Characterization of the *Bacillus anthracis* SleL Protein and its Role in Spore Germination

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ABSTRACT

Bacillus anthracis is a spore-forming bacterium that is included on the list of select agents compiled by the Centers for Disease Control. When a *B. anthracis* spore germinates, a protective layer of peptidoglycan known as the cortex must be depolymerized by germination-specific lytic enzymes (GSLEs) before the bacterium can become a metabolically active vegetative cell. By exploiting cortex lytic enzymes it may be possible to control germination. This could be beneficial in elucidating ways to enhance current decontamination methods.

In this work we created in-frame deletion mutants to study not only the role of one GSLE, SleL, but by creating multi-deletion mutants, we were able to analyze how the protein cooperates with other lytic enzymes to efficiently hydrolyze the cortical PG. We determined that SleL plays an auxiliary role in complete peptidoglycan hydrolysis, secondary to cortex lytic enzymes CwlJ1, CwlJ2, and SleB. The loss of *sleL* results in a delay in the loss of optical density during germination. However, spores are capable of completing germination as long as CwlJ1 or SleB remains active. HPLC analysis of muropeptides collected from *B. anthracis* Δ*sleL* strains indicates that SleL is an N-acetylglucosamidase that acts on cortical PG to produce small muropeptides which are quickly released from the germinating spore.

By analyzing the *in vitro* and *in vivo* activities of SleL we confirmed the enzymatic activity of the protein, characterized its substrates, and studied the roles of its putative LysM domains in substrate binding and spore-protein association. We were able to show that purified SleL is capable of depolymerizing partially digested spore PG resulting in the production of N-acetylglucosaminidase products that are readily released as small muropeptides. *In vitro*, loss of

the LysM domain(s) decreases hydrolysis effectiveness. The reduction in hydrolysis is likely due to LysM domains being involved in substrate recognition and PG binding. When the SleL derivatives are expressed *in vivo* those proteins lacking one or both LysM domains do not associate with the spore, suggesting that LysM is involved in directing protein localization.

DEDICATION

I would like to dedicate this work to my wonderful family. Each member of my family contributed to my success, and I am grateful for them all. To my mom, Jean, and my dad, Tom, I am especially indebted. Mom, thank you for always taking the time to listen to me. You continually encouraged me throughout this long process. Dad, thanks for knowing when I had had enough. Thanks for shutting my books and just giving me a big bear hug. To Gram I say thank you for teaching me the importance of education and following your dreams. Gramps you were always a special friend to me and if there was one person that influence my decision to pursue science it was you. I will be forever thankful. To my sister and brother, Stephanie and Nathan, you have always known when to call me and plan time to watch Mountaineer football so I could get my mind off of work.

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ATTRIBUTION

Jared Heffron and conntributed equally to the research including multi-deletion construction and writing involved in the material presented in Chapter 3. Nora Sherry contributed by creating some of the multi-deletion mutant strains used in Chapter 3 and made peptidoglycan substrates used in Chapter 4, and David Popham was the principle investigator. Each coauthor has given written permission to reproduce the material in this dissertation.

Chapter 1

Introduction and Literature Review

Bacterial endospores are the most resilient biological entity known. These stout structures can persist in nature for thousands, if not millions of years (10). Bacteria of the genera *Bacillus* and *Clostridium* are capable of producing dormant, highly resistant endospores that are known to contaminate food processing centers and hospitals (17, 89). *Bacillus* and *Clostridium* species are frequently the subjects of scientific research because the bacterial spores and cells are notorious for causing illnesses including histotoxic diseases (58), colitis (81), food poisoning (29), and anthrax (1). For spores of these pathogenic bacteria to cause disease they must germinate into metabolically and enzymatically active cells (21). This transition requires one particular group of enzymes known as germination-specific cortex lytic enzymes that help degrade the spore cortical PG (53). The identification and analysis of these enzymes is of interest because an understanding of their actions will promote the development of germination inhibitors that can be used to prevent disease. Likewise, germination stimulants could be developed and their implementation would promote decontamination and foster disease prevention (9, 35, 74). By studying the processes of sporulation and germination we can build a better understanding of the bacterial lifecycle and learn how to manipulate the biological machinery of *Bacillus* and *Clostridium*.

Bacterial sporulation

Bacillus and *Clostridium* cells sporulate as a step in their lifecycles. In some cases sporulation is a biological adaptation to starvation (20). During sporulation a vegetative cell divides asymmetrically into two sister compartments and, over the course of development, one compartment commits its resources to packaging the second in a structure surrounded by multiple protective layers before it self-destructs (19, 20, 49). This process is outlined in Figure 1.1.

Sporulation is divided into seven stages, each of which is characterized by its own morphological transition (20). Stage I of sporulation begins when a dividing vegetative cell detects environmental conditions including nutrient depletion and commits to cellular differentiation. During stage I the bacterial chromosome partially replicates resulting in an axial chromatin filament that spans the length of the cell (49). Stage II in the cell's transition includes completion of chromosome replication and formation of a septum that produces two sister cells: a large mother cell compartment and a smaller forespore compartment. During septum formation one complete copy of the chromosomal DNA is partitioned to the forespore (49). The mother cell membrane progressively engulfs the forspore until a protoplast is encased within the mother cell. These events are defined as stage III (19). Stage IV involves the synthesis and deposition of two layers of peptidoglycan called the germ cell wall and cortex between the two forespore membranes (19). Next, stage V occurs when a thick layer of spore proteins surrounds the periphery of the developing spore to produce the inner and outer coats (20). As stage VI progresses there are few morphological changes, but the spore matures and develops resistance properties. At the completion of sporulation, stage VII, the spore has fully developed and the mother cell lyses, releasing the metabolically dormant structure which is capable of surviving for centuries (19, 20, 49). The development of the spore's multiple protective shells allows it to withstand otherwise harmful environmental conditions including radiation, desiccation, starvation, pH extremes, and toxic chemicals (28, 91).

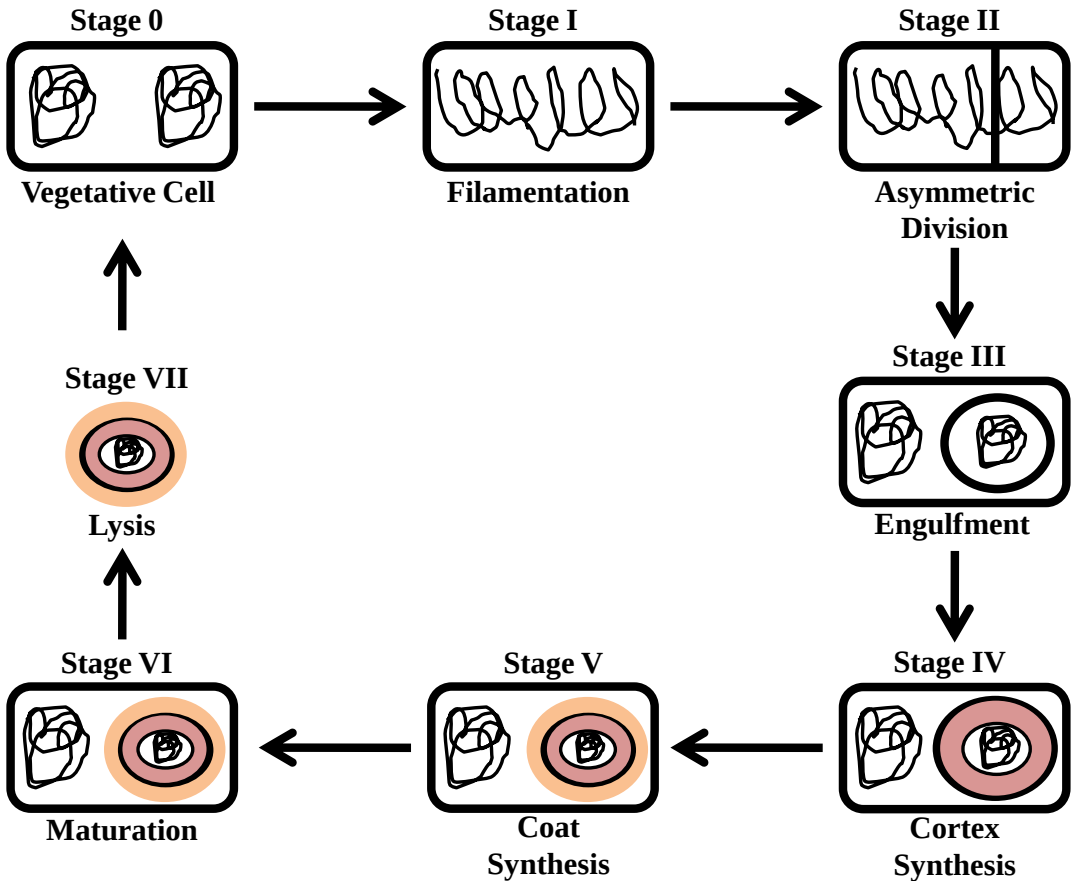

Figure 1.1: Bacterial sporulation. During sporulation *Bacillus* and *Clostridium* cells advance through a series of developmental steps. First, a vegetative cell detects environmental conditions and commits to sporulation. The vegetative cell replicates its chromosome and partitions one copy to a specialized compartment called the forespore. Meanwhile, a second compartment known as the mother cell commits its resources to producing cortical peptidoglycan and proteins that link to form protective layers for the spore. After the spore matures, the mother cell's job is complete and it lyses releasing a highly resistant, dormant structure that can withstand environmental assaults for many years.

The development of a bacterial spore is the result of a vast array of genes whose expression is harmoniously turned on and off at different stages of sporulation. These genes are regulated by a group of compartmentalized sigma factors. σ^E and σ^F are found in the pre-septational cell, and they become active in the mother cell and forespore respectively upon the formation of the septum. Included in the genes regulated by σ^E and σ^F are σ^G and σ^K, respectively. σ^G and σ^K regulate expression of genes in the forespore and mother cell, respectively, during the later stages of sporulation (20).

Spore germination

When bacterial spores are introduced into an environment conducive to sustenance they germinate into metabolically active, dividing vegetative cells (Figure 1.2). Like sporulation, germination consists of multiple steps that ultimately lead to the formation of a metabolically and physiologically distinct structure. Germination is initiated when a spore encounters non-nutrient or nutrient stimuli in the environment. Non-nutrient stimuli consist of lysozyme, salts, high pressure, Ca^{2+}-DPA and dodecylamine (91). Nutrient stimuli, termed germinants, can consist of amino acids, ribosides, purine nucleosides and/or sugars (27, 91). The germinants contact Ger receptors located at the inner membrane, and the spore becomes committed to germination (22). After activation, cations including H^+, Na^+, and Zn^{2+} are released from the spore core resulting in an increase in pH from ~6.5 to 7.7 (62, 91). Shortly thereafter, the large cache of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) chelated to Ca^{2+} is expelled from the spore core and replaced by inflowing water. As the core partially rehydrates it begins to lose its heat resistance properties but is still metabolically inactive (87). Next, a group of proteins called germination-specific lytic enzymes (GSLEs) begin to digest the cortex peptidoglycan (PG) that is responsible

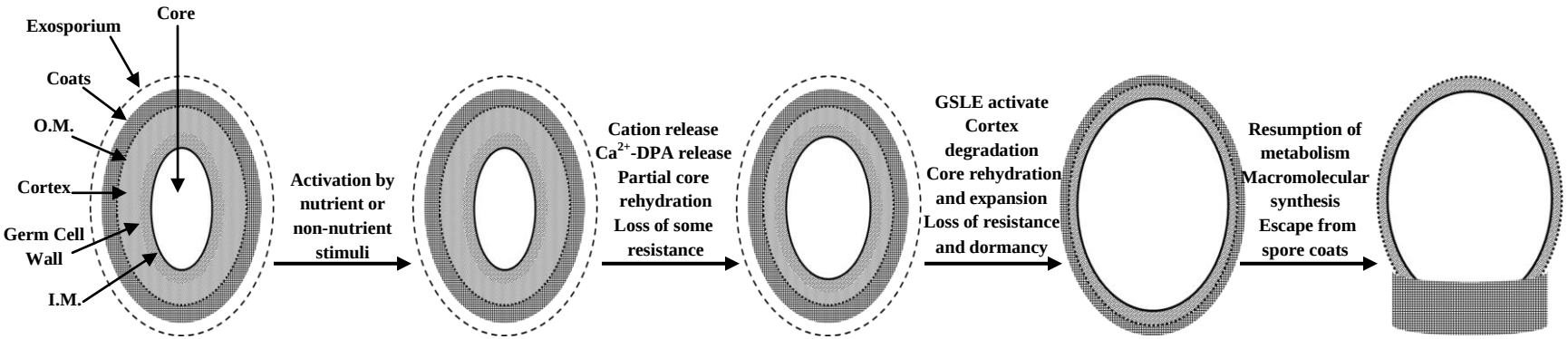

Figure 1.2: Bacterial spore germination. When a *Bacillus* or *Clostridium* spore encounters nutrient or non-nutrient stimuli within its environment it germinates. Water influx partially rehydrates the spore core while replacing the cache of Ca^{2+}DPA that is simultaneously released. Germination-specific cortex-lytic enzymes are activated and degrade the cortical peptidoglycan. As the spore core expands it becomes completely rehydrated allowing mobilization of proteins and the resumption of metabolism. The spore escapes the proteinacous coats and outgrows into a vegetative cell. (O.M.: outer membrane, I.M.: inner membrane).

for promoting resistance by maintaining dehydration (3, 11, 24, 53, 78). Hydrolysis of the cortical PG is essential to spore germination because it permits more water to penetrate the spore core until it is completely rehydrated (78). This allows proteins to become mobile, and metabolism can resume (16, 91). The morphological and biochemical processes that occur during germination are common among many species of spore-forming bacteria. For this reason the molecular events that take place during the developmental course may be conserved among endospore formers (45).

Cortex peptidoglycan structure

As mentioned, a critical step in spore germination is the degradation of the cortical PG. The cortex has structural characteristics that distinguish it from the germ cell wall PG (Figure 1.3) thus allowing GSLEs to preferentially digest it without disrupting the integrity of the germ cell wall (3, 76, 79). Cortical PG precursors are synthesized in the mother cell and then polymerized between the membranes of the developing forespore. Initially, the cortex is likely produced by the same components involved in vegetative PG synthesis. However, during stage IV of sporulation, specific genes included in the σ^E regulon including *spoVB*, *spoVD*, *spoVE*, *sporVG*, *gerJ*, and *gerM* are expressed, and their protein products are thought to be involved in cortex synthesis and development. Like the germ cell wall, cortical PG consists of alternating N-acetyl-glucosamine and N-acety-muramic acid residues linked by a β,1-4 bond. Unlike the germ cell wall, every other N-acetyl-muramic acid residue within the cortex is converted to a muramic-δ-lactam by the enzymes CwlD and PdaA (26, 79). It is the muramic-δ-lactam residue of the cortical PG that is thought to be a recognition determinant for the GSLEs (79). The remaining N-acetyl-muramic acid residues of the cortex are bound to a tetrapetide side chain or a single L-alanine (*Bacillus*) (78), dipeptide or tripeptide (*Clostridium perfringens*) (70). These

modifications to the cortex cause it to be less highly cross linked than the germ cell wall, a characteristic that promotes core dehydration and spore heat resistance (78).

Bacillus germination-specific lytic enzymes

GSLEs are subdivided into two groups: spore cortex lytic enzymes (SCLEs) and cortical fragment lytic enzymes (CFLEs). SCLEs act on intact cortical PG partially digesting it into large fragments. CFLEs then act on the partially hydrolyzed cortex and further fragment it into small muropeptides that are readily released from the spore (53). GSLEs have been characterized in many spore forming bacteria including *Bacillus anthracis* (35, 47, 51), *Bacillus cereus* (11, 52, 66), *Bacillus megaterium* (23, 88), *Bacillus subtilis* (38, 42, 65), *Bacillus thuringiensis* (36), *Clostridium difficile* (9), and *Clostridium perfringens* (96). In the *Bacillus* species two SCLEs, CwlJ and SleB, and one CFLE, SleL, have been described. In *Clostridium* SleC acts on intact cortical PG while SleM further digest the fragmented PG. Although these enzymes cooperate to promote the breakdown of the cortex, they each play different roles.

