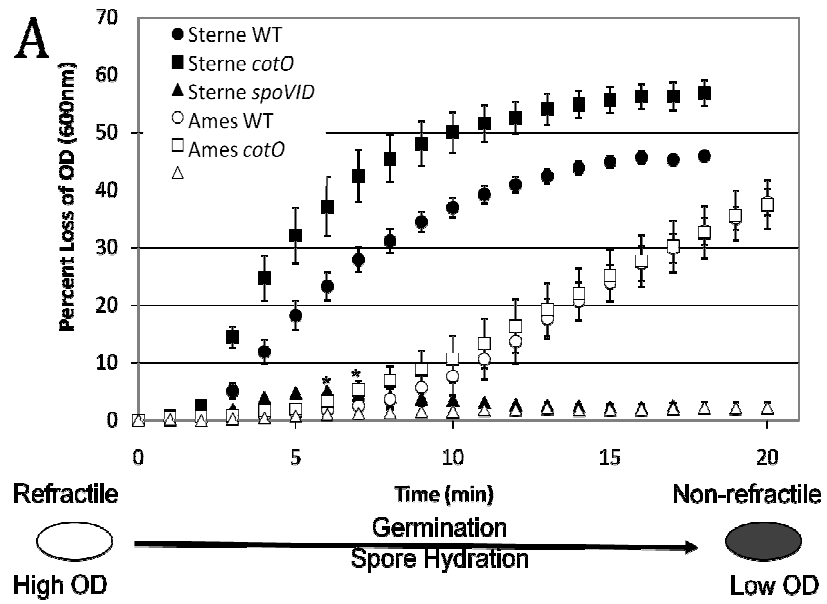
Each of ten female Hartley guinea pigs were infected with the equivalent of 20 spores equivalents of a 50% lethal dose ( $LD_{50}$ ) of either the wild type Ames strain (closed circles), *cotO* mutant Ames spores (closed boxes), *spoVID ysxE* mutant Ames spores (closed diamonds), or *spoVID* mutant Ames spores (closed triangles) and were monitored for morbidity for three days post-infection.

### *Germination and Resistance Properties of Mutant Spores*

Given the severe morphological phenotypes of *cotO* and *spoVID* mutant spores we hypothesized that it would be very likely that the spores would also have functional defects. We therefore tested *cotO* mutant, *spoVID* mutant Sterne and Ames spores for their ability to germinate, and the ability of mutant Sterne spores to resist lysozyme. To test the ability of *cotO* mutant spores to germinate we used an assay which measures the loss of refractility of spores, an intermediate step during germination (Moir, 2006). Interestingly, we observed that, in general, all strains derived from the Ames background were delayed in germination as compared to the Sterne background-strains in this assay, suggesting a possible strain-to-strain variation in germination efficiency (Fig 16A). Furthermore, we found that *cotO* mutant spores germinated modestly better than wild type spores, as *cotO* mutant spores lost refractility more rapidly and more completely than wild type spores at every time point measured (Fig. 16A). This finding is consistent with recent reports that show that germination-inhibiting enzymes, namely nucleoside hydrolase (IunH) and alanine racemase (Alr), are present in the exosporium of *B. cereus* group spores (Todd *et al.*, 2003, Chesnokova *et al.*, 2008, Redmond *et al.*, 2004, Lai *et al.*, 2003, Giorno *et al.*, 2009). In the absence of these enzymes, germination is more efficient (Liang *et al.*, 2008, Chesnokova *et al.*, 2008); this is also likely to be the case when the exosporium fails to assemble to the spore. To test the hypothesis that the *cotO* mutant spores germinated more efficiently than the wild type due to a lack of Alr Dr.



Susan Welkos (USAMRIID) analyzed the ability of wild type and mutant spores to germinate in response to L-alanine, she found that *cotO* mutant spores germinated much more efficiently in the presence of L-alanine than wild type spores (Fig. 16B). This suggests that *cotO* mutant spores lack Alr (consistent with the lack of an exosporium). In contrast to the increased germination efficiency we saw with the *cotO* mutant spores, *spoVID* mutant spores (from strains MGM84 and JABAmess*spoVID**dysxE*) showed a severe germination defect, and germination remained below the limit of detection throughout the assay (Fig 16A). This finding is consistent with previous reports of germination defects in spores with severe coat defects (Ghosh *et al.*, 2008, Beall *et al.*, 1993, Zheng *et al.*, 1988, Cutting *et al.*, 1989). The exact reason for the defect is unclear, but one explanation could be the lack of cortex lytic enzymes present in the coat which are required for efficient germination (Heffron *et al.*, 2009, Dowd *et al.*, 2008, Bagyan *et al.*, 1998, Chirakkal *et al.*, 2002, Giebel *et al.*, 2009).



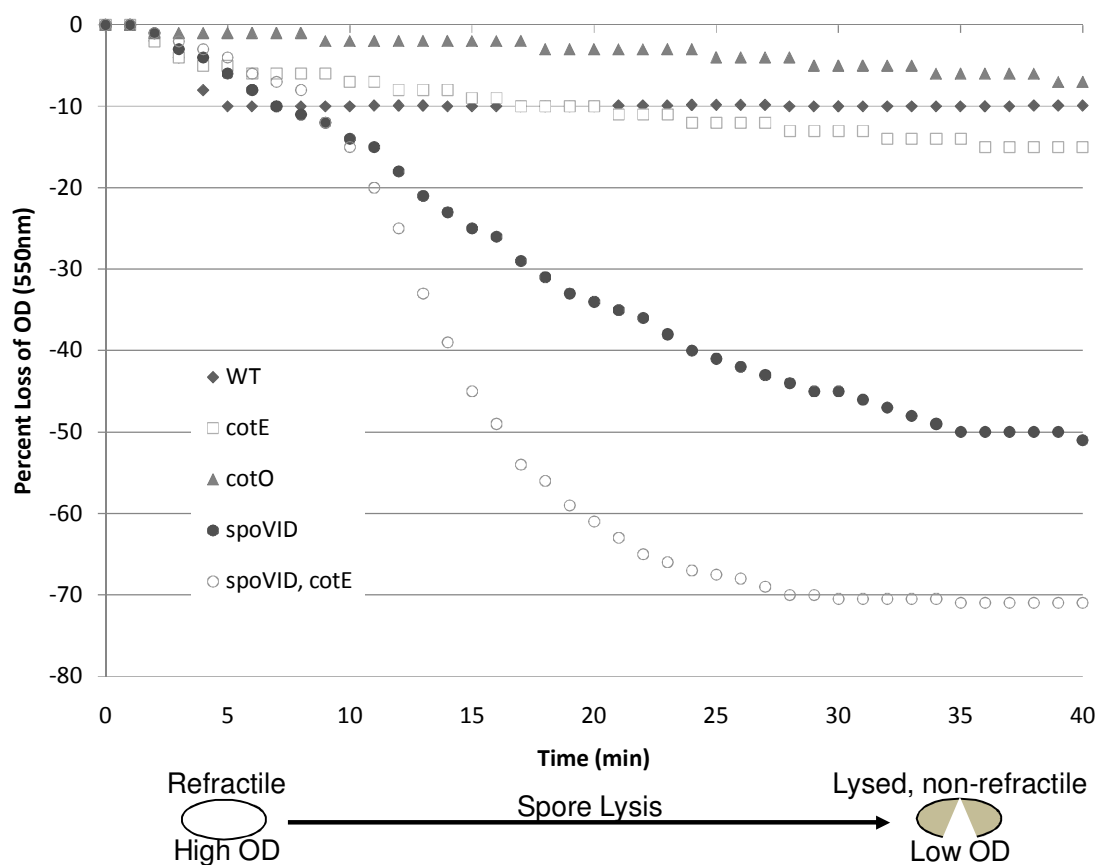
**Figure 16. Germination analyses of wild type and mutant *B. anthracis* spores.**

A) Loss of spore optical density due to germination. Wild type and mutant, Sterne and Ames spores, (see legend (inset)) were resuspended in germination buffer, heat activated, and germination was initiated by the addition of BHI (see Methods for details). The data are reported as percent of the optical density at 550nm at T=0 min. The diagram below the graph indicates the relationship between germination and the loss of optical density. Error bars represent the standard error of the mean from three independent germination experiments. B) This experiment was conducted by Susan Welkos at USAMRIID.

Percent increase in fluorescence due the fluorescence dye uptake. Heat activated wild type Sterne (closed squares) or *cotO* mutant spores (closed diamonds) were resuspended in germination buffer and exposed to 50mM L-alanine. The increase in fluorescence is expressed as the percent increase of fluorescence of each sample at T=0. The diagram below the graph indicates the relationship between germination and the increase in spore fluorescence. Error bars represent the standard error of the mean from three experiments.

Given the changes in morphology, germination, and protein composition we observed in *cotO* and *spoVID* mutant spores I wished to test the ability of the mutant spores to exclude lysozyme. To assay lysozyme resistance I monitored the loss of optical density as a result of spore lysis (Moir, 1981). We found that *cotO* mutant spores were just as resistant to lysozyme as wild type spores (Fig. 17), suggesting that the loss of the exosporium did not affect the ability of *cotO* mutant spores to exclude lysozyme. In contrast, *spoVID* mutant spores (from strain MGM84) had a severe lysozyme defect (Fig. 17), and the optical density of the *spoVID* spores dropped rapidly over the 40min time course of the experiment. However, the near-linear drop in optical density was somewhat unexpected from previous reports of *B. subtilis* spores with severe coat defects (Moir, 1981), which showed a precipitous drop in optical density at the onset of the assay. I hypothesized that the exosporium might act as a kinetic barrier to lysozyme and would therefore be responsible for the observed linear (and not logarithmic) drop in optical density. To test this hypothesis I introduced the *cotE* mutation from strain RG56 (Giorno *et al.*, 2007), into the *spoVID* mutant strain (MGM84) via transduction to create MGM145. We previously showed that CotE is required for exosporium assembly, and importantly, that *cotE* mutant spores do not have a lysozyme resistance defect (Fig. 17). I then tested the ability of the *cotE spoVID* mutant (MGM145) to resist lysozyme (Fig. 17). As expected the loss of optical density due to spore lysis was more precipitous for the *spoVID cotE* mutant strain (MGM145) than I observed for the *spoVID* mutant strain (MGM84) (Fig 17), suggesting that the exosporium is porous to relatively large toxic

molecules, consistent with classical and modern structural analyses of the exosporium indicating the presence relatively large pores present in the crystalline-like basal layer (Gerhardt & Ribi, 1964, Ball *et al.*, 2008, Holt & Leadbetter, 1969).



**Figure 17. Lysozyme resistance analysis of wild type and mutant spores.**

Spores suspended in phosphate-buffered saline were exposed to 50ug/ml lysozyme. The diagram below the graph illustrates the relationship between optical density and the lysis of spores by lysozyme. The optical density at 550nm was monitored every minute for 40min using a plate reader. The percent of optical density at T=0min is reported for each sample.

## CHAPTER 5

### DISCUSSION

A few of the most fundamental questions in biology are: How do cells transform themselves into different cell-types? What makes each cell-type unique? And what are the functions of specialized cells? The process of bacterial sporulation represents an ideal model for investigating these questions. Sporulating cells undergo a highly-regulated developmental process that results in the formation of a hardy cell-type surrounded by spore-specific organelles. Furthermore, each organelle has unique functions that ensure the survival of the cell thru long periods of severity. The relatively small genome of *Bacillus subtilis*, and the ease with which it can be manipulated, makes sporulation the best-understood developmental process in biology. However, little was known about how broadly the process of sporulation (as it's known in *B. subtilis*) applies to other species. The variety of environments in which spores reside and the myriad of elaborate structures that spores display suggest that each species' spores have evolved specific adaptations to flourish in different environmental niches. We wished to analyze spore assembly in one of these species to begin to determine the function of novel spore structures and to dissect the assembly pathway that created them. We chose to focus our efforts on *Bacillus anthracis* since it has long been the subject of human health and security concerns and is amenable to genetic manipulation.