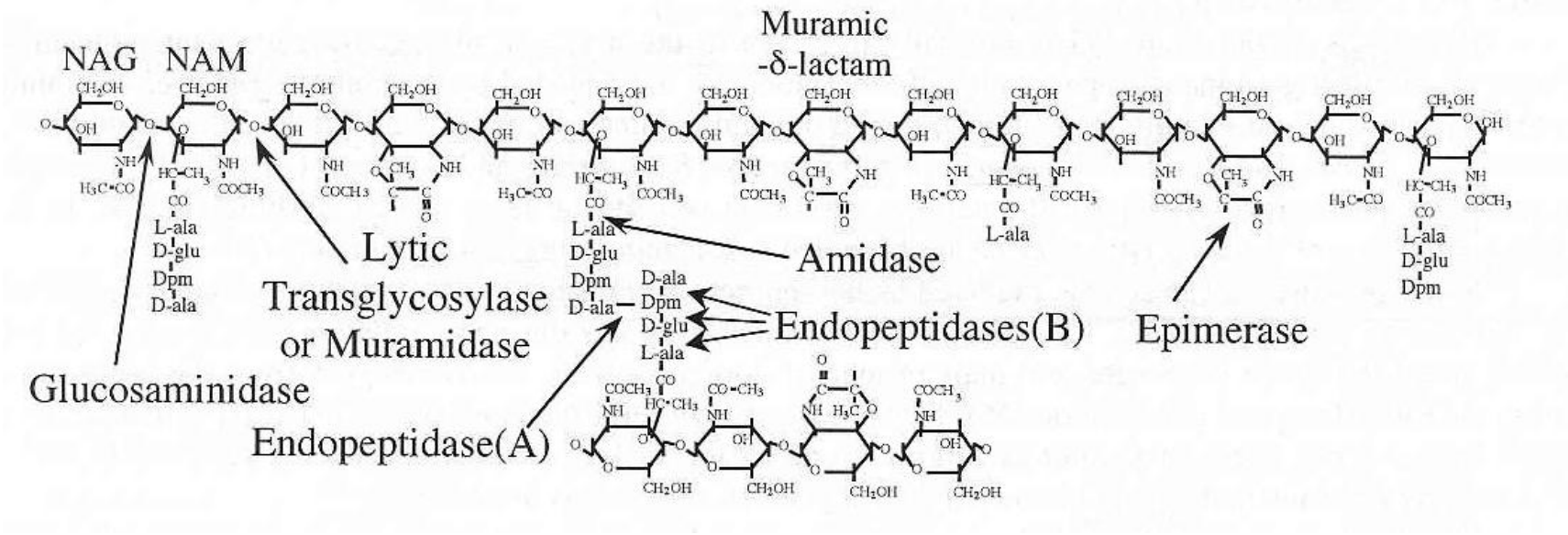

Figure 1.3: Structure of *Bacillus* cortex peptidoglycan. The cortical peptidoglycan of *Bacillus* spores consist of alternating N-acetyl-glucosamine and N-acetyl-muramic acid residues. Every other N-acetyl-muramic acid residue is converted to a muramic-δ-lactam residue. Of the remaining N-acetyl-muramic acid residues half have only a single alanine side chain. These changes not only decrease the amount of cross-linking within the cortex, but they also allow germination-specific lytic enzymes to preferentially digest the cortex and leave the germ cell wall peptidoglycan intact. The cortex peptidoglycan can be potentially digested by an array of enzymes as depicted above.

CwlJ

The least understood of all the GSLEs is CwlJ (Table 1.1). *B. subtilis* contains one *cwlJ* homolog that is found in a monocistronic operon. *B. anthracis*, on the other hand, contains two homologs termed CwlJ1 and CwlJ2 which are 62 and 58% identical to the *B. subtilis* CwlJ protein. *cwlJ1* is the first gene in a bicistronic operon with *gerQ* while *cwlJ2* is located in a monocistronic operon. β-galactosidase activity of a *cwlJ-lacZ* fusion strain suggests that the genes are expressed between t_2-t_7 (2-7 hours after the onset of sporulation) (38). The expression of *B. subtilis cwlJ* is regulated by σ^E within the mother cell as determined by northern blotting and primer extension analysis (38). Whether or not the gene is expressed monocistronically or bicistronically, its protein product relies on the product of *gerQ* for proper localization and function within the mature spore (38). Upon localizing to the spore, CwlJ is thought to be contained at the periphery of the spore. Because CwlJ is removed from decoated spores and it is absent from *cotE* spores with severe coat defects, the protein is likely located within the proteinaceous coat layer (5, 35). CwlJ is less likely associated with the cortex or inner membrane because, not only does it lack a signal sequence or PG recognition domain, but it cannot be extracted from spores treated with lysozyme, high salt, or Triton (5). CwlJ can be activated during germination by endogenous or exogenous Ca^{2+}-DPA and is therefore thought to be involved in Ca^{2+}-DPA induced germination (72). Although the role of CwlJ has been studied in multiple *Bacillus* species, its enzymatic activity has yet to be definitively elucidated. The 18 kDa protein is thought to be a SCLE that is partially redundant to SleB, a protein with which CwlJ shares 30% sequence identity within the catalytic hydrolase family 2 domain (Figure 1.4) (38). In the absence of CwlJ, nutrient-induced germination is delayed but will proceed as a result of the remaining GSLEs (35, 38).

SleB

A second SCLE in *Bacillus* species is SleB (Table 1.1). Although no single GSLE is essential for spore germination, SleB and CwlJ are collectively essential for *Bacillus* germination. Expression of *sleB* is regulated by σ^G. Primer extension analysis and *lacZ*-fusion studies show that *sleB* is transcribed as a bicistronic or polycistronic mRNA along with *ypeB* in *B. subtilis* or *ypeB* and *ylaJ* in *B. anthracis* within the developing forespore between t_3-t_6 (7, 35). YpeB is required for the proper localization of SleB and may be involved in inhibition of SleB prior to germination (7, 13). The 24-kDa SleB protein is composed of an N-terminal signal sequence followed by two PG binding domains and a C-terminal hydrolase family 2 catalytic domain (Figure 1.4) (64). Within the forespore, SleB is thought to translocate across the inner membrane via its secretion signal peptide (64). The final location of SleB is controversial. Immunoelectron microscopy indicates that SleB localizes to the outer membrane or cortex region (64). It has also been shown that some SleB may localize to the inner forespore membrane (13). At some point during sporulation or germination, the signal sequence of SleB is cleaved by an unknown protease. After germination initiation, SleB becomes activated although the mechanism has yet to be determined. Some speculate that stress on the spore cortex as a result of partial core hydration may create a suitable substrate for the enzyme (13, 23). Upon activation SleB digests intact cortical PG. Early studies investigating the role of the *B. cereus* SleB protein indicate that because amino groups, but no sugar reducing groups, are released during germination, the enzyme is likely an amidase or peptidase (64, 65). However, more recent studies involving the *B. subtilis* (7) and *B. anthracis* (35) SleB proteins indicate that the enzymes are lytic transglycosylases that digest the cortical PG resulting in the release of anhydromuropeptides.

Loss of SleB due to genetic mutations results in a delay in germination (7, 35, 38). However, vegetative cells eventually emerge as a result of the remaining active GSLEs.

SleL

The only CFLE characterized in *Bacillus* species is SleL (known as YaaH is *B. subtilis*) (Table 1.1). The SleL proteins of *B. anthracis* and *B. cereus* share 98% sequence identity. The *B. anthracis* SleL and *B. subtilis* YaaH proteins share only 48% sequence identity. Although the time and location of the *B. cereus sleL* gene has not been evaluated, studies show that *yaaH* is expressed as a monocistronic mRNA in the mother cell. Northern blot analysis and *yaaH-lacZ* transcription fusion studies indicate that the gene is regulated by σ^E and expression in the mother cell occurs between t_1-t_5 (42, 51). Unlike CwlJ and SleB there are no known proteins involved in proper localization or activation of either SleL or YaaH. Both proteins are composed of three domains; two N-terminal LysM domains and a C-terminal glycosyl hydrolase family 18 catalytic domain (Figure 1.4). Fluorescent microscopy has shown that during sporulation YaaH translocates from the mother cell to the developing forespore (42). The final location of both CFLEs is thought to be the periphery of the spore. Chen et al. (11) suggest that the *B. cereus* SleL protein probably localizes at the exterior of the cortex. A recent study on spore coat proteins indicates that the *B. subtilis* YaaH protein localizes to the inner coats. This inference is based upon the ideas that YaaH localization is CotE independent, and the protein is further from the spore core than YhcN, a cortex associated protein (37). Like CwlJ, SleL and YaaH are inactivated by decoating (35, 37). SleL is considered a CFLE because unlike CwlJ and SleB, SleL is incapable of digesting intact cortical PG (11). SleL does not become active within the germinating spore until the cortex has been partially degraded. This could be why the mature protein is capable of being sustained within the spores without causing lysis of the cortex.

Digestion of the cortical PG by activated SleL results in muropeptides produced by an N-acetylglucosaminidase (11). Studies involving *B. subtilis* also indicate that YaaH may be responsible for the production of epimerase products (4, 13). The roles of SleL and YaaH during germination are dispensable since loss of the genes and their products do not affect the overall outcome of germination.

***Clostridium* germination-specific lytic enzymes.**

Although there are significant differences between *Clostridium* and *Bacillus* spores the germination pathways are relatively similar. Like *Bacillus*, *Clostridium* species contain at least one SCLE and one CFLE that are required for cortex lysis. The enzymes are equally as important to the resumption of metabolism although their activation and enzymatic activities differ somewhat from those seen in *Bacilli.* Among the *Clostridia* studied including *C. acetobutylicum, C. difficile, C. perfringins,* and *C. tetani,* the GSLEs SleC and SleM are conserved (57).

SleC

The SCLE of *Clostridium* is known as SleC (Table 1.1). RT-PCR and northern hybridization indicates that the monocistronic expression of *sleC* occurs around t_4 and is probably regulated by σ^E in the mother cell (57). *sleC-gfp* (green fluorescent protein) fusions are detectable between t_4-t_8 in the mother cell of developing sporangia of *Clostridium* when analyzed by fluorescent microscopy. However, no fluorescence is detected in the forespore. Immunoblot analysis using anti-GFP suggests that the resultant SleC-GFP fusion protein is present in dormant spores signifying that SleC may be masked by the outer protein coats (57). This idea is supported by the fact that decoated *Clostridium* spores do not respond to germinants possibly because SleC is either removed or inactivated (45). SleC is initially composed of four domains. Originally, it is expressed as a precursor whose N-terminal pre-region and C-terminal pro-regions are cleaved.

The inactive proform known as proSleC then consists of an N-terminal pro-sequence and a mature domain (60). The N-terminal pro-sequence of the zymogen is cleaved by CspB during germination resulting in a mature active SleC protein (Figure 1.4) (60, 70, 96). Mature SleC is thought to act as a bifunctional enzyme with lytic transglycosylase and N-acetyl-muramoyl-L-alanine amidase activities (45). Spores lacking SleC as a result of mutagenesis have severe germination defects suggesting that the protein is essential to *Clostridium* cortex degradation (45).

SleM

After SleC partially digests *Clostridium* cortex PG, the CFLE SleM further hydrolyzes the fragments to small pieces (Table 1.1). Expression of *sleM* occurs between t_2-t_6 and is probably regulated by σ^E in the mother cell (57). Like SleC, fluorescent microscopy and immunoblotting using anti-GFP suggests that the protein translocates from the mother cell to the spore coats (57). Unlike SleC, SleM is expressed as a mature protein (Figure 1.4) whose digestion pattern is indicative of a muramidase (12). SleM is likely maintained as a mature protein in the spore without digesting the cortex PG because it only recognizes partially digested substrate (12, 96). Removal of SleM by decoating or genetic manipulation does decrease the rate of germination but not the final outcome. Therefore, SleM is a non-essential GSLE (45).

Bacillus anthracis

Bacillus anthracis is a gram-positive, nonmotile, aerobic, facultative anaerobic, spore-forming, rod-shaped bacterium. *B. anthracis* spores are the contagion of anthrax. There are three forms of anthrax: inhalational, cutaneous, and gastrointestinal. During inhalational anthrax, inhaled spores are engulfed by macrophages deep in the lungs (86). These macrophages detach from the lungs and are transported to the regional lymph nodes. During this time the endospores

germinates into a metabolically active vegetative bacilli (86). The bacilli replicate, kill the macrophage, and disseminate in the blood stream (32). Once in the bloodstream the bacilli respond to the host's physiology and produce virulence factors including a capsule and toxins (44). The toxins that are produce by the bacteria act on macrophages causing them to release proinflammatory cytokines that are responsible for fatal shock (31). There have been no documented cases of anthrax caused by vegetative *B. anthracis* bacilli or live-animal-to-live animal transmission (33).

Because *B. anthracis* can cause widespread illness and death, which may impair a city or region, the Centers for Disease Control (CDC) have classified the bacteria as a category A organism (84). This means that *B. anthracis* can be easily disseminated and/or transmitted from person to person resulting in a high mortality rate (83). It is estimated that at least 17 nations have offensive biological weapons programs that include anthrax (83). For these reasons it is important to have very effective, rapid methods to decontaminate areas that may be tainted with *B. anthracis* spores. Although spores are highly resistant, bacilli are poor survivors (50). Thus, by studying *B. anthracis* germination we will be able to elucidate ways to exploit the physiological process in order to enhance current decontamination methods and prevent disease transmission.

Study objectives

The role of SleL in *Bacillus anthracis* germination and how it cooperates with CwlJ and SleB has yet to be sufficiently studied. Because *B. anthracis* has been used as a biowarfare agent in recent history and remains on the list of select agents, we have established a list of objectives that upon investigation will help formulate a better understanding of the *B. anthracis* germination process. This will help in establishing better decontamination procedures to help promote

bacterial eradication and disease prevention. These objectives include genetically manipulating *sleL* and evaluating the effects of the mutation on spore germination. Analyses of spore viability, germination, and cortex PG hydrolysis indicate that *B. anthracis* SleL functions as a CFLE and is an *N*-acetylglucosaminidase. The second objective involves analyzing the effects of multiple deletion mutations affecting GSLEs on spore germination efficiency and kinetics of cortex hydrolysis. The data confirm the dominant roles played by CwlJ1 and SleB in initiation of cortex hydrolysis and the major role of SleL in release of small cortex fragments. A minor role of CwlJ2 in nutrient-triggered germination and the contributions of CwlJ1 and CwlJ2 to Ca^{2+}-dipicolinic acid (DPA) triggered germination were revealed. Finally, the *in vitro* and *in vivo* enzymatic activities of *B. anthracis* SleL was evaluated, and the roles of the LysM domains during protein localization and substrate recognition were characterized. These studies demonstrate *N*-acetylglucosaminidase activity *in vitro* and show that both LysM domains are required for protein translocation to the spore. Although the LysM domains improve enzymatic digestion they are not essential for substrate recognition and degradation.