To begin our analysis of spore assembly in *B. anthracis* we chose to investigate the roles that two classes of proteins have in spore development. The first: a group of the master-regulators of spore coat assembly in *B. subtilis*, the so-called morphogenetic proteins. And the second: a group of proteins which appear to be specific to the *B. anthracis* clade of species (also known as the *B. cereus* group). My project focused on two of the morphogenetic proteins CotO and SpoVID and the group-specific proteins Cot $\beta$ , ExsFA, ExsFB, and IunH (Mallozzi *et al.*, 2008, Giorno *et al.*, 2009).

### **Coat Assembly and the Role of SpoVID and YsxE**

SpoVID is a morphogenetic protein shown to be critical for the assembly of the coat in *B. subtilis* (Beall *et al.*, 1993). SpoVID acts early during coat assembly and helps attach the coat to the spore. The *B. anthracis* homologue of SpoVID is 39% identical (63% similar) to its *B. subtilis* counterpart and is part of a bioinformatically-predicted two-gene operon containing the downstream gene *ysxE* (Price *et al.*, 2005a, Price *et al.*, 2005b). In order to rapidly generate a mutation of the *spoVID* gene in *B. anthracis* I engineered a plasmid that would integrate into and inactivate the *spoVID* gene allowing for the recombination of the plasmid DNA into the *spoVID* locus. This type of mutations tends to be polar on genes downstream of the integration site especially if they are part of an operon (as this will often result in the formation of a truncated transcript lacking the downstream gene). However, in the case of SpoVID, previous studies in *B. subtilis* indicated that the



downstream gene (*ysxE*) was not important for spore development in *B. subtilis* (Beall *et al.*, 1993). Therefore we proceeded to analyze this mutant strain in the context of a single mutation in *spoVID*. We found that the *B. anthracis spoVID* mutant shared a variety of characteristics with its *B. subtilis* counterpart. First, we found that *B. anthracis* spores lacked or had very thin coats (Fig. 4). Secondly, the *B. anthracis spoVID* mutant spores were sensitive to lysozyme and had a severe germination defect (Figs. 16 and 17). Taken together, these findings suggest that SpoVID has a highly conserved role in spore development, consistent with its position near the beginning of the model hierarchy of morphogenetic proteins (Fig. 1).

These severe phenotypes drove us to ask if the spores might also have a defect in their ability to cause disease in an animal model (Fig. 15). Interestingly, we found there was an approximate one-day delay in the onset of disease in animals infected with *spoVID* mutant spores as compared to the wild type. While these findings were exciting we suspected (given previous experiences – personal communication Joel Bozue) that the delay in the onset of disease might have been due to the *spoVID* mutant reverting to wild type via a recombination event that removes the plasmid from the chromosome. To determine if this was the case, Dr. Bozue generated a marker replacement mutation (which is unlikely to be polar) in the *spoVID* gene generating strain JABAmess*spoVID*. Unexpectedly, this mutant did not have the day delay in the onset of disease (Fig. 15). Thin section electron microscopy revealed that this mutant did not have a defect in spore

coat assembly, and the spores were indistinguishable from wild type (Fig. 4). These results suggested that the phenotypes we observed, at least in large part, were due to the effect of the polar mutation on *ysxE*.

The conclusion that *ysxE* is responsible for many of the phenotypes previously attributed to *spoVID* (in both *B. anthracis* and *B. subtilis*) is supported by several lines of evidence. First, YsxE is a CotE-independent coat protein (like SpoVID) (Kim *et al.*, 2006), suggesting it is in the right position in the spore to be able to control coat attachment. Second, YsxE is highly conserved and is found in all sequenced species bearing SpoVID (data not shown). Third, the *ysxE* mutant in *B. subtilis* is sensitive to lysozyme suggesting the presence of a significant coat defect (Klobutcher *et al.*, 2006). Fourth, the original *B. subtilis spoVID* mutant strain (generated by Campbell integration) leaves open the question of whether or not the phenotypes observed are also a result of a polar effect on *ysxE*. Taken together, along with the *spoVID* mutant phenotypes in *B. anthracis*, the data suggests that the mutant phenotypes previously attributed to *spoVID* are due to *ysxE* or a mutation in both *spoVID* and *ysxE*. A complementation analysis (currently underway) will distinguish between these two possibilities.

### **Exosporium Attachment and Assembly of the Interspace.**

The second morphogenetic protein I analyzed in *B. anthracis* was CotO, a protein shown to be important for the assembly and organization of the outer coat in *B. subtilis* (McPherson *et al.*, 2005). *B. anthracis* CotO is only distantly related to the *B. subtilis* protein (24% identical 39% similar), and resides on the outer portion of the model hierarchy of morphogenetic proteins. Interestingly, *B. anthracis* CotO is located in a cluster of genes that are involved in the assembly of the exosporium (Boydston *et al.*, 2006, Sylvestre *et al.*, 2002, Johnson *et al.*, 2006). We wished to determine if CotO might also play a role in spore assembly in *B. anthracis* and insertionally inactivated CotO. We found that a significant number of *cotO* mutant spores lacked exosporia, which had detached from the spores. These results contribute to a growing body of evidence indicating that at least two genes (*cotO*, *cotE*) play a role in attaching the exosporium to the spore (rather the formation of the exosporium itself). We wished to try to clarify the role *cotO* and *cotE* play in attaching the exosporium. To do this we generated a strain bearing a mutation in *cotO* and *cotE* and analyzed exosporium assembly by staining the exosporium via immunofluorescence microscopy and quantified the number of spores with exosporia. Interestingly, only about 30% of the doubly mutant spores had exosporia (the same as each of the individual mutants), suggesting that CotO and CotE have redundant functions in exosporium attachment. It's not immediately clear which genes are responsible for the attachment of the exosporium in the *cotO cotE*

mutant spores. Possibly, still other genes may be involved in attachment. SafA in *Bacillus cereus* (where it is called ExsA) has been shown to affect exosporium assembly (Bailey-Smith *et al.*, 2005). However, *exsA* mutant spores have a severe coat defect that may indirectly affect the ability of the exosporium to attach to the coat material present in those spores, making it unclear whether or not ExsA has a direct role in exosporium attachment.

While it seems clear that CotO, CotE, and possibly ExsA have roles in exosporium attachment, it remains ambiguous exactly how the exosporium is attached to the coat. In fact, little is known about the composition of the space between the coat and the exosporium (known as the interspace). So far only two proteins have been suggested to localize there (Giorno *et al.*, 2009). And neither of those appears to be required for the attachment of the exosporium (Giorno *et al.*, 2009). Given its electron sparsity, it is tempting to speculate that protein is a minor constituent of the interspace. Nevertheless, it's clear that some proteins are involved in determining interspace morphology. CotY in *B. cereus* has been shown to have a dramatic effect on the shape of the interspace (Johnson *et al.*, 2006). Without CotY, spores lack the typical single lobe of the interspace which gives the exosporium its balloon like appearance (Johnson *et al.*, 2006). Instead the interspace (and the exosporium) is distributed evenly around the circumference of the spore (Johnson *et al.*, 2006). While, the composition of the connections linking the exosporium to the coat is still unclear, a relatively small number

of mutant strains have given us some important insights into how the exosporium attaches to the coat. The same can be said for the formation of the exosporium, in which a few key mutants have given us insight into the assembly of this structure.

### **Exosporium Assembly**

The assembly of the exosporium is one representative of a whole host of structures that probably evolved to fulfill a specific function. As mentioned previously (see literature review) the exosporium has a variety of functions including those involved with host interactions, germination, and resistance. Interestingly, exosporium proteins are predominately made up of poorly-conserved group-specific proteins (Driks & Mallozzi, 2009). This suggests that evolution of the exosporium assembly pathway probably evolved independently of the coat. Although CotO and CotE appear to be involved in the attachment of the exosporium, they are not required for exosporium formation since fragments of what appears to be fully assembled exosporium material can be found in the mother cell cytoplasm and extracellular milieu (see results) (Giorno *et al.*, 2007).

However, recently it was shown that ExsY is responsible for initiating the polymerization of the exosporium around the spore. In the absence of ExsY a small biochemically-distinct fragment (or cap) of exosporium material can be seen but no other fully assembled material can be seen (Boydston *et al.*, 2006). The most plausible interpretation is that the remainder of the exosporium polymerizes around the spore under

the direction of ExsY. This mode of assembly also appears to direct the assembly of BclA into hair-like projections that surround the spore (Steichen *et al.*, 2005, Sylvestre *et al.*, 2005, Giorno *et al.*, 2009). We have shown that in the absence of ExsFA, BclA still assembles to the cap of exosporium but does not encompass the spore and does not result in the formation of hair-like fibers (Giorno *et al.*, 2009). This suggests ExsFA directs both the polymerization of BclA around the spore as well as the assembly of BclA monomers into collagen-like coiled-coils. Finally, ExsFA also appears to direct the polymerization of another exosporium protein (ExsK) around the spore (Severson *et al.*, 2009). We've shown that in the absence of ExsFA, ExsK remains confined to one side of the spore (Severson *et al.*, 2009). Interestingly, Severson *et al.* also showed that the formation of high-molecular-weight species of ExsK depended on BclA (Severson *et al.*, 2009). Taken together these data suggest that ExsK, ExsFA, and BclA dynamically interact to direct exosporium polymerization and the formation high-molecular weight protein-complexes. The lack of both hair like projections and ExsK complexes in *bclA* mutant spores suggest an intriguing possibility that ExsK may be assembled into the projections in wild type spores. This may explain why ExsK is detectable on the spore surface by IFM, whereas ExsFA can only be detected in the absence of BclA (Giorno *et al.*, 2009, Severson *et al.*, 2009). Given the relative small number of proteins that make up the exosporium (~20 exosporium protein vs. ~80 coat proteins), it may be possible to resolve how each protein is involved in the assembly of the exosporium and where each protein is within the high-resolution structures that are available (Ball *et al.*, 2008).

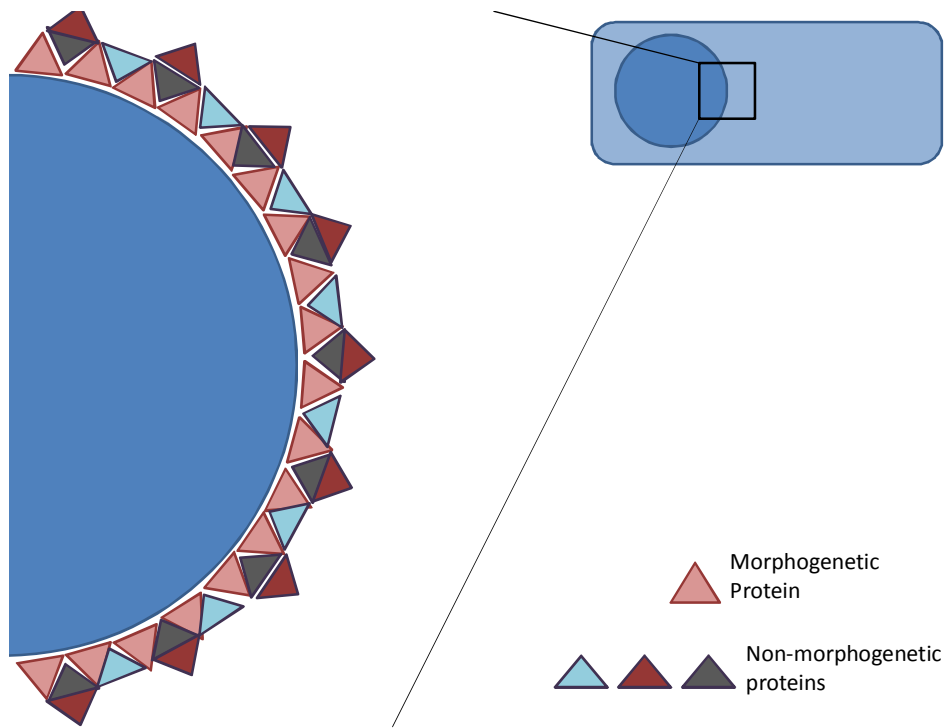
## Speculation on the Mechanism of Morphogenetic Proteins

So far we have considered the role that individual proteins play in the assembly of the *B. anthracis* spore. However, there are two important questions regarding the role that both morphogenetic proteins and clade-specific proteins play in spore assembly in all species.