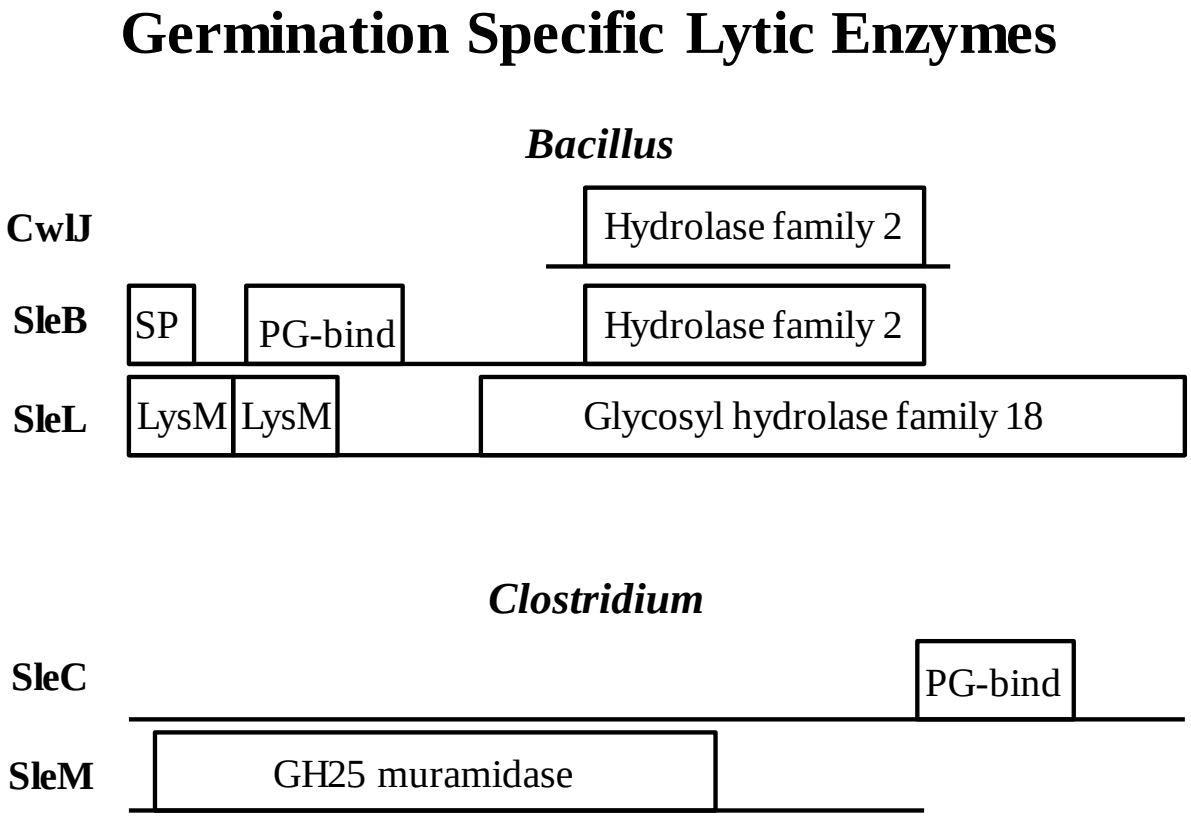

Figure 1.4: *Bacillus* and *Clostridium* germination-specific lytic enzymes' domains. Both CwlJ and SleB contain hydrolase family 2 catalytic domains that share 30% sequence identity. However, SleB also has an N-terminal signal peptide (SP) and two peptidoglycan binding domains (PG-bind). SleL also has two peptidoglycan binding domains (LysM) followed by a catalytic glycosyl hydrolase family 18 domain. The mature *Clostridium* SleC protein contains a peptidoglycan binding domain while SleM is composed largely of a glycosyl hydrolase family 25 (GH25) muramidase.

Table 1.1 Endospore germination-specific lytic enzymes.

	Bacillus			*Clostridium*	
	SCLE		**CFLE**	**SCLE**	**CFLE**
	CwlJ	**SleB**	**SleL**	**SleC**	**SleM**
Gene Expression Regulation	σ^E	σ^G	σ^E	σ^E	σ^E
Protein Biosynthesis	18 kDa mature protein	28-kDa protein with signal peptide 24-kDa mature protein	48-kDa mature protein	50-kDa precursor 38-kDa intermediate 35-kDa pro-sequence 31-kDa mature protein	38-kDa mature protein
Localization in Dormant Spore	Spore coats	Spore cortex, IFM, OFM	Spore coats or cortex	Spore coats	Spore coats
State in Dormant Spore	Mature protein inactive	Mature protein inactive	Mature protein active	Precursor inactive	Mature protein active
Substrate	Intact cortex PG	Intact cortex PG	Hydrolyzed cortex PG	Intact cortex PG	Hydrolyzed cortex PG
Predicted Enzymatic Activity	Unknown	Lytic transglycosylase and amidase	N-acetylglucosaminidase and epimerase	Lytic transglycosylase and amidase	Muramidase

CHAPTER 2:

The *Bacillus anthracis* SleL (YaaH) Protein is an *N*-acetylglucosaminidase Involved in Spore Cortex Depolymerization

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COAUTHORS CONTRIBUTIONS

Emily Lambert conducted all of the research involved in the following study, and she drafted the manuscript. David Popham served as the principle investigator and revised the manuscript.

ABSTRACT

Bacillus anthracis spores, the infectious agent of anthrax, are notoriously difficult to remove from contaminated areas because they are resistant to many eradication methods. These resistance properties are due to the spore dehydration and dormancy, and to multiple protective layers surrounding the spore core, one of which is the cortex. In order for *B. anthracis* spores to germinate and resume growth, the cortex peptidoglycan must be depolymerized. This study reports analyses of *sleL* (*yaaH)*, which encodes a cortex lytic enzyme. Inactivation of *sleL* does not affect vegetative growth, spore viability, or the initial stages of germination including dipicolinic acid release. However, mutant spores exhibit a slight delay in loss of optical density when compared to wild-type spores. Mutants also retain more diaminopimelic acid and N-acetyl-muramic acid during germination than wild-type spores, suggesting that SleL plays an auxiliary role in complete peptidoglycan hydrolysis. This finding is supported by HPLC analysis of peptidoglycan structure used to confirm that SleL acts as an N-acetylglucosaminidase. When *sleL* is inactivated, the cortex peptidoglycan is not depolymerized into small muropeptides but instead is retained within the spore as large fragments. Spores germinating in the absence of the *sleL*-encoded N-acetylglucosaminidase must be relying on essential cortex lytic enzymes, SleB and CwlJ, to break down the cortex peptidoglycan.

INTRODUCTION

The Gram-positive rod *Bacillus anthracis* transitions between two cellular morphologies, the spore and vegetative cell, during its intricate lifecycle. The spore is the infectious agent that causes all three types of anthrax: cutaneous, gastrointestinal, and inhalational (61). Unlike vegetative cells, spores are resistant to many environmental insults including extreme pH, high temperatures, chemical treatment, radiation, desiccation and starvation (90). These characteristics allow the metabolically inactive, dormant morphotype to survive outside a host for centuries (27) and hinder clean-up efforts in contaminated areas. Environmental conditions inside the *B. anthracis* host support the resumption of metabolic activity triggering the spores to germinate into vegetative cells. It is the vegetative cell that is responsible for the fatal symptoms associated with certain types of anthrax because it produces a protective capsule and deadly toxins.

Germination is the transition of a spore to a vegetative cell. It is stimulated when the spore encounters nutrient or nonnutrient germinants in its environment (91). Nutrient germinants interact with germinant receptors within the inner membrane of the spore. This initiates a cascade of events including release of cations and dipicolinic acid (DPA) from the spore core, partial core rehydration, hydrolysis of the cortex peptidoglycan (PG), and further hydration causing swelling of the spore and loss of dormancy (63, 73, 87, 91).

Bacillus spores contain two types of PG, the germ cell wall and the cortex PG. The germ cell wall is adjacent to the inner forespore membrane, and this layer may act as a template for vegetative cell wall synthesis after germination (3, 14). The cortex PG is assembled between the germ cell wall and the outer forespore membrane (59). The cortex PG is composed of glycan strands made of alternating N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM), the latter of which may be modified to muramic-δ-lactam or whose side chain may be cleaved to

a single L-alanine (3, 78, 100). These distinct modifications result in a loosely cross-linked cortex that helps maintain dehydration promoting spore heat resistance and dormancy (49, 77, 99).

As spores germinate, the cortex PG is hydrolyzed by germination-specific lytic enzymes (GSLEs). GSLEs can differentiate between cortex and germ cell wall PG by identifying the unique muramic-δ-lactam residues of the cortex (3, 11, 53, 79). These types of enzymes have been subclassified as either spore cortex lytic enzymes (SCLE) or cortical fragment lytic enzymes (CFLE) which recognize intact or partially hydrolyzed fragments of cortex PG, respectively (53). Studies have identified SCLEs and CFLEs in several *Bacillus* species. SleB is an SCLE with orthologs in *B. thuringiensis* (36), *B. cereus* (52, 66), *B. subtilis* (65), and *B. anthracis* (51). The *sleB* gene is expressed in the forespore under the control of σ^G (7, 64), and the protein is then translocated to the cortex or the inner membrane in its mature form (13, 56, 64). After recognizing PG containing muramic-δ-lactam, SleB is thought to act as either a lytic transglycosylase or an amidase (7, 64, 66). CwlJ has sequence similarity to one domain of SleB, but this enzyme is apparently associated with the proteinaceous coats of the spore (5, 38). The enzymatic activity of CwlJ has yet to be conclusively determined, but it has been speculated to be an amidase (38). In *B. subtilis*, SleB and CwlJ are redundant in an essential role during germination. In the absence of both enzymes, spores cannot hydrolyze the cortex PG and complete germination (13).

The product of the *B. cereus sleL* gene functions as a CFLE (11). Like *cwlJ*, *sleL* is expressed in the mother cell under the regulation of σ^E (42). SleL localizes to the periphery of the dormant spore where its LysM domains likely anchor it to its probable substrate; cortex peptidoglycan (8, 11, 43). The enzymatic function of SleL has been suggested to be either an N-

acetylglucosaminidase (11) or an epimerase (4, 13). We have characterized the role of *sleL* in *B. anthracis* spore germination. Analyses of spore viability, germination, and cortex PG hydrolysis indicate that SleL functions as a CFLE and is an N-acetylglucosaminidase.

MATERIALS AND METHODS

Strains and spore preparation. *B. anthracis* strains and plasmids used in this study are described in Table 2.1. *Escherichia coli* strains used to propagate plasmids were grown in LB broth or agar media with appropriate antibiotics at 37ºC. *B. anthracis* Sterne strain 34F2 ($pXO1^+$, $pXO2^-$) was grown on brain heart infusion (BHI, Difco) with antibiotics when indicated. The following concentrations of antibiotics were added to either LB or BHI to select resistance markers when necessary: tetracycline 10 μg ml^{-1} (Jersey Lab Supply), kanamycin sulfate 50 μg ml^{-1} (Jersey Lab Supply), erythromycin 500 μg ml^{-1} (*E. coli*) or 5 μg ml^{-1} (*B. anthracis*) (Fisher). *B. anthracis* endospores were prepared by growth in Modified G broth (41) at the appropriate temperatures for 3-4 days. The spores were harvested by centrifugation and washed repeatedly in deionized water. Any remaining vegetative cells were heat killed at 65ºC for 20 minutes. The spores were further purified with a 50% sodium diatrizoate (Sigma-Aldrich) gradient as previously described (69). Spores were stored in deionized water at 4ºC until analysis.

Table 2.1: Bacterial strains and plasmids.

Strain/Plasmid	Relevant Genotype[a]	Source or Reference
B. anthracis		
Sterne 34F2	$pX01^+$ $pX02^-$	P. Hanna
DPBa27	*sleL-lacZ*::pDPV350 Kan^R	This study
DPBa35	*ΔsleL*	This study
DPBa36	*ΔsleL*, pBKJ236 Er^R	This study
DPBa37	*ΔsleL*, pDPV352 Er^R	This study
Plasmids		
pDONRtet	Tet^R	(22)
pNFd13	Kan^R, Ts pE194 ori, P_{spac}	(22)
pBKJ236	Er^R, Ts ori,	(39)
pBKJ223	Tet^R, P_{amy}	(39)
pDPV350	pNFd13::*sleL'*	This study
pDPV351	pBKJ236::*ΔsleL*	This study
pDPV352	pBKJ236::*sleL*	This study

[a] Abbreviations for antibiotic resistance: Kan^R, kanamycin; Er^R, erythromycin; Tet^R, tetracycline.

Mutant construction. An *sleL-lacZ* transcriptional fusion was created as described previously (22) with slight modifications. Briefly, primers SleLgatFor and SleLgatRev (sequences available upon request) were used to PCR amplify a 442 base pair region of the *B. anthracis* Sterne genome including the Shine-Dalgarno sequence and first 142 codons of *sleL*. The *sleL* truncation was introduced into pDONRtet and then pNFd13 using the Invitrogen Gateway Cloning system (22), and the resulting plasmid was named pDPV350. Plasmid constructs were verified after each step by restriction enzyme digestion and sequencing. pDPV350 was prepared from *E. coli* INV110 (*dam*$^-$, *dcm*$^-$) (Invitrogen) and was electroporated into *B. anthracis* Sterne as described previously (82). To select for integration of the plasmid into the *B. anthracis* chromosome, a broth culture of the resulting strain was shifted to 39ºC. The correct chromosomal construct of the strain denoted as DPBa27 was verified by Southern blotting and by PCR using primers homologous to regions up and downstream of *sleL* (SleL1 and SleL4 respectively), within *lacZ*, and upstream of the P_{spac} promoter. Southern blots were performed using the Gene Images Random Prime Labeling Kit and Gene Images ECL Detection Kit (GE Healthcare) following the manufacturer's protocols.

The *sleL* gene was deleted by markerless gene replacement as described previously (39) with the following modifications. PCR was used to amplify a 2,377 base pair fragment including *sleL* and its flanking regions. Primers SleL1 and SleL4 were designed to add a *Not*1 restriction site upstream and a *BamH*I site downstream of *sleL*, respectively. The PCR product and pBKJ236 (39) were digested with *Not*I and *BamH*I, and the products were ligated resulting in pDPV352. Inverse PCR of the plasmid using primers SleL2 and SleL3 resulted in a linear 7,373 base pair product with *Bgl*II sites at both ends. The linear product was digested with *Bgl*II and ligated to produce pDPV351. The plasmid was transformed into INV110 and then introduced

into *B. anthracis* via conjugation. Selection at 37°C resulted in insertion of the plasmid via a single crossover at the *sleL* locus. pBKJ223 (39) isolated from *E. coli* INV110 was electroporated into the resulting strain to allow expression of I-SceI, and strains in which pDPV351 was deleted from the chromosome by a second recombination event were identified by screening for antibiotic sensitivity. A strain carrying the in-frame *sleL* deletion mutation, denoted DPBa35, was verified by PCR using primers SleL1 and SleL4 and sequencing of the resulting PCR product. Complementation studies involved the introduction of pBKJ236 (empty vector) or pDPV352 (complementing plasmid) into DPBa35 to produce DPBa36 and DPBa37, respectively. Plasmids were maintained in the replicative form by conducting complementation studies at 27ºC.

Phenotypic analyses. Spore viability was analyzed by conducting colony formation assays. Equivalent amounts of spores of each strain were heat activated at 70ºC for 30 minutes to synchronize germination. After activation, the initial optical density (OD_{600}) of the spore solutions was measured. The suspensions were then serially diluted, and spores were plated on BHI without antibiotics. Plates were incubated overnight at 39ºC, and were used to calculate colony-forming units per OD_{600} (cfu/OD_{600}).

Starter cultures for growth and sporulation assays were cultivated on BHI plates with the appropriate antibiotics. Isolated colonies were inoculated into Modified G broth without antibiotics and incubated at 39ºC, unless otherwise noted, until the OD_{600} was ~0.5. The cultures were back-diluted 1:25, and optical density readings were recorded over 8 hours. To determine the activity of the wild-type *sleL* promoter, 1 ml culture samples were collected, and cells were pelleted and stored at -80ºC. β-galactosidase activity was assayed as previously described (69).

For germination and outgrowth studies, spores were heat activated in water at 70ºC for 30 minutes and then briefly cooled on ice. Germination was initiated by diluting spores to an OD_{600} of 0.2 in BHI broth and shaking at 39ºC. Germination and outgrowth were monitored as changes in OD_{600} over time and are graphically depicted as the percent initial OD relative to incubation time.