1) How do morphogenetic proteins interact with all of the proteins that they direct to the developing spore surface? 2) How are the more recently-evolved (clade-specific) proteins integrated into the primordial/prototypical program of spore assembly? Let us consider the first of these questions. Morphogenetic proteins have a surprising number of cognate partners that they direct to the nascent spore surface. How does one morphogenetic protein of finite size interact with so many partners? Does each morphogenetic protein interact with all of its cognate partners? If so, how can one protein maintain so many interactions using a finite amount of surface exposed residues? Here I propose three models that answer these questions and are consistent with the data regarding morphogenetic proteins. To begin constructing such models I think it's important to make some assumptions. First, it's unlikely that any one morphogenetic protein can directly interact with more than a handful of its cognate partners since any given morphogenetic protein has a finite amount of surface area to interact with. Therefore, if one subunit of a morphogenetic protein interacts with all of its cognate partners it is likely to do so through multiple *indirect* interactions. Second, an exposed layer of morphogenetic protein is likely to be saturated once a subset of its interacting

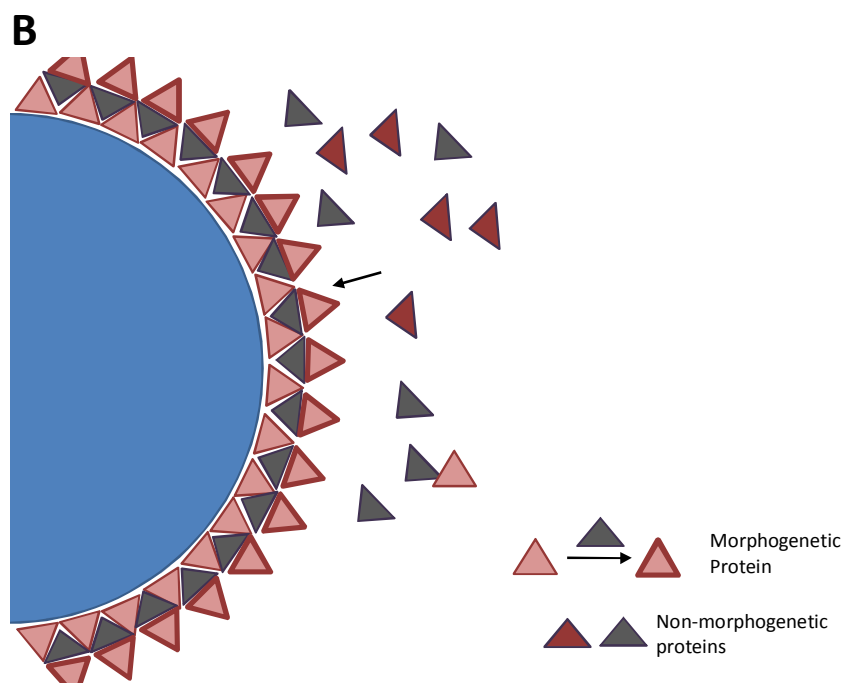
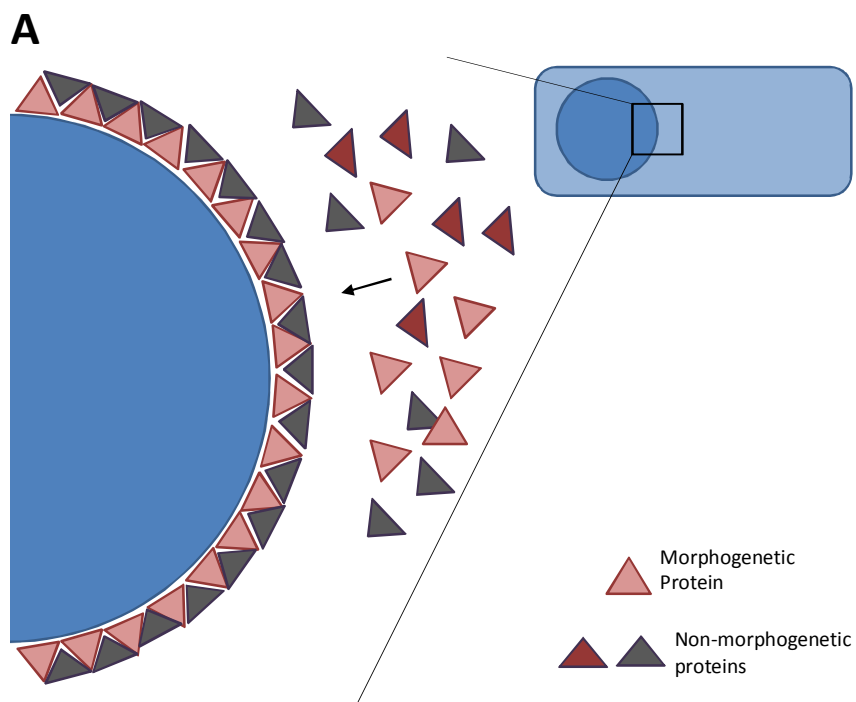
partners has bound to the developing spore surface. This is illustrated in the first model (Fig. 18). Here the morphogenetic protein bound to the forespore surface interacts with three non-morphogenetic proteins. Two of these interactions are direct while a third is indirect. This model is limited in its explanatory power since the surface of the morphogenetic protein layer is easily saturated preventing the morphogenetic protein from interacting the remaining subset of its interacting partners. In the second model (Fig. 19) the affinity of the morphogenetic protein for any given cognate partner is altered in response to the partner(s) it is already interacting with (Fig. 19). This model explains how a morphogenetic protein can interact with multiple partners (in a progressive fashion). Further, the model also explains the robust nature of the assembly process since the absence of any given non-morphogenetic protein can be compensated for by another protein which will still bind (albeit it with less affinity). In the last model (Fig. 20) morphogenetic proteins and non-morphogenetic proteins form dimers. Dimer formation by non-morphogenetic proteins allows for the formation of multiple layers of morphogenetic proteins (preventing the saturation of any given layer). Dimer formation by morphogenetic proteins also acts to prevent saturation, while morphogenetic protein monomers allow for a novel interaction to be made with a different cognate partner. Nevertheless, these models aren't mutually exclusive and it is likely that a combination of these three mechanisms contribute to the capacity of morphogenetic proteins to interact with a large number of cognate partners.

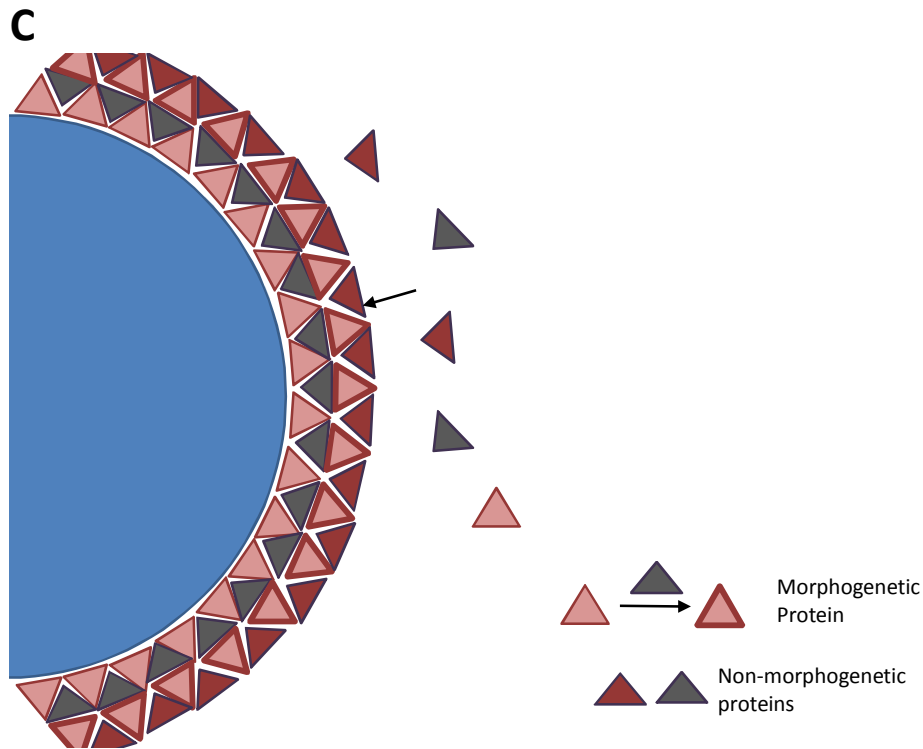




**Figure 18. A simplistic model of morphogenetic proteins.**

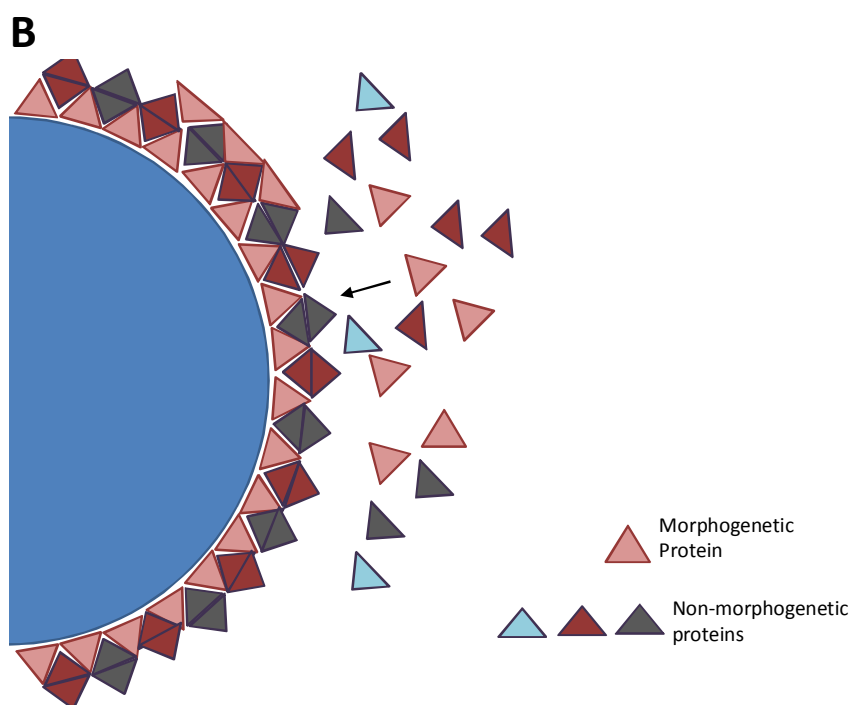
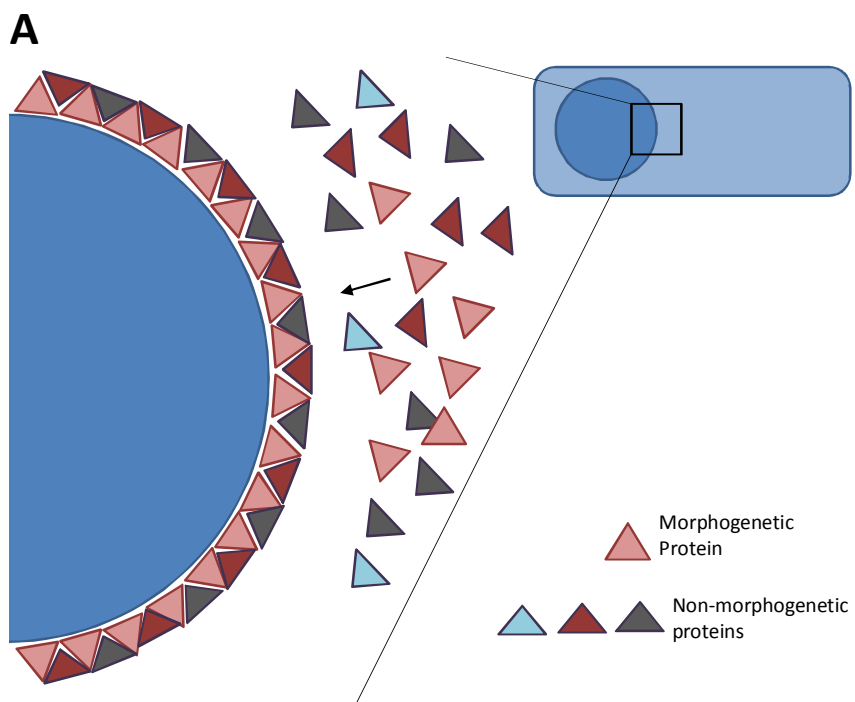
A model of morphogenetic proteins in which interactions with cognate partner proteins can be both direct and indirect. The region of the sporulating cell (upper right hand corner) is enlarged to show detail. In this example the morphogenetic protein (pink triangles) interacts directly with two partner proteins (pink and grey triangles), and indirectly with another (maroon triangles). The loss of any one of the non-morphogenetic proteins does not dramatically affect the structure because the remaining cognate partners can fill in the gaps to preserve the gross structure. However, this model is limited because the morphogenetic protein layer is quickly saturated and cannot interact with a large number of different cognate proteins.