Assays of biochemical changes during germination. 1 ml of purified spores at an OD_{600} of 50 were heat activated at 70ºC for 30 minutes. $NaPO_4$ pH = 7.0 was added to a final concentration of 40 mM and the mixture was incubated at 37ºC for 5 minutes. Germination was initiated by the addition of L-alanine (Fisher) to 10 mM and inosine (Sigma) to 1 mM. Spores were incubated at 37ºC until the OD_{600} decreased at least 40% (approximately 4 minutes) at which time samples were collected for muropeptide, NAM, diaminopimelic acid (Dpm), and DPA analyses. The germinating spore pellets and exudates were separated by centrifugation and PG was prepared and analyzed as previously described (18). Both spore exudates and pellet samples were assayed for DPA content as described previously (69). Briefly, spore pellets were disrupted by boiling the samples resuspended in 10 mM Tris HCl, pH=8.0 for 20 minutes. After cooling on ice, both the pellet and exudate samples were centrifuged to remove any insoluble material. The supernatants were combined with DPA assay reagent, and the absorbance at 440 nm was measured. The amount of DPA in the pellets or exudates was compared to a standard curve produced using purified DPA (Sigma). Muramic acid and amino acid analysis was done as previously described (59) on pellet and exudate samples collected during germination.

RESULTS

Identification and expression of *sleL*. Database searches revealed only a single strong homolog of the *B. subtilis* (49% amino acid identity) and *B. cereus* SleL (YaaH) (98% amino acid identity) proteins encoded in each of the available *B. anthracis* genome sequences. Locus BAS3402 was identified as the *B. anthracis* Sterne ortholog of *sleL*. The genes flanking BAS3402 are oriented in the opposite direction, indicating that, as in *B. subtilis* (42) and *B. cereus* (11), *B. anthracis sleL* is a monocistronic locus. By creating a transcriptional fusion between the native *sleL* promoter and *lacZ*, we determined the activity of the promoter. While wild-type *B. anthracis* had only background levels of β-galactosidase activity, the native *sleL* promoter in DPBa27 was inactive during growth, became active at t_1 during sporulation, was most active at $t_{2.5,}$ and became inactive by t_5 (Figure 2.1).

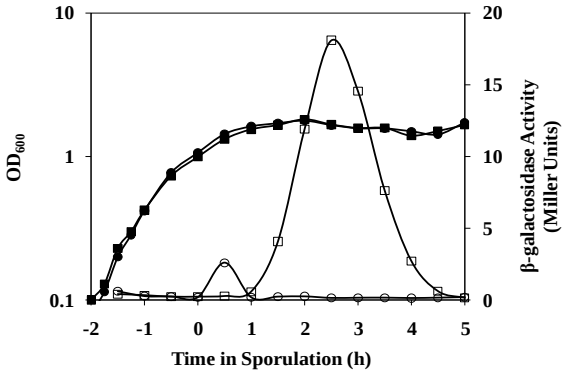

Figure 2.1. Sporulation-specific expression of *sleL*. Strains were incubated in Modified G medium at 39°C for 7 hours during which samples were collected for β-galactosidase assays. Growth (filled symbols) and β-galactosidase activity (open symbols) were assayed for wild-type (circles) and DPBa27 (*sleL-lacZ*::pDPV350) (squares) *B. anthracis*. The graph is representative of three independent experiments.

Construction and characterization of *sleL* mutant strains. Vegetative growth assays (Figure 2.1) indicated that the wild-type and both the *sleL* plasmid-insertion (DPBa27) and in-frame *ΔsleL* (DPBa35) mutant strains had similar doubling rates in Modified G broth. Both

mutants also produced equivalent amounts of viable, heat resistant spores as compared to the wild-type (data not shown). Purified spores were analyzed to determine the role of *sleL* in germination and outgrowth. When spores were germinated in BHI, both wild-type and mutant strains reached 70% of their maximum drop in OD_{600} within 4 minutes after the addition of germinants (Figure 2.2). However, wild-type spores reached their maximum drop in OD_{600} 20 minutes after germination initiation at which point they lost an average of 65±1% of their initial optical density. Plasmid-insertion *sleL* mutant spores, however, reached their maximum OD decrease slightly later, 30 minutes after induction, and lost only 60±3% of their initial OD_{600}. Repeated measures two-way ANOVA indicates that there is a significant difference ($p \leq 0.0098$) between the two strains from 10-35 minutes after initiation of germination. Each strain then progressed through outgrowth at approximately the same rate (Figure 2.2), reaching the point of the first cell division ~110 minutes after germination initiation (data not shown). Similar results were obtained with the in-frame *ΔsleL* mutant strain (data not shown).

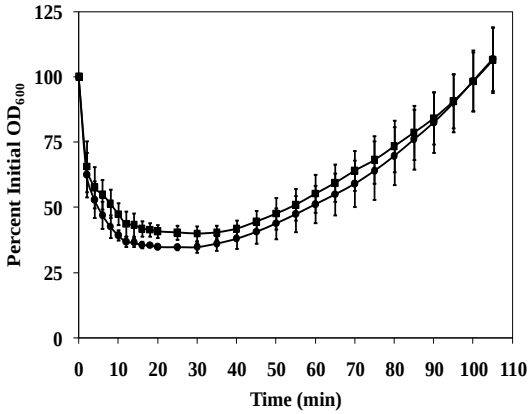

Figure 2.2. Germination of *sleL* spores is slightly delayed. Heat-activated spores were germinated in BHI medium at 39°C. Germination and outgrowth of wild-type (●) and DPBa27 (*sleL-lacZ*::pDPV350) (■) *B. anthracis* spores were followed as changes in OD_{600} value over time. Error bars represent 1 S.D. of the mean of three independent assays. The difference in loss of OD_{600} between the two strains is significant ($p <= 0.0098$) from 10-35 minutes after germination initiation.

DPA, NAM, and Dpm release during germination. Heat activated *B. anthracis* spores were induced to germinate with L-alanine and inosine in buffered solution as described in materials and methods. Germination was allowed to proceed until the spores lost 40% of their initial OD_{600} value, which occurred within 5 minutes. Samples were collected and analyzed for release of DPA, NAM, and Dpm from the spores. The amount of DPA released from all strains studied was similar (Figure 2.3). Nearly all of the DPA contained within the spore core was released within ten minutes of germination initiation.

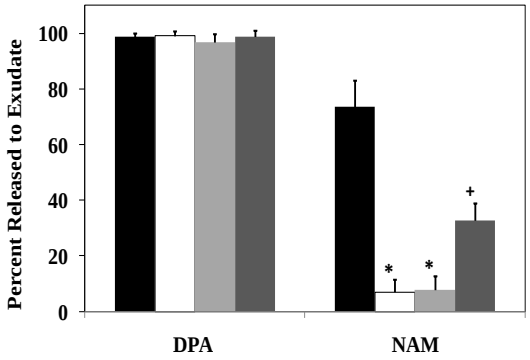

Figure 2.3. Release of DPA and NAM during germination. Heat-activated spores were germinated with L-ala and inosine in buffer at 37°C for 10 minutes, and samples were removed and centrifuged for assay of exudate and spore pellet contents. The percents of DPA and NAM released from wild-type (black), *ΔsleL* (DPBa35, white), *ΔsleL* with pBKJ236 vector control (DPBa36, light gray), and *ΔsleL* with pDPV352 complementing vector (DPBa37, dark gray) *B. anthracis* spores are shown. Error bars represent 1 S.D. of the mean of three independent experiments. Asteriks indicate a statistically significant difference ($p \leq 0.0001$) as compared to wild-type *B. anthracis*. A cross indicates a statistically significant difference ($p \leq 0.007$) as compared to either the wild-type or DPBa35.

However, the amount of NAM released differed significantly (Tukey adjusted $p \leq 0.0001$ as determined using ANOVA with transformed data) between wild-type and *ΔsleL* mutant spores (Figure 2.3). Wild-type spores that had germinated for approximately 10 minutes released 73±10% of their NAM, whereas *ΔsleL* spores released 7±5% which is likely the result of SleB and CwlJ lytic activity. When the *ΔsleL* was complemented by the addition of a vector

containing the full-length gene under the control of the native promoter (DPBa37) the amount of hexosamine released from the spore increased significantly (Tukey adjusted $p \leq 0.007$) to 33±6%. Partial complementation was likely the result of sporulating DPBa37 in Modified G broth without antibiotics, so there was no selective pressure for maintaining the complementation vector. Screening of cells for the plasmid antibiotic resistance revealed that only ~20% of cells entering sporulation (at t_0) still carried pDPV352. Only the cells carry the complementation vector express SleL in the mother cell which then localizes to the spore. The combine lytic activity of reduced levels of SleL plus baseline levels of SleB and CwlJ leads to the partial complementation seen for DPBa37. Sporulation in the absence of antibiotics was done due to concerns about changes in sporulation efficiency and spore physiology in the presence of varied media components for different strains. Subsequent sporulation in the presence of erythromycin to select for the complementing plasmid resulted in no significant change in the number of heat resistant spores produced per unit of culture. Germination studies of these spores revealed stronger complementation, leading to release of 90% of the NAM observed for the wild type during early germination (data not shown). The same pattern of diminished release in the *ΔsleL* mutant and partial complementation was seen for Dpm, another component of the cortex PG (data not shown).

Muropeptide structural dynamics. In order to analyze the muropeptide composition of wild-type, *ΔsleL*, and complemented spores, heat activated spores were germinated in buffer with L-alanine and inosine. Germination proceeded for five minutes before spore pellets and exudate samples were separated by centrifugation. To inactivate lytic enzymes, the spore pellets were then disrupted by boiling in a solution containing detergent and dithiothreitol, while exudate samples were simply boiled. The pellet samples and half of the exudate sample volumes

were digested with Mutanolysin, which cleaves the PG between NAM and NAG leaving NAM as the terminal reducing sugar. Finally, all samples were reduced and muropeptides were separated using HPLC (Figure 2.4). The identities of the muropeptides represented by labeled peaks were previously determined by amino acid analysis and mass spectrometry (18) and are listed in Table 2.2.

Wild-type exudate-associated muropeptide profiles differed from *ΔsleL* profiles in that the former contained peaks G2, G3, G6, G7, G10u and G12 representing tetrasaccharide (TS) or hexasaccharide (HS) muropeptides with NAG as the terminal reducing sugar (N-acetylglucosaminitol when reduced) (Fig. 2.4B). The loss of *sleL* resulted in the disappearance of these products (Fig. 2.4C). Therefore, the majority of germination-associated muropeptides released from the *ΔsleL* spores were aG7a and aG7b, which appear to be the result of SleB lytic transglycosylase activity (unpublished data). When *ΔsleL* was complemented with pDPV352 the exudate muropeptide profile was more similar to that of the wild-type (Fig. 2.4E). aG7a and aG7b were still released, but like wild-type, considerable amounts of G2, G6 and G7 muropeptides were released from the spore, as well as small amounts of G3, G10u, and G12. This muropeptide profile is consistent with the enzymatic digestion pattern of a glucosaminidase (18). The profile changes were not the result of the pBKJ236 vector because only aG7a and aG7b germination-associated muropeptides were present in the chromatogram of DPBa36 containing the empty vector (Fig. 2.4D).

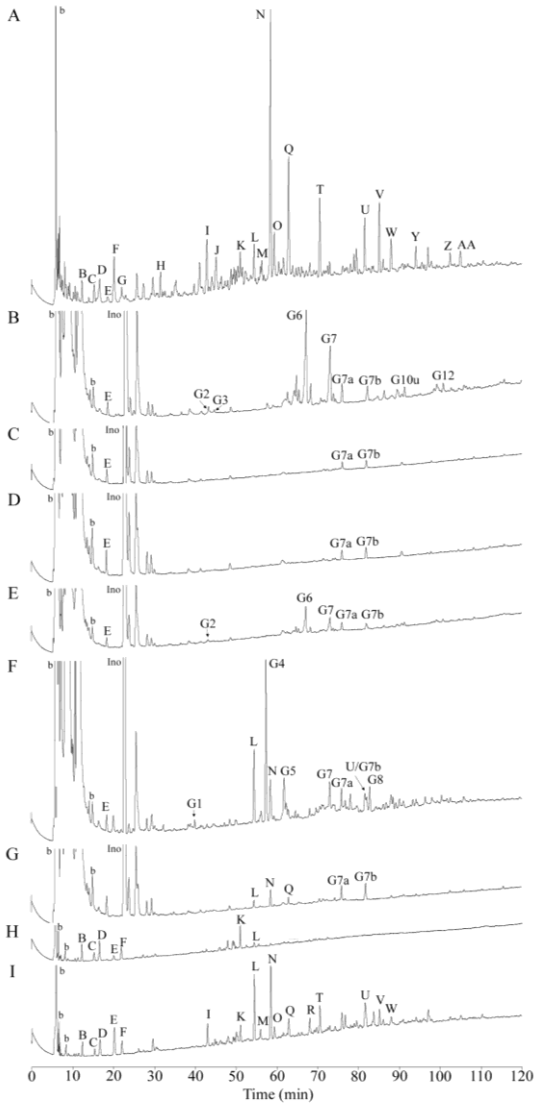

Figure 2.4. HPLC analysis of wild-type and Δ*sleL B. anthracis* muropeptides. Heat-activated spores were germinated for 5 minutes with L-ala and inosine in buffer at 37°C. Samples were removed and centrifuged for analysis of peptidoglycan in the exudate and spore pellet. Peptidoglycan was collected, digested, and reduced, and muropeptides were separated using a methanol gradient as previously described (18). Muropeptides were derived from dormant spores (A), from the exudates of germinating spores (B-E), from the exudates of germinating spores digested with Mutanolysin (F, G), and from germinating spore pellets (H, I). Samples were from wild-type (A, B, F, H), from Δ*sleL* (DPBa35) (C, G, I), from *ΔsleL* with pBKJ236 vector control (DPBa36) (D), and from *ΔsleL* with pDPV352 complementing vector (DPBa37) (E) spores. Peaks G3, G10u, and G12 are clearly visible upon magnification of panel E, but are not marked for clarity. Peaks are labeled as in (18) and Table 2, except the first "a" in the germination specific peak names are omitted for space considerations. Identities of peaks aG7a and aG7b are unpublished data. Ino indicates the added germinant inosine. Large unlabeled peaks eluting between 10 and 30 minutes for exudate samples are other small molecules released from germinating spores that were analyzed and found not to contain peptidoglycan components. Peaks labeled b are buffer components.

Table 2.2. Muropeptide peak identification.

Name	Structure
Muropeptides produced from dormant spore peptidoglycan	
A	DS-TriP
B	DS-Ac-TriP
C	DS-TriP+Am
D	DS-Ac-TriP+Am
E	DS-Ala
F	DS-TP
G	DS-Ac-TP
H	TS-TP open lactam
I	TSred-TP
J	TSred-Ala
K	DS-Ac-TP x TriP+Am-DS-Ac
L	TS-TP-Ac
M	DS-Ac-TP+Am x TriP+Am-DS-Ac
N	TS-TP
O	TS-TP x TP
P	DS-TP x TP-TSred
Q	TS-Ala
R	HSred-Ac-TP (right lactam reduced)
S	HSred-Ac-TP (left lactam reduced)
T	DS-TP x TP-TS
U	HS-TP-Ac
V	TS-TP x TP-TS
W	HS-Ala-Ac
X	HS-Ala-Ac
Y	HS-TP
Z	HS-Ala
AA	TS-TP x TP-HS
Muropeptides produced from germinated spore exudate following Mutanolysin digestion	
aG1	TriS-TP Red
aG4	TriS-TP
aG5	TriS-Ala
aG8	PS-TP-Ac
Muropeptides produced from germinated spore exudate with no Mutanolysin digestion	
aG2	TS-TP NAGr Red
aG3	TS-Ala NAGr Red
aG6	TS-TP NAGr
aG7	TS-Ala NAGr
aG7a	TS-TP anhydro
aG7b	TS-Ala anhydro
aG10u	HS-TP-Ac NAGr
aG12	HS-Ala-Ac NAGr

Abbreviations: DS, disaccharide (NAG-NAM); TS, tetrasaccharide (NAG-lactam-NAG-NAM); HS, hexasaccharide (NAG-lactam-NAG-lactam-NAG-NAM); TriS, trisaccharide (lactam-NAG-NAM); PS, pentasaccharide (lactam-NAG-lactam-NAG-NAM); TriP, tripeptide (Ala-Glu-Dpm); TP, tetrapeptide (Ala-Glu-Dpm-Ala); -Ac, deacetylated glucosamine; +Am, amidated Dpm; Red, reduced lactam (an artifact of sample preparation (101); NAGr, NAG at the reducing end; x, crosslink between two peptides; anhydro, the NAM at the reducing end is in the anhydro form.

The muropeptide profile of wild-type spore exudate digested with Mutanolysin reveals the presence of high levels of G1, G4, G5 and G8 (Fig. 2.4F). These trisaccharides (TriS) and pentasaccharides (PS) result when NAG is cleaved from the reducing ends of G2, G6, G7 and G10u, respectively. When *ΔsleL* exudate samples were digested and analyzed there were no peaks at the positions of G1, G4, G5 or G8 (Fig. 2.4G). The muropeptides that were released from the mutant spores: L, N, and Q; must be produced by Mutanolysin digestion of larger PG fragments that have been released from the spores, but are present in lower amounts than for the wild-type, because most of the cortex PG is retained within the mutant spores (Fig 2.4I). There was no difference in the release of aG7a or aG7b between wild-type and *ΔsleL* spores, however, suggesting that these products of cortex digestion are small enough to be released to the exudate and are unaffected by Mutanolysin digestion.

The major differences between the muropeptide profiles of wild-type and *ΔsleL* germinated spore-associated material is that wild-type spores retain very few muropeptide constituents five minutes after germination initiation (Fig. 2.4H). Notably, essentially undetectable amounts of the predominant cortex-associated muropeptides N and Q were identified in wild-type profiles. *ΔsleL* spores, however, had much higher amounts of not only N and Q but also other dormant spore-associated muropeptides including other TS, HS and cross-linked muropeptides (Fig. 2.4I). Therefore, more cortex PG was retained in *ΔsleL* mutant spores as a result of the loss of SleL enzymatic activity.

DISCUSSION

The *B. anthracis sleL* gene encoding a GSLE was mutated and evaluated for its role in germination. Loss of *sleL* slightly delays germination because mutant spores take ten minutes longer to reach their maximum drop in OD_{600} value. In refined studies of the progression of germination, our findings indicate that SleL is involved in the depolymerization of cortex PG. Stage I of germination appears to proceed normally in the *sleL* mutant. Both wild-type and mutant spores release equivalent amounts of DPA, and their drop in optical density is equivalent for the first five minutes after germination initiation. However, mutants progress more slowly within stage II of germination, which includes cortex hydrolysis (91). At this point mutant spores are not hydrolyzing their cortex PG as efficiently. Because *sleL* mutants enter and complete outgrowth like wild-type *B. anthracis* suggests that they are not impaired in rehydration and outgrowth.

It was previously proposed that SleL may degrade partially digested cortex PG (11). In the absence of SleL the cortex continues to be depolymerized by SCLE, presumably SleB and CwlJ, but the spore retains these large fragments. This would explain why we see ~65% more NAM and ~50% more Dpm being retained in germinating *sleL* mutant spores. The retention of these cortex-associated products indicates that SleL is involved in their release and may also explain why *sleL* spores continue to be more optically dense than wild-type *B. anthracis*. Despite these alterations, *sleL* mutants depolymerize the cortex sufficiently using the essential lytic enzymes SleB and CwlJ so that germination is completed and outgrowth begins.

Chen *et al*. (11) subjected *B. cereus* PG fragments to purified SleL and found that the cortex lytic enzyme was capable of digesting partially disrupted cortex PG and is therefore classified as a CFLE. By using RP-HPLC they also determined that the enzyme acts as an *N*-

acetylglucosaminidase involved in spore cortex depolymerization. Sequence studies reveal that the SleL protein is part of a large family of glycosyl hydrolases that includes chitinases (55), which cleave a polymer of N-acetyl-glucosamine. Despite the fact that *B. cereus* and *B. anthracis* SleL have 48% amino acid identity to *B. subtilis* SleL, reports of muropeptide structure and composition analyses in the latter species suggested that SleL is a putative muramic δ-lactam epimerase (13). Atrih *et al.* (4) first proposed the role of an epimerase after concluding that the cortex PG was modified in a way uncharacteristic of an amidase or hydrolytic enzyme. Instead the enzymatic activity occurred only at muropeptides containing muramic-δ-lactam and possibly altered the stereochemistry of muramic acid residues. Chirakkal *et al*. (13) evaluated a *B. subtilis sleL* null mutant for cortex PG muropeptide dynamics of dormant and germinated spores. They reported that the *sleL* strain did not produce epimerase products but continued to release glucosaminidase products to the exudate. We cannot explain the results of Chirakkal *et al.* (13), but our results are consistent with those of Chen *et al.* (11) in predicting that SleL is an *N*-acetylglucosaminidase.

In our analyses, peaks G2, G3, G6, G7, G10u, and G12 were absent from the *ΔsleL* mutant's germination muropeptide profile. Muropeptides with similar retention times and identical masses were previously identified as epimerase products (2, 4). However, Dowd *et al*. (18) recently reported that the most evident lytic activity detected during *B. anthracis* germination is that of a glucosaminidase. When the exudate fractions were digested with Mutanolysin and reduced, the original TS and HS were converted to tri- and penta-saccharides indicating that the native exudate muropeptides had NAG at their reducing termini rather than NAM. Dowd *et al*. (18) went on to suggest that in *B. subtilis*, the glucosaminidase products were

misidentified as epimerase products. All of our data would indicate that the enzymatic activity of SleL is not an epimerase but actually an *N*-acetylglucosaminidase.

By creating an *sleL-lacZ* transcriptional fusion we were able to show that *B. anthracis* expresses *sleL* around t_2 or mid-sporulation. This result is consistent with microarray analysis of *B. anthracis* that indicates *sleL* is transcribed during wave 4 of gene expression which includes $T_{1.5\text{-}4}$ (6, 51). Because both *B. anthracis* and *B. subtilis* express *sleL* around t_2, and given that Kodama *et al.* (42) have shown that *B. subtilis sleL* is under the control of σ^E, it is reasonable to predict that *B. anthracis sleL* is most likely expressed in the mother cell under the regulation of σ^E. The protein is then apparently localized to the developing forespore where it is incorporated into the mature spore (11, 42).

The SleL *N*-acetylglucosaminidase plays a major role in determining the structure of muropeptides released into the medium by germinating *B. anthracis*. These muropeptides may have a major role in modulating the host immune system during an anthrax infection (30). To fully understand the contributions of GSLEs during *B. anthracis* germination it will be imperative to create and analyze combinatorial double and triple mutants. This will allow us to determine if and how these enzymes cooperate to produce particular muropeptide products and to allow for such a fast, efficient morphological transition.

ACKNOWLEDGEMENTS

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CHAPTER 3:

Contributions of Four Cortex Lytic Enzymes to Germination of *Bacillus anthracis* Spores

Jared D. Heffron, Emily A. Lambert, Nora Sherry and David L. Popham. 2010.

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COAUTHOR CONTRIBUTIONS

Emily Lambert and Jared Heffron contributed equally to the research, including multi-deletion strain construction, and writing of the material presented herein. Jared also conducted spore germination assays in response to nutrient and non-nutrient stimuli, and muropeptide analyses. Emily prepared spores, conducted colony formation assays, and cortex fragment release tests. Nora Sherry assisted in the creating of the multi-deletion mutant strains used in the study, and David Popham was the principle investigator. Each coauthor has given written permission to reproduce the material in this dissertation.

ABSTRACT

Bacterial spores remain dormant and highly resistant to environmental stress until they germinate. Completion of germination requires the degradation of spore cortex peptidoglycan by germination-specific lytic enzymes (GSLEs). *Bacillus anthracis* has four GSLEs: CwlJ1, CwlJ2, SleB, and SleL. In this study the cooperative action of all four GSLEs *in vivo* was investigated by combining in-frame deletion mutations to generate all possible double, triple, and quadruple GSLE mutant strains. Analyses of mutant strains during spore germination and outgrowth combined observations of optical density loss, colony-producing ability, and quantitative identification of spore cortex fragments. The lytic transglycosylase SleB alone can facilitate enough digestion to allow full spore viability and generates a variety of small and large cortex fragments. CwlJ1 is also sufficient to allow completion of nutrient-triggered germination independently and is a major factor in Ca^{2+}-DPA-triggered germination, but its enzymatic activity remains unidentified because its products are large and not readily released from the spore's integuments. CwlJ2 contributes the least to overall cortex digestion, but plays a subsidiary role in Ca^{2+}-DPA-induced germination. SleL is an *N*-acetylglucosaminidase that plays the major role in hydrolyzing the large products of other GSLEs into small, rapidly released muropeptides. As the roles of these enzymes in cortex degradation become clearer, they will be targets for methods to stimulate premature germination of *B. anthracis* spores, greatly simplifying decontamination measures.

INTRODUCTION

The gram-positive bacterium *Bacillus anthracis* is the etiologic agent of cutaneous, gastrointestinal, and inhalational anthrax (61). An anthrax infection begins when the host is infected with highly resistant, quiescent *B. anthracis* spores (1, 61). Within the host, the spore's sensory mechanism recognizes chemical signals, known as germinants, and triggers germination which leads to the resumption of metabolism (91). Spores that have differentiated into vegetative cells produce a protective capsule and deadly toxins. These virulence factors allow the bacteria to evade the host's immune system and establish an infection resulting in septicemia, toxemia and frequently death (61). Although vegetative cells produce virulence factors that are potentially fatal, these cells cannot initiate infections and are much more susceptible to antimicrobial treatments than spores (61). Therefore, efficient triggering of spore germination may enhance current decontamination methods.

Spores are highly resistant to many environmental insults because the spore core (cytoplasm) is dehydrated, dormant, and surrounded by multiple protective layers including a modified layer of peptidoglycan (PG) known as the cortex (91). The cortex functions to maintain dormancy and heat resistance by preventing core rehydration (24). It is composed of alternating N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) sugars (Figure 3.5). Peptide side chains on the NAM residues are either involved in interstrand cross-linking, cleaved to a single L-alanine side chain, or are fully removed with accompanying formation of muramic-δ-lactam (3, 78, 100). After germination is initiated by either nutrient or non-nutrient germinants, the cortex is depolymerized resulting in complete core rehydration, resumption of metabolic activity, and outgrowth (87, 91).

Cortex hydrolysis is driven by autolysins called germination-specific lytic enzymes (GSLEs) that recognize the cortex-specific muramic-δ-lactam residues (3, 11, 53, 79). GSLEs fall into two classes; spore cortex lytic enzymes (SCLEs) are thought to depolymerize intact cortical PG, and cortical fragment lytic enzymes (CFLEs) which further degrade partially hydrolyzed cortex (53). Both SCLEs and CFLEs have been identified in a variety of spore forming species including *B. anthracis* (35, 47, 51), *Bacillus cereus* (11, 52, 65), *Bacillus megaterium* (23, 88), *Bacillus subtilis* (38, 42, 65), *Bacillus thuringiensis* (36), and *Clostridium perfringens* (12, 60). Of the four GSLEs identified in *B. anthracis* CwlJ1, CwlJ2 and SleB are predicted to be SCLEs (35) whereas SleL is thought to be a CFLE (47).

Recently, independent studies showed that CwlJ1 and the lytic transglycosylase SleB play partially redundant roles and that either is sufficient for spore germination and outgrowth (25, 35). However, these same studies report conflicting results concerning the role of CwlJ2 during germination. Heffron *et al.* found no effect of CwlJ2 on the biochemistry of cortex hydrolysis or on colony forming efficiency of spores (35). Giebel *et al.* reported that loss of CwlJ2 caused a minor defect in germination kinetics and that in the absence of SleB and CwlJ1, further loss of CwlJ2 had a major effect on colony forming efficiency (25). SleL in *Bacillus anthracis* is proposed to be an *N*-acetylglucosaminidase whose role is to further degrade cortex fragments resulting from SCLE hydrolysis (47). SleL is not essential for the completion of germination but does promote the release of small muropeptides to the spore's surrounding environment (47).

This study reports the effects of multiple deletion mutations affecting GSLEs on spore germination efficiency and kinetics of cortex hydrolysis. The data confirm the dominant roles played by CwlJ1 and SleB in initiation of cortex hydrolysis and the major role of SleL in release

of small cortex fragments. A minor role of CwlJ2 in nutrient-triggered germination and the contributions of CwlJ1 and CwlJ2 to Ca^{2+}-dipicolinic acid (DPA) triggered germination were revealed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. anthracis* strains and plasmids used are listed in Table 3.1. *Escherichia coli* strains used to propagate plasmids were grown in LB with 500 μg/ml erythromycin (Fisher) or 100 μg/ml ampicillin (Jersey Lab Supply) and incubated at 37°C. *B. anthracis* strains were grown in brain heart infusion (BHI, Difco) containing 5 μg/ml erythromycin or 10 μg/ml tetracycline (Jersey Lab Supply) and incubated at 22°C prior to allelic exchange or 37°C afterwards.

Table 3.1. *B. anthracis* strains and plasmids

Strain or plasmid	Relevant genotype[a]	Construction[b]	Source or reference
Strains			
Sterne 34F2	pXO1$^+$, pXO2$^-$		P. Hanna
DPBa35	*ΔsleL*	pDPV351→34F2	(47)
DPBa38	*ΔsleB*	pDPV383→34F2	(35)
DPBa55	*ΔsleB ΔsleL*	pDPV383→DPBa35	This study
DPBa60	*ΔcwlJ2*	pDPV344→34F2	This study
DPBa61	*ΔcwlJ1*	pDPV347→34F2	(35)
DPBa66	*ΔcwlJ2 ΔsleL*	pDPV344→DPBa35	This study
DPBa69	*ΔcwlJ1 ΔsleL*	pDPV347→DPBa35	This study
DPBa72	*ΔcwlJ1 ΔsleB ΔsleL*	pDPV347→DPBa55	This study
DPBa73	*ΔcwlJ2 ΔsleB*	pDPV383→DPBa60	This study
DPBa74	*ΔcwlJ1 ΔsleB*	pDPV383→DPBa61	This study
DPBa78	*ΔcwlJ1 ΔcwlJ2*	pDPV347→DPBa60	This study
DPBa82	*ΔcwlJ2 ΔsleB ΔsleL*	pDPV344→DPBa55	This study
DPBa83	*ΔcwlJ1 ΔcwlJ2 ΔsleL*	pDPV344→DPBa69	This study
DPBa84	*ΔcwlJ1 ΔcwlJ2 ΔsleB ΔsleL*	pDPV344→DPBa72	This study
DPBa85	*ΔcwlJ1 ΔcwlJ2 ΔsleB*	pDPV347→DPBa73	This study
Plasmids			
pBKJ223	Tet^R, P_{amy}-*I-SceI*		(39)
pBKJ236	Er^R, ori^{ts}		(39)
pDPV344	*ΔcwlJ2*	pBKJ236	This study
pDPV347	*ΔcwlJ1*	pBKJ236	(35)
pDPV351	*ΔsleL*	pBKJ236	(47)
pDPV383	*ΔsleB*	pBKJ236	(35)

[a] Abbreviations: Tet^R, tetracycline resistance; Er^R, erythromycin resistance; ori^{ts}, temperature-sensitive origin of replication.

[b] Strains were constructed by conjugation followed by the published series of steps required for recombination of the deletion mutation into the chromosome. The designation preceding the arrow is the plasmid and the designation following the arrow is the recipient strain. Single plasmid designations indicate the vector in which the deletion construct was created.

In-frame deletion mutagenesis of *cwlJ1*, *cwlJ2*, *sleB*, and *sleL* has previously been published (35, 47). Each deletion encoded only 6-9 codons of the original gene. In-frame deletions were integrated into the *B. anthracis* chromosome using markerless gene replacement as previously described (39). The deletion mutations were verified in each new strain by PCR amplification of each locus and sequencing the regions including >250 base pairs both up and downstream.

Spore preparation. *B. anthracis* strains were incubated at 37°C with shaking in Modified G broth (41) for 3 days. Spores were harvested by repeated centrifugation and water washing. Vegetative cells were heat killed at 65°C for 30 minutes. Spores were purified by centrifugation through 50% sodium diatrizoate (Sigma) (69). Purified spores were ~99% free of vegetative cells and were stored in deionized water at 4°C until analysis.