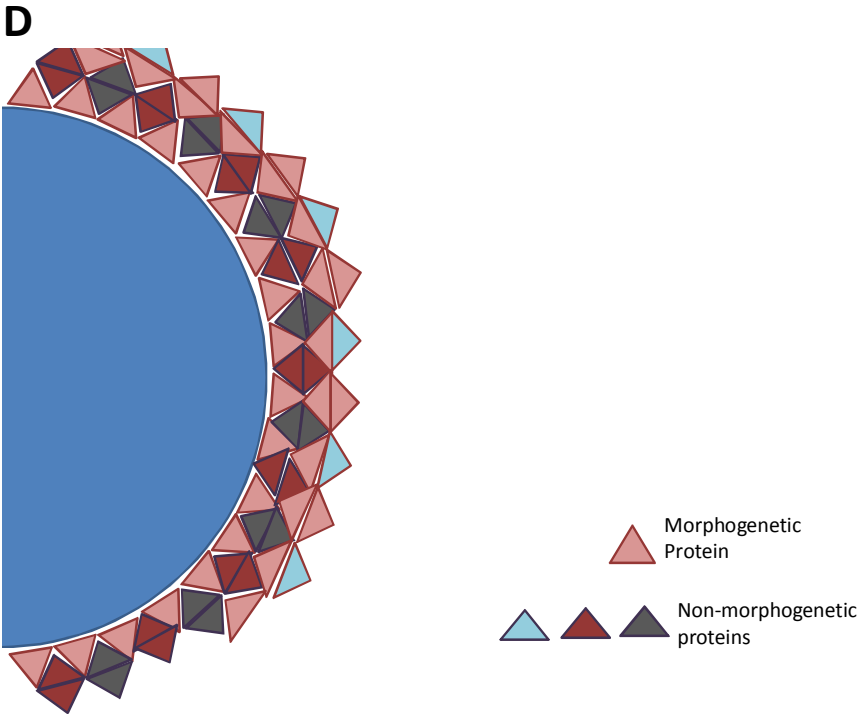
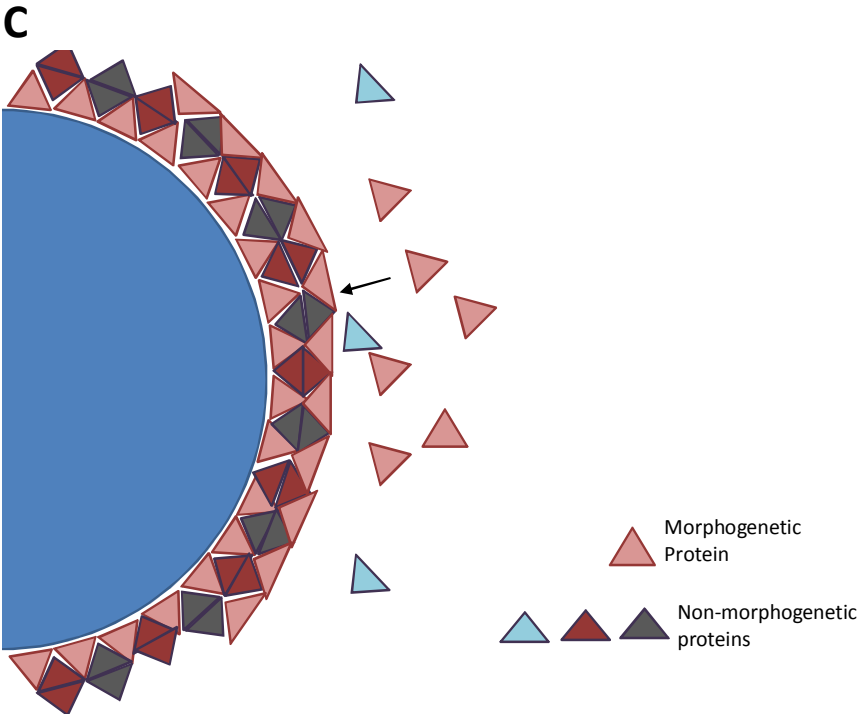




**Figure 19. A model of morphogenetic proteins in which the affinity for cognate partner-proteins varies.**

A) In this example a morphogenetic protein (pink triangles) deposited on the forespore surface allows for the binding a non-morphogenetic protein (grey triangles). B) The deposited grey now protein forms a surface that is compatible for binding to more morphogenetic protein. C) This novel surface may further interact with the pink morphogenetic protein, a different morphogenetic protein, or other non-morphogenetic proteins. This model explains the robust nature of spore coat assembly since in the absence of the grey protein the maroon protein can still bind to the morphogenetic protein, preserving the integrity of the assembly process.





**Figure 20. The possible role of dimer formation in morphogenetic protein function.**

A) In this example the morphogenetic proteins (pink triangles) deposited on the forespore surface allow for the binding of non-morphogenetic proteins (grey and maroon triangles). B) Dimer formation by non-morphogenetic proteins effectively doubles their interaction faces. C) Because the faces of the non-morphogenetic proteins that interact with the pink (morphogenetic) protein are exposed via dimer formation, a new layer of morphogenetic protein can bind to the developing surface. D) This novel surface of morphogenetic protein can either homodimerize (allowing regeneration of the previous layer of coat) or heterodimerize with different non-morphogenetic proteins (blue triangles) to form novel interactions.