Spores at an optical density at 600nm (OD) = 2 were decoated at 70°C for 30 minutes in 0.1 N NaOH, 0.1 M NaCl, 1% (wt/vol) sodium dodecylsulfate, and 0.1 M dithiothreitol. After decoating, spores were washed 5 times in sterile deionized water. To evaluate the effects of decoating on viability, decoated and intact spores were serially diluted and plated on BHI.

Spore germination assays. To evaluate colony formation efficiency, intact or decoated spores were resuspended at an OD = 0.2. After heat activation at 70°C for 30 minutes, the suspensions were serially diluted, spotted on BHI with or without 1 μg/ml lysozyme (Sigma), and incubated at 30°C overnight.

Spore germination and outgrowth in liquid BHI was assayed by monitoring OD as previously described except incubation was done at 37°C (35, 47). Ca^{2+}-DPA treatment to trigger germination was carried out essentially as previously described (72). Spores were suspended at an OD = 1 in water or 50 mM Ca^{2+}-DPA solution and incubated at 25°C for 60

minutes. Spore suspensions were then heated at 70°C for 20 minutes, serially diluted in water, spotted on BHI plates, and incubated at 37°C overnight to determine heat-resistant titers.

Biochemical analyses of PG hydrolysis and release. After heat activation, spores were induced to germinate in a buffered solution containing 10 mM L-alanine and 1 mM inosine as previously described (47). Spore-associated (pellet) and exudate (supernatant) fractions collected throughout germination were assayed for muramic acid and diaminopimelic acid (Dpm) content as described previously (59). PG was purified and prepared for reverse-phase high-pressure liquid chromatography (RP-HPLC) analysis as previously described (18). Briefly, the germinated spore suspension was separated into pellet and supernatant fractions. Lytic enzymes were inactivated with heat (supernatant) or with heat and detergent (pellet), and PG was purified. The PG material from the pellets and half of each exudate fraction was then digested with the muramidase Mutanolysin (Sigma). All fractions were reduced with $NaBH_4$ prior to HPLC separation.

RESULTS

Effects of GSLEs on nutrient-triggered germination. Wild-type and mutant spores were allowed to germinate and produce colonies on BHI plates overnight (Table 3.2). All strains containing either CwlJ1 or SleB produced an equivalent number of colonies per OD unit as the wild-type strain. However, all strains lacking both CwlJ1 and SleB exhibited a $>10^3$ fold decrease in colony formation. There was no further decrease in colony formation when other GSLEs, CwlJ2 and/or SleL, were also absent. Colony formation efficiency was restored to near wild-type levels in strains lacking both CwlJ1 and SleB when decoated spores were plated on BHI supplemented with 1 μg/ml of lysozyme (Table 3.2). Spores were decoated to facilitate the penetration of lysozyme to the cortex, but this treatment did not affect colony formation (data not shown).

Table 3.2. Spore plating efficiency and lysozyme recovery

		CFU/ml/OD on[a]	
Strain	Genotype	BHI	BHI+Lyso[b]
34F2	Wild-type	$1.5x10^8$	$1.1x10^8$
DPBa35	*ΔsleL*	$1.3x10^8$	ND
DPBa38	*ΔsleB*	$9.2x10^7$	ND
DPBa55	*ΔsleB, ΔsleL*	$1.2x10^8$	ND
DPBa60	*ΔcwlJ2*	$1.2x10^8$	ND
DPBa61	*ΔcwlJ1*	$1.4x10^8$	ND
DPBa66	*ΔcwlJ2, ΔsleL*	$1.0x10^8$	ND
DPBa69	*ΔcwlJ1, ΔsleL*	$1.1X10^8$	ND
DPBa72	*ΔcwlJ1, ΔsleB, ΔsleL*	$3.7x10^4$	$1.2x10^8$
DPBa73	*ΔcwlJ2, ΔsleB*	$1.3x10^8$	ND
DPBa74	*ΔcwlJ1, ΔsleB*	$4.3x10^4$	$1.5x10^8$
DPBa78	*ΔcwlJ1, ΔcwlJ2*	$1.0x10^8$	ND
DPBa82	*ΔcwlJ2, ΔsleB, ΔsleL*	$9.9x10^7$	ND
DPBa83	*ΔcwlJ1, ΔcwlJ2, Δ sleL*	$9.3x10^7$	ND
DPBa84	*ΔcwlJ1, ΔcwlJ2, ΔsleB, ΔsleL*	$2.9x10^4$	$7.9X10^7$
DPBa85	*ΔcwlJ1, ΔcwlJ2, ΔsleB*	$1.3x10^5$	$6.1x10^7$

[a] Values are averages for three independent spore preparations.
[b] Spores were decoated and plated on BHI containing 1 μg/ml of lysozyme. ND, not determined.

Spores lacking GSLEs in various combinations were germinated in liquid BHI and monitored by measuring the change in OD. Germinating spores rapidly lose approximately 50% of their OD in the first few minutes, and the OD then increases as the population continues into vegetative growth. As previously demonstrated (35, 47), spores of the wild-type strain and strains lacking any single GSLE were successful at synchronous germination, and those strains without functional CwlJ1, SleB, or SleL had germination delays (Fig. 3.1A and 3.1B). When both *cwlJ1* and *sleB* were deleted, spores were capable of initiating germination and lost OD for only ten minutes, but at that point germination was arrested and no further changes were observed (Fig. 3.1A and (35)). This was the most dramatic phenotype, and adding any number of additional GSLE-eliminating mutations to the Δ*cwlJ1 ΔsleB* background did not produce further significant changes ($P > 0.05$, as determined by a Tukey-Kramer HSD analysis) (Fig. 3.1A and data not shown).

Adding a *ΔsleL* mutation to Δ*cwlJ1*, Δ*sleB*, Δ*cwlJ1* Δ*cwlJ2*, and Δ*sleB* Δ*cwlJ2* strains generated spores with increased delays during germination that were evident by shallower curves (Fig. 3.1A and 3.1B and data not shown). Despite this consistent trend, these differences were not statistically significant ($P > 0.05$, as determined by a Tukey-Kramer HSD analysis) which is likely the result of variability inherent in assay of multiple independent spore preparations. Placing a *ΔcwlJ2* deletion into any other GSLE mutant strains, whether single, double, or triple, did not impact the preexisting germination phenotypes except in the case of Δ*cwlJ1* Δ*cwlJ2* spores. This strain exhibited a slowed OD increase compared to Δ*cwlJ1* spores with statistically significant delays from 60 minutes onward ($P \leq 0.01$, as determined by a Tukey-Kramer HSD analysis) (Fig. 3.1B).

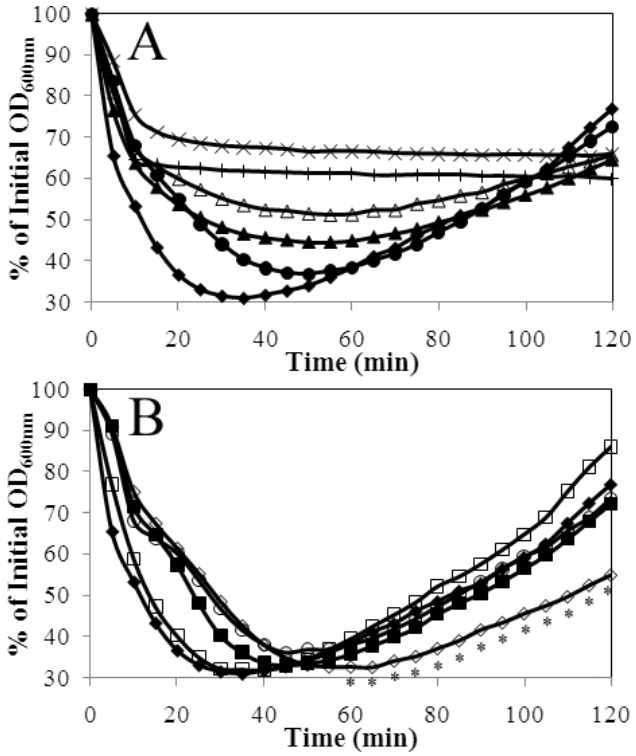

Figure 3.1. Germination and outgrowth of spores in BHI. Spores were heat activated in water and germinated in BHI at 37°C, and OD was monitored. Data shown are averages for three independent spore preparations; error bars are omitted for clarity. A) Germination of wild-type (♦), Δ*sleB* (▲), Δ*sleL* (●), Δ*sleB* Δ*sleL* (△), Δ*cwlJ1* Δ*sleB* (+), and Δ*cwlJ1* Δ*cwlJ2* Δ*sleB* Δ*sleL* (×) spores. B) Germination of wild-type (♦), Δ*cwlJ1* (■), Δ*cwlJ2* (□), Δ*cwlJ1* Δ*cwlJ2* (◇), and Δ*cwlJ1* Δ*sleL* (○) spores. Asterisks indicate those time points when Δ*cwlJ1* Δ*cwlJ2* spores were significantly different ($P \leq 0.01$) from Δ*cwlJ1* spores.

Effects of GSLEs on Ca^{2+}-DPA-triggered germination. It was previously demonstrated that *B. subtilis* and *B. megaterium* spores that lack CwlJ are extremely unresponsive to the non-nutrient germinant Ca^{2+}-DPA (72, 88). Spores that germinate in response to Ca^{2+}-DPA become heat sensitive. For example, after treatment with Ca^{2+}-DPA, our control wild-type *B. subtilis* spores lost a significant level of heat resistance indicating that 99% of the spores had germinated while *B. subtilis* spores carrying a *cwlJ* mutation were not affected (Fig. 3.2).

Wild-type *B. anthracis* spores incubated with exogenous Ca^{2+}-DPA exhibited a 99% decrease in heat-resistant colony forming units (Fig. 3.2). Surprisingly, Δ*cwlJ1* spores were also

significantly heat sensitive after Ca^{2+}-DPA exposure, suffering a 79% lowered titer. While Δ*cwlJ2* spores performed similarly to those of the wild-type strain, spores lacking both *cwlJ1* and *cwlJ2* had no significant change in heat resistance after Ca^{2+}-DPA treatment (Fig. 3.2). Phase-contrast microscopy of spores during their Ca^{2+}-DPA incubation was also carried out since germinating spores transition from phase bright to phase dark. Consistent with the heat resistance assay, 98% of wild-type and Δ*cwlJ2* spores became phase dark after 60 minutes, 60% of Δ*cwlJ1* spores transitioned, but only 6% of spores carrying both *cwlJ1* and *cwlJ2* deletions became phase dark (data not shown). Together these observations show that, in *B. anthracis* spores, both CwlJ1 and CwlJ2 independently contribute to a Ca^{2+}-DPA germination response, and that CwlJ1 is responsible for the majority of the activity. Deletions of either *sleB* or *sleL* had no impact on the spore response to Ca^{2+}-DPA (data not shown).

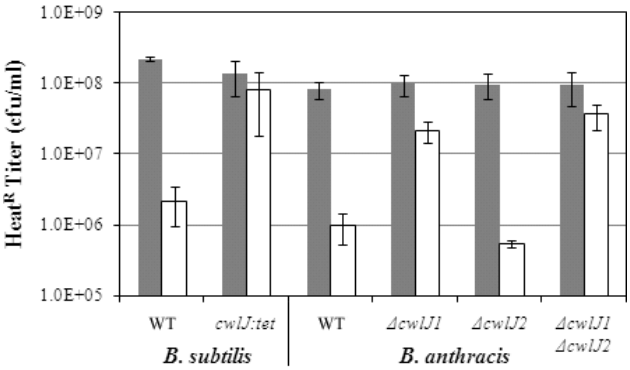

Figure 3.2. Germination of spores in response to Ca^{2+}-DPA. Spores from *B. subtilis* strains PS832 (wild-type) and FB111 (Δ*cwlJ::tet*) (72), and *B. anthracis* strains DPBa2 (wild-type), DPBa61 (Δ*cwlJ1*), DPBa60 (Δ*cwlJ2*), and DPBa78 (Δ*cwlJ1* Δ*cwlJ2*) were incubated in water (gray bars) or 50 mM Ca^{2+}-DPA pH 8.0 (white bars) for 60 minutes at 25°C before being heated at 70°C for 20 min, serially diluted, and plated on BHI. Values are averages of at least three independent spore preparations. Error bars represent one standard deviation of the mean.

Release of cortex fragments from germinating spores. During germination, spores release cortex fragments that can be quantified based upon muramic acid and Dpm content. Data is presented only for muramic acid release, but in all cases Dpm release was analyzed and paralleled the release of muramic acid (data not shown). Wild-type *B. anthracis* spores released

nearly 90% of their muramic acid within 15 minutes of germination initiation (Fig. 3.3). The *ΔcwlJ1* spores had an early delay in muramic acid release, but these spores still released nearly as much muramic acid as those of the wild-type strain after 15 minutes (Fig. 3.3 and (35)). While the *ΔcwlJ2* mutant was indistinguishable from the wild-type strain (data not shown and (35)), *ΔcwlJ1 ΔcwlJ2* spores exhibited an additive decrease in muramic acid release which was significant during the first 15 minutes of germination in comparison to both the wild-type and *ΔcwlJ1* strains (Fig. 3.3). However, by 30 minutes the double mutant was still able to release a normal amount of cortex fragments.

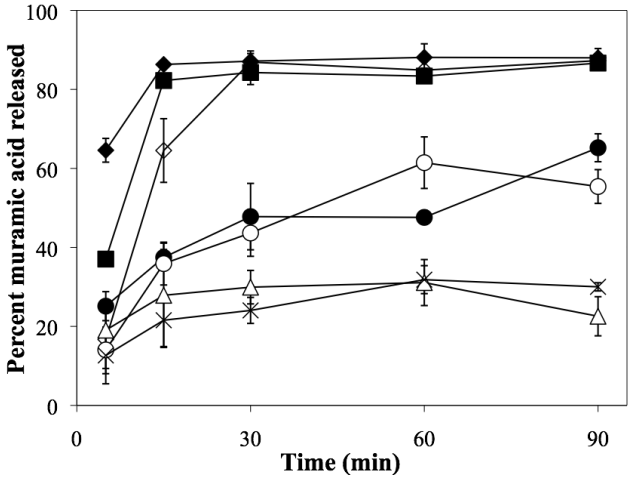

Figure 3.3. Release of cortex fragments from germinating spores. Dormant wild-type (♦), Δ*cwlJ1* (■), Δ*sleL* (●), Δ*cwlJ1* Δ*sleL* (○), Δ*sleB* Δ*sleL* (Δ), Δ*cwlJ1* Δ*cwlJ2* (◇), and Δ*cwlJ1* Δ*cwlJ2* Δ*sleB* Δ*sleL* (×) spores in buffer were sampled for assay of the release of muramic acid following exposure to the germinants L-alanine and inosine. Error bars represent one standard deviation of the mean for three independent spore preparations. All points have error bars, but in some cases, these are too small to be visible.

The Δ*sleL* mutation caused dramatic effects on muramic acid release (Fig. 3.3 and (47)). These spores released ≤40% of their muramic acid within 15 minutes, and even after 90 minutes expelled a maximum of only 65% of this cortex component. The Δ*sleL* Δ*cwlJ1* double mutant spores displayed kinetics similar to the Δ*sleL* single mutant over the course of the assay. However, Δ*sleB* Δ*sleL* spores were more severely impaired at discharging cortical fragments.

While Δ*sleB* spores have only a minor delay in cortex release (35), Δ*sleB* Δ*sleL* double mutant spores released considerably less muramic acid throughout the assay than did those of the single mutants (Fig. 3.3 and (35)). In fact, the Δ*sleB* Δ*sleL* spores released muramic acid with kinetic similar to the quadruple mutant lacking all four GSLEs. These two strains released a maximum ~30% of their muramic acid, however, the Δ*sleB* Δ*sleL* spore accomplished this within 15 minutes whereas the quadruple mutant required 60 minutes (Fig. 3.3).

GSLE effects on cortex hydrolysis. Cortex structural changes during germination of spores lacking multiple GSLEs were analyzed using a RP-HPLC technique that was previously employed to identify the cortex lytic activities of SleB and SleL in *B. anthracis* spores (35, 47). In these earlier studies, spores lacking CwlJ2 were indistinguishable from those of the wild-type. Spores of strains carrying mutations in *cwlJ1*, *sleB*, or *sleL* released lower amounts of muropeptides, and in the cases of *sleB* and *sleL* specific muropeptides indicative of those gene products' activities were lacking. It was shown that Δ*cwlJ1* Δ*sleB* spores released essentially no measurable muropeptides, consistent with their germination block. For the present study, spores with a combination of *cwlJ1* and *sleB* deletions were not investigated for muropeptide release due to this severe phenotype.

The muropeptides released from Δ*cwlJ1* Δ*sleL* spores were anticipated to be the consequence of SleB's lytic transglycosylase activity. After germinating for two hours this strain released more than 60% of cortex PG into the exudate and, as expected, generated the lytic transglycosylase products aG7a and aG7b (Fig. 3.4B), which are tetrasaccharides terminating in N-acetyl-anhydromuramic acid (Table 3.3 and (35)). Further digestion of the exudate with the muramidase Mutanolysin increased the relative amounts of aG7a and aG7b by 44% and 114% respectively, and generated a large amount of muropeptide N (Fig. 3.4E). Muropeptide N is a

tetrasaccharide that is the predominant product produced by Mutanolysin digestion of intact cortex strands (Table 3.3 and (18)). Based on the sizes of peaks aG7a, aG7b, and N released from these spores, we calculate that 42% of SleB products are anhydrotetrasaccharides (aG7a and aG7b) and that the remaining 58% are larger fragments. Also after Mutanolysin digestion, the increase of anhydrotetrasaccharides compared to muropeptide N is in a ratio of nearly 1:1, which suggests the average large muropeptide after SleB digestion is eight sugars in length. Mutanolysin digestion of the PG retained in the germinated Δ*cwlJ1* Δ*sleL* spore pellet released a small amount of aG7a and aG7b, demonstrating that a few SleB products remained too large to be released from the spore (data not shown).

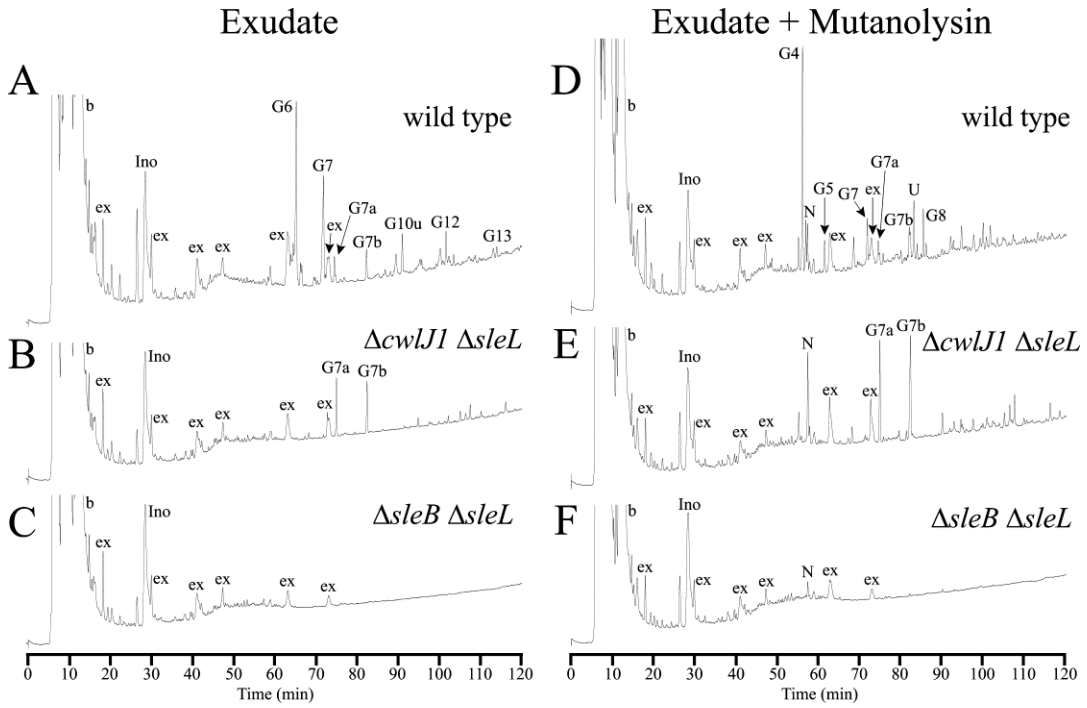

Figure 3.4. RP-HPLC separation of muropeptides released from germinating spores. PG was prepared from germinating spore suspensions as described in Materials and Methods. Samples were collected after spores were allowed to germinate for 120 minutes. 50% of each exudate (D-F) sample was digested with muramidase, reduced, and separated as previously described (59). The other 50% of each exudate (A-C) was reduced and separated without muramidase digestion. Peaks are numbered as in (35) and Table 3.3, but the initial "a" in the germination-specific-peak names were omitted for space considerations. Early-eluting peaks labeled "b" are buffer components present in blank samples. Peaks labeled "ex" are spore

exudate components that are not derived from PG. Peaks labeled “Ino” are from the inosine used as germinant.

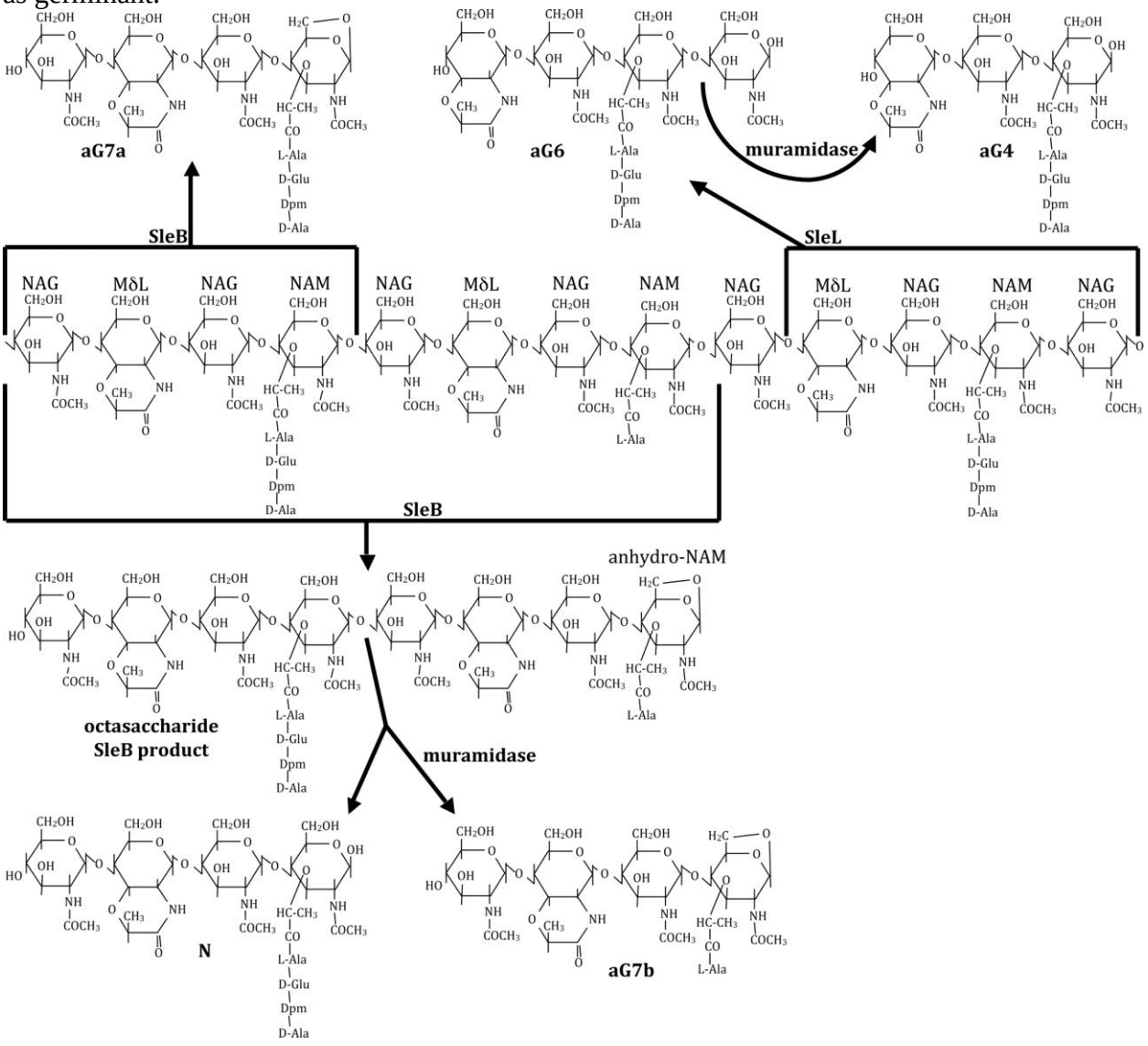

Figure 3.5: Spore PG structure and hydrolysis. The central structure shows a representative spore PG strand with alternating NAG and NAM or muramic-δ-lactam (MδL) residues and with tetrapeptide or L-Ala side chains on the NAM residues. Forked arrows originate at sites of hydrolysis by the indicated enzymes and point to muropeptide products. The indicated “aG” muropeptide names are previously published (18, 35). SleB lytic transglycosylase activity produces muropeptides terminating in anhydro-NAM. Cleavage at adjacent NAM residues produces the tatrasaccharide aG7a or aG7b, while cleavage further apart can produce octasaccharides or larger fragments. These can be further cleaved by muramidase treatment, resulting in the production of tetrasaccharide N, which terminates in NAM. The *N*-acetylglucosaminidase activity of SleL produces tetrasaccharides terminating in NAG, which can be further cleaved by muramidase to trisaccharides terminating in NAM.

Table 3.3. Muropeptide peak identification

Name[a]	Structure[b]	Enzymatic origin
N	TS-TP	muramidase
U	HS-TP-Ac	muramidase
aG4	TriS-TP	N-acetylglucosaminidase + muramidase
aG5	TriS-Ala	N-acetylglucosaminidase + muramidase
aG6	TS-TP NAGr	N-acetylglucosaminidase
aG7	TS-Ala NAGr	N-acetylglucosaminidase
aG7a	TS-TP anhydro	Lytic transglycosylase
aG7b	TS-Ala anhydro	Lytic transglycosylase
aG8	PS-TP-Ac	N-acetylglucosaminidase + muramidase
aG10u	HS-TP-Ac NAGr	N-acetylglucosaminidase
aG12	HS-Ala-Ac NAGr	N-acetylglucosaminidase
aG13	HS-TP NAGr	Cortex, N-acetylglucosaminidase

[a] Muropeptide names are as previously published (18, 35). Muropeptide names preceded by "a" indicate those generated by *B. anthracis* in order to differentiate them from those generated by other species.

[b] Abbreviations: TS, tetrasaccharide (NAG-lactam-NAG-NAM); HS, hexasaccharide (NAG-lactam-NAG-lactam-NAG-NAM); TriS, trisaccharide (lactam-NAG-NAM); PS, pentasaccharide (lactam-NAG-lactam-NAG-NAM); TP, tetrapeptide (Ala-Glu-Dpm-Ala); -Ac, deacetylated glucosamine; NAGr, NAG at the reducing end. "Anhydro" indicates that the NAM at the reducing end is in the anhydro form.

The chromatograms of spore germination exudates from Δ*sleB* Δ*sleL* (Fig. 3.4C) and Δ*cwlJ2* Δ*sleB* Δ*sleL* strains (data not shown) were indistinguishable; any cortex fragments released from these strains are expected to be products of CwlJ1 activity. These spores released no detectable small muropeptides (Fig. 3.4C), however, treatment of the exudates with Mutanolysin produced a small quantity of muropeptide N (Fig. 3.4F). This suggests that depolymerization of cortex catalyzed by CwlJ1 alone produced large muropeptides that are not resolved with this method. In fact, assays of released cortex fragments (Fig. 3.3) and of cortex released from the germinated spore pellet by Mutanolysin (data not shown) indicate that ≥75% of CwlJ1-generated cortex fragments are too large to be released from the spore.

DISCUSSION

In the simplest measure of spore germination kinetics, tracking of OD loss, slight delays in germination are observed in single mutants lacking *cwlJ1*, *sleB*, or *sleL*, and combinations of these mutations result in additive effects. In the cases of *sleB* and *sleL* mutants, germination is indistinguishable from that of the wild-type strain during the first 10 minutes, and delays are obvious only after that time. A *cwlJ1* mutation affects OD loss even earlier, though it has the least effect on the overall kinetics of cortex hydrolysis and release, suggesting that this mutation has an additional effect on some other aspect of the germination process. A combination of *cwlJ1* and *sleB* mutations renders spores essentially incapable of completing germination (25, 35); cortex hydrolysis is completely blocked and OD decrease ceases after 10 minutes. This indicates that OD decrease during the first 10 minutes of germination is primarily due to loss of spore refractility, release of spore solutes, and uptake of water. Studies in other species demonstrated that *cwlJ* mutations slowed the process of Ca^{2+}-DPA release (38, 75, 88), and we suggest that the early delay in OD loss in a *cwlJ1* mutant is due to a slowed process of spore solute exchange. How might a cortex lytic enzyme affect solute movement into and out of the spore? We propose that CwlJ1 (and CwlJ2) acts initially on the outer layers of the cortex, due to their apparent location at or near the cortex/coat interface (5, 13, 35, 46). Outer cortex layers are more highly cross-linked than the inner layers (59) and thus may produce a slightly greater diffusion barrier and/or might exert a greater influence on the ability of the spore core to expand and take up water in the first moments of germination. Such water uptake may be critical for solubilization and movement of Ca^{2+}-DPA, for dissociation of SASP proteins from the spore DNA, and for activation of a germination-specific protease in the core (89, 91).

Giebel *et al*. 2009 found that spores of a *cwlJ2* mutant lost OD slightly more slowly than those of the wild-type strain during germination, but with an overall effect less dramatic than those of *cwlJ1* and *sleB* mutants (25). While our analyses with single mutants have never demonstrated a significant phenotypic change due to a single *cwlJ2* mutation (35), we and Giebel *et al*. (25) did observe that a *cwlJ2* mutation had additive effects to those of a *cwlJ1* mutation. In particular, we find that *cwlJ1 cwlJ2* spores exhibit a delay in outgrowth that is significantly greater than might be expected based on the relative delays in OD loss and cortex fragment release among all the mutant strains analyzed. We also found that *cwlJ2* plays a role in Ca^{2+}-DPA-stimulated germination. Studies in other species have demonstrated roles of CwlJ proteins in response to this non-nutrient germinant, and release of Ca^{2+}-DPA from the germinating spore is apparently the mechanism by which this lytic enzyme is normally activated (72). In *B. anthracis*, Ca^{2+}-DPA-triggered germination utilizes CwlJ1 and CwlJ2 working in concert, since loss of both enzymes is required to completely eliminate the response. Together, the data suggest that CwlJ2 is a functional GSLE that has the lowest level of activity among known GSLEs in *B. anthracis*, consistent with its apparent low expression level (35). Furthermore, we assert that CwlJ1 and CwlJ2 carry out identical functions given: 1) the high level of sequence identity (58%) between the proteins, 2) that deletion of the former is needed in order to demonstrate activity for the latter, and 3) the requirement of both enzymes for maximum response to Ca^{2+}-DPA.