## **The Role of Coat and Exosporium Proteins in Germination**

The process of germination must be highly regulated in order to ensure that spores do not germinate during development, and that they only reactivate when conditions will support growth of the organism. Since spores are completely dormant the spore relies on passive means of regulating germination. First, germination receptors which are located in the inner membrane (and sit under the spores outer-structures) detect the presence of small molecule nutrients (see (Moir, 2006, Setlow, 2003) for reviews). Binding of these receptors to their cognate nutrients allows for the first steps of rehydration to occur via the release of  $\text{Ca}^{2+}$ -DPA release and the influx of water. Interestingly,  $\text{Ca}^{2+}$ -DPA subsequently activates a cortex-lytic enzyme (CwlJ) located in the coat which helps to hydrolyze the spore's peptidoglycan leading to the complete rehydration of the core and the eventual re-activation of metabolism. However, beyond these simple regulatory steps little is understood about how the spore's outer-structures facilitate and/or regulate germination. We therefore wished to better understand the role that the coat and the exosporium play in regulating germination by analyzing germination in our mutant spores.

To begin our germination analysis we wished to determine if the exosporium played a role in regulating germination. The exosporium has been shown to harbor alanine racemase (Alr) and an inosine-uridine preferring nucleoside hydrolase (IunH) (Todd *et*

*al.*, 2003, Redmond *et al.*, 2004, Lai *et al.*, 2003). Both of these enzymes can downregulate germination by inactivating the germinant molecules presumably by inactivating them as they pass thru the exosporium (Liang *et al.*, 2008, Chesnokova *et al.*, 2008). Therefore, we analyzed germination in the *cotO* mutant and *cotE* mutant strains which lacked the exosporium (MGM76 and RG56 respectively), and analyzed early (dye uptake), intermediate (loss of OD), and late events (tetrazolium metabolism) in germination (Fig. 16 and data not shown) (Moir, 2006, Setlow, 2003). Interestingly, we found that the *cotO* mutant germinated modestly more-efficiently than wild type in both the dye uptake and loss of optical density assays, while *cotE* spores germinated less efficiently in these assays (Fig. 16 and (Giorno *et al.*, 2007)). These results at first seem to contradict one another since both mutant spores lack an exosporium; however a distinguishing feature of *cotE* mutant spores might explain the decreased efficiency. We have found that *cotE* mutant spores have coats that are pulling away from the cortex. Possibly the space between the coat and the cortex prevents CwlJ (the cortex lytic enzyme in the coat) from reaching its target once it's activated. There is some supportive evidence for this hypothesis. First, gaps develop between the coat and the cortex as the spore dehydrates (reducing the core volume), and folds form in the coat, probably to accommodate the change in volume (Chada *et al.*, 2003, Holt *et al.*, 1975). Secondly, mutations which appear to affect the formation of these folds also appear to affect germination (Giorno *et al.*, 2007). Alternatively, CotE may control the assembly of CwlJ as it does in *B. subtilis* (Bagyan & Setlow, 2002). This would explain why *cotE* mutant



spores lack an exosporium but germinate less efficiently than wild type, but does not explain why *cotH* mutant spores (which have an exosporium) germinate faster than wild type (Giorno *et al.*, 2007). Localizing the cortex lytic enzymes in the *B. anthracis* spore in the dormant and germinating states should help to resolve this question.

**TABLE 2 List of Strains and Plasmids****Strains**

<i>Strain Name</i>	<i>Genotype</i>
<b>RG56</b>	Sterne <i>cotE</i> ::kan (Giorno <i>et al.</i> , 2007)
<b>MGM76</b>	Sterne <i>cotO</i> ΩpMGM3 (This study)
<b>MGM84</b>	Sterne <i>spoVID</i> ΩpMGM2 (This study)
<b>MGM155</b>	Sterne <i>ba5149</i> ΩpSAM1 (This study)
<b>MGM68</b>	Sterne <i>cotβ</i> ::kan (Mallozzi <i>et al.</i> , 2008)
<b>MGM170</b>	Sterne <i>cotJC</i> ΩpSAM2 (This study)
<b>MGM193</b>	Sterne <i>yrbB</i> ΩpSAM3 (This study)
<b>JABAmescotO<sup>1</sup></b>	Ames <i>cotO</i> ΩpMGM3 (This study)
<b>JABAmesspoVIDysxE<sup>1</sup></b>	Ames <i>spoVID</i> ΩpMGM2 (This study)
<b>JABAmesspoVID<sup>1</sup></b>	Ames <i>spoVID</i> ::kan (This study)

<sup>1</sup> Ames mutants constructed by Joel Bozue at the United States Army Medical Research Institute for Infectious Disease, Ft. Deterick, MD

**Plasmids**

<i>Plasmid Name</i>	<i>Genotype</i>
<b>pKS1</b>	<i>kan<sup>r</sup> erm<sup>r</sup></i> (Shatalin & Neyfakh, 2005)
<b>pEO3</b>	Bacillus <i>E. coli</i> shuttle vector (Mendelson <i>et al.</i> , 2004)
<b>pMGM6</b>	pKS1- <i>gfp</i> (Severson <i>et al.</i> , 2009)
<b>pMGM2</b>	pKS1 bearing an internal fragment of <i>spoVID</i> (This study)
<b>pMGM3</b>	pKS1 bearing an internal fragment of <i>cotO</i> (This study)
<b>pSAM1</b>	pMGM6 bearing a 3' fragment of <i>ba5149</i> (This study)
<b>pSAM2</b>	pMGM6 bearing a 3' fragment of <i>cotJC</i> (This study)
<b>pSAM3</b>	pMGM6 bearing a 3' fragment of <i>yrbB</i> (This study)
<b>pJAB<i>spoVID</i>::kan</b>	pEO3 3' and 5' regions of <i>spoVID</i> flanking <i>kan<sup>r</sup></i> (This study)

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## VITA

Michael Mallozzi graduated from Coronado High School in Colorado Springs, CO in 1997, and attended Colorado State University in Fort Collins, CO that fall. In 2002 Michael graduated from CSU with a degree in Microbiology and Immunology and a minor in Biochemistry. In August of 2002 Michael started graduate school at Loyola University Chicago in the Dept. of Microbiology and Immunology.

After joining the lab of Dr. Adam Driks in late 2003, Mike began his investigation of *Bacillus anthracis* spore structure and function. In the summer of 2009 Mike left to take a position at the University of Arizona in Tucson, under the direction of Dr. Gayatri Vedantam studying the pathogenesis of *Clostridium difficile* (another spore-forming bacteria).