Colony formation is unaffected in *B. anthracis* spores that contain at least SleB or CwlJ1 (25, 35), supporting the idea that either one of these SCLEs is sufficient to degrade the cortex to a great enough degree that metabolic activity resumes and the cell can grow out of its integuments. When both SleB and CwlJ1 are absent from spores, in strains with or without other

GSLEs present, we observe a 1000-fold decrease in colony-forming efficiency (Table 2 and (35)). In each case, colony forming ability can be rescued by the addition of lysozyme, indicating that the block to germination is due to incomplete cortex hydrolysis. Contrary to our results, a recent report indicated that the additional loss of CwlJ2 from a *ΔcwlJ1ΔsleB* double mutant resulted in a much more profound loss of colony-forming efficiency (25). To explain this difference, we must ask why 0.1% percent of spores that lack all known GSLEs are able to produce colonies in our studies, but not in those of Giebel *et al*. One potential explanation is that small amounts of exogenous lytic enzymes in our growth media might be hydrolyzing the cortex PG enough to allow this small percentage of spores to complete germination, despite the fact that we have used BHI medium from the same manufacturer as Giebel *et al*. (25). The potential for exogenous lytic enzymes to allow germination of GSLE-deficient spores was also cited as an explanation for the virulence of these spores at a level greater than would be expected from their colony forming efficiency on laboratory medium (25).

Cortex fragment release from germinating *B. anthracis* spores follows three different kinetic paths depending on which proteins are actively digesting the cortex PG. Maximum total release occurs when both SleB and SleL are functional, and CwlJ1 and CwlJ2 only affect the initial rate of release. SleB digests the PG into fragments of varying sizes, some of which are released, but the majority of which are initially retained within the spore. SleL then acts on these fragments, thus increasing the proportion that are small enough for rapid release. In the absence of SleL, muropeptides found in the exudates are significantly reduced. Given enough time, SleB alone can digest the PG to fragments small enough, primarily tetra- and octa-saccharides, for release of >50% of the cortex from the spore. In the absence of both SleB and SleL, cortex degradation can still be accomplished by CwlJ1 and CwlJ2 to allow outgrowth. However, the

cortex fragments are apparently so large that they are not released, and muramic acid release by *sleB sleL* spores is indistinguishable from that of spores lacking all four GSLEs.

The fact that the products of CwlJ1 activity are so large has prevented determination of the site of PG cleavage by this enzyme. Muramidase digestion of germinated *cwlJ2 sleB sleL* spore PG, which presumably has been cleaved only by CwlJ1, has yielded only muramidase products (data not shown). This suggests that CwlJ1 is either a muramidase or that its cleavage sites are so few as to be undetectable by our current methods. The possibility of CwlJ1 muramidase activity is consistent with the significant sequence homology between CwlJ and SleB lytic transglycosylase proteins. Lytic transglycosylases and muramidases cleave the same bond in PG, but differ in the chemistry of their products, the former producing anhydro-N-acetylmuramic acid and the latter producing N-acetyl-muramic acid. Similar protein folds can result in these two enzymatic activities (95). Ongoing efforts to produce significant CwlJ1 activity *in vitro* may allow cleavage of a purified substrate to an extent sufficient to directly identify the enzymatic products.

While the roles played by the *B. anthracis* GSLEs and their requirements for germination both in and out of the host (25, 35, 47) are becoming clear, many questions remain concerning the mechanisms by which they are held inactive in the dormant spore and activated during germination. Future studies of the localization, processing, and interaction partners of these enzymes may answer these questions. A strategy for efficient external activation of GSLEs, and therefore initiation of germination, will allow the development of simpler methods for decontamination of sites of spore release.

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CHAPTER 4:

In vitro and *In vivo* Analyses of the *Bacillus anthracis* Protein SleL

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COAUTHOR CONTRIBUTIONS

Emily Lambert conducted the majority of the research presented herein. Nora Sherry contributed by creating peptidoglycan substrates for protein assays, and David Popham was the principle investigator. Although the manuscript has yet to be published, each coauthor has given written permission to print the material in this dissertation.

ABSTRACT

The bacterial endospore is the most resilient biological structure known to man. Multiple protective integument layers shield the spore core and promote spore dehydration and dormancy. Dormancy is broken when a spore germinates and becomes a metabolically active vegetative cell. Germination requires the breakdown of the spore layers including a modified layer of peptidoglycan known as the spore cortex. This study reports on *in vitro* and *in vivo* analyses of the *Bacillus anthracis* SleL protein. SleL is a spore cortex lytic enzyme composed of three conserved domains; two N-terminal LysM domains and a C-terminal glycosyl hydrolase family 18 domain. Derivatives of SleL containing both, one, or no LysM domains were purified and characterized. SleL is incapable of digesting intact cortical PG of either decoated spores or purified spore sacculi. However, SleL derivatives can hydrolyze partially depolymerized PG substrates containing muramic-δ-lactam recognition determinants. The muropeptides that result from SleL hydrolysis are the products of *N*-acetylglucosaminidase activity. These muropeptide products are small and readily released from the cortex matrix. However, the loss of the LysM domain(s) decreases hydrolysis effectiveness. The reduction in hydrolysis is likely due to the role of LysM domains in substrate recognition and PG binding. When the SleL derivatives are expressed *in vivo* those proteins lacking one or both LysM domains do not associate with the spore. Instead, the protein remains in the mother cell during sporulation. SleL with both LysM domains localizes to the coat or cortex of the endospore. The information revealed by elucidating the role of SleL and its domains to *B. anthracis* sporulation and germination is important in designing new spore decontamination methods. By exploiting germination-specific lytic enzymes, eradication techniques can be greatly simplified.

INTRODUCTION

Inhalational, gastrointestinal, and cutaneous anthrax infections result when a host becomes infected with *Bacillus anthracis* spores (1, 61). Establishment of the bacterial infection requires spore germination. This morphological transition leads to the development of metabolically active vegetative cells that produce virulence factors and can initiate a fatal illness (61, 91). Not only is the process of germination critical to infection progression, but an understanding of the biological transition will allow for the development of more effective decontamination methods. Spores are highly resistant structures that can withstand extremes of pH, high temperatures, noxious chemicals, radiation, desiccation, and starvation (68, 90). However, vegetative bacilli are relatively easy to eradicate. Therefore, by manipulating proteins involved in germination the process can be constructively exploited.

Germination has been studied in an array of gram-positive spore-forming species including *Clostridium perfringens* (12, 60), *Bacillus thuringiensis* (36), *Bacillus subtilis* (38, 42, 65), *Bacillus megaterium* (23, 88), *Bacillus cereus* (11, 52, 66), and *B. anthracis* (34, 35, 47, 51). The *B. anthracis* germination cascade begins when nutrient or non-nutrient germinants contact receptors within the spore. A cache of chelated dipicolinic acid-Ca^{2+} is released from the spore core as water influx partially rehydrates the spore. Shortly thereafter, germination-specific lytic enzymes (GSLEs) are activated (63, 73, 79, 87). The first subset of these enzymes termed spore cortex lytic enzymes (SCLEs) initiate the degradation of a modified layer of peptidoglycan (PG) known as the cortex, which is involved in the maintenance of spore core dehydration and resistance (3, 11, 24, 53, 79). *B. anthracis* contains three SCLEs: CwlJ1, CwlJ2 and SleB. The enzymatic activities of CwlJ1 and CwlJ2 have yet to be elucidated, but the proteins likely play the same role. SleB is a lytic transglycosylase that plays a dominant role in cortex hydrolysis (34,

35). A second subset of GSLEs termed cortical fragment lytic enzymes (CFLEs) advances the germination cascade (53). One such protein, SleL, further digests the cortex PG, and small muropeptide fragments are released from the spore (34, 47). This allows for complete core rehydration and loss of spore associated resistance characteristics.

This study investigates the *in vitro* and *in vivo* activities of the CFLE, SleL, in an effort to further characterize the protein and its domains. SleL is an *N*-acetylglucosaminidase that breaks β-1,4 glycosidic bonds found in cortical PG (47). Previous studies show that *sleL* is expressed under the control of σ^E, which is active in the mother cell during early stages of sporulation (11, 42). As sporulation progresses, SleL translocates from the mother cell to the developing forespore (8, 11, 37, 43). SleL finally localizes to the outer periphery of the spore. Chen et al. (11) report that in *B. cereus* SleL is probably at the exterior of the cortex. A study by Imamura et al. (37) indicates that the SleL homolog of *B. subtilis*, YaaH, localizes to the inner coats. This inference is based upon the observation that YaaH localization is CotE independent and the protein is further from the spore core than YhcN, a cortex-associated protein (37).

On the basis of sequence homologies, SleL is composed of three conserved domains; two N-terminal LysM domains and a C-terminal glycosyl hydrolase family 18 domain (94, 97). LysM domains are a common element in many PG binding proteins (8). The LysM domains of the *B. subtilis* YaaH protein have been shown to play a role in development by directing proteins to the surface of the developing spore during sporulation (43). For these reasons we hypothesize that the LysM domains of SleL may be involved in protein localization and/or substrate recognition (8, 82, 83).

By studying SleL derivatives containing both, one, or no LysM domains *in vitro* and *in vivo* we were able to determine that *in vitro* SleL does have *N*-acetylglucosaminidase activity,

and the protein recognizes and digests partially hydrolyzed spore PG containing the muramic-δ-lactam recognition determinant. LysM domains play a role in not only substrate recognition and binding but are also required in directing the SleL protein to the developing forespore.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. anthracis* and *Escherichia coli* strains and plasmid vectors used in this study are summarized in Table 4.1. *B. anthracis* strains were cultured in brain heart infusion (BHI, Difco) containing 5 μg ml^{-1} erythromycin to select for resistance markers when necessary. Endospores were prepared by culturing strains in Modified G broth (41) containing erythromycin for selection when necessary. Sporulating cultures were incubated at 37°C for 3 days at which point spores were harvested and purified by water washing and centrifugation through 50% sodium diatrizoate as previously described (69). *E. coli* strains were cultured at 37°C in LB containing 50 μg ml^{-1} ampicillin (Jersey Lab Supply) and 30 μg ml^{-1} chloramphenicol (Jersey Lab Supply).

Overexpression of His_6-MBP-SleL in *E. coli*. Overexpression vectors encoding SleL and its truncated derivatives were constructed using the Invitrogen Gateway Cloning system and restriction free cloning as previously described (67, 98). Briefly, regions of *sleL* including base pairs 4-1290, 151-1290, or 295-1290 were amplified by PCR using primers SleL-A+SleL-D, SleL-B+SleL-D or SleL-C+SleL-D, respectively (primer sequences available upon request). The amplicons were introduced into the entry vector pDonR201 and then transferred to the destination vector pDest-HisMBP (85). The resulting plasmids encoded an N-terminal His_6-tagged maltose binding protein (MBP) fused to a tobacco etch virus (TEV) cleavage site and an SleL derivative. Because the full-length SleL fusion protein could not be successfully cleaved using TEV, restriction free cloning (98) using primer SleL-A2+SleL-D was used to integrate three additional glycine residues at the N-terminus of SleL. Plasmid constructs pDPV405, pDPV381, and pDPV382 were verified by restriction enzyme digestion and sequencing.

Table 4.1: Bacterial strains and plasmid vectors.

Strain	Relevent Genotype	Source or Reference	Plasmid	Relevent Genotype	Source or Reference
E.coli			**Vector**		
DPVE13	BL21, λDE3, pLysS, Cm^R	Novagen	pDonR201	Kn^R, Cm^R	Schubot
DPVE396	pDPV405, Amp^R, Cm^R	This Study	pDPV377	pDONR201::$SleL_{2-429}$	This Study
DPVE366	pDPV381, Amp^R, Cm^R	This Study	pDPV378	pDONR201::$SleL_{45-429}$	This Study
DPVE367	pDPV382, Amp^R, Cm^R	This Study	pDPV379	pDONR201::$SleL_{95-429}$	This Study
B. anthracis			pDest-HMBP	His6-MBP, Amp^R, Cm^R	Schubot
DPBa35	*ΔsleL*	(47)	pDPV405	pDest-HMBP::$SleL_{2-429}$	This Study
DPBa84	*ΔcwlJ1 ΔcwlJ2 ΔsleB ΔsleL*	(34)	pDPV381	pDest-HMBP::$SleL_{45-429}$	This Study
DPBa96	*ΔsleL*, pDPV406, Er^R	This Study	pDPV382	pDest-HMBP::$SleL_{95-429}$	This Study
DPBa97	*ΔsleL*, pDPV407, Er^R	This Study	pBKJ236	Er^R, ori^{ts}	(39)
DPBa98	*ΔsleL*, pDPV408, Er^R	This Study	pDPV406	pBKJ236::$SleL_{2-429}$	This Study
DPBa99	*ΔsleL*, pDPV409, Er^R	This Study	pDPV407	pBKJ236::$SleL_{51-429}$	This Study
			pDPV408	pBKJ236::$SleL_{99-429}$	This Study
			pDPV409	pBKJ236::*ΔsleL*	This Study

Overexpression vectors were transformed into DPVE13 (BL21 (DE3) pLysS). *E.coli* strains were cultured at 37°C until the optical density at 600 nm (OD) reached ~0.8 at which point isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.7 mM. The culture temperature was reduced to 10°C and incubation continued for 16 hours.

Purification of SleL derivatives. Cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl pH 7.5, , 5% glycerol, 25 mM imidizole, 50 mM NaCl ($SleL_{2-429}$) or 125 mM NaCl ($SleL_{51-429}$ and $SleL_{99-429}$) and lysed using a French press. Soluble and insoluble protein fractions were separated by high speed centrifugation; 117,000x*g* for 1 hour. Soluble His_6MBP-SleL protein fusions were purified using a nickel-charged HisTrap HP column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.5, 5% glycerol, 25 mM imidizole and 50 mM NaCl ($SleL_{2-429}$) or 125 mM NaCl ($SleL_{51-429}$ and $SleL_{99-429}$). Proteins were eluted with 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol, 250 mM imidizole and fractions were dialyzed in 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol. Proteins at a concentration of ~1.5 mg/ml were incubated with 0.5 mg/ml His-tagged TEV (S219V) protease (40) and allowed to digest at 15°C for 16 hours. Cleavage was verified by SDS-PAGE analysis. Digested proteins were separated using a HiTrap SP-HP cation exchange column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 7.5, 5% glycerol. Proteins were eluted with a linear gradient to 1 M NaCl in the same buffer. Fractions containing SleL proteins were dialyzed in 25 mM Tris-HCl pH 7.5, 250 mM NaCl, 5% glycerol and further purified using a Superdex 200 gel filtration column equilibrated with the same buffer. After elution the concentration of each pure protein was determined by measuring the absorbance (A) at 280 nm and using the Beer-Lambert law:

$$A=\varepsilon lC$$

where ε is the molar absorption coefficient (M^{-1} cm^{-1}), *l* is the pathlength (cm), and *C* is the protein concentration (M) (71).

Raising of antibodies and western blot analysis. Polyclonal anti-SleL antibodies were raised by injecting rabbits (Open Biosystems) with purified $SleL_{99-429}$. SleL derivates were identified after western blotting using colorimetric detection. Primary anti-SleL (Open Biosystems) and secondary goat anti-rabbit antibody-Horse Radish Peroxidase (Biorad) antibodies were generally used at a 1:5000 dilution.

***In vivo B. anthracis* derivative strains.** *B. anthracis* strains encoding SleL with both, one or no LysM domains were constructed using plasmid insertion mutagenesis. Briefly, *sleL* along with 600 base pairs upstream of the gene was amplified by PCR using primers SleL-1+SleLDown00 which were designed to introduce *NotI* and *BamHI* restriction sites to the 5' and 3' ends of the product, respectively. The amplicon and pBKJ236 were digested with the endonucleases and the products were ligated to produce pDPV406. Inverse PCR of this vector using primers SleLInvUpNcoI+SleLInvDown1NcoI, SleLInvUpNcoI+SleLInvDown2NcoI, or SleL-2+SleL-3 resulted in linear fragments that were digested with *NcoI* or *BglII* and ligated to produce pDPV407, pDPV408, and pDPV409, respectively. Constructs were verified by restriction enzyme digestion and sequencing. Each plasmid was transformed into *E. coli* INV110 (*dam*$^-$, *dcm*$^-$) and then transformed into *B. anthracis* strain DPBa35 (Δ*sleL*) by conjugation as previously described (39). The plasmids were integrated into the *B. anthracis* chromosome upstream of Δ*sleL* by increasing the culturing temperature to 37°C. Strains DPBa96, 97, and 98 were verified by PCR using primers SleL-1+SleLDown00 and pBKJ236SeqA+SleL-4 while DPBa99 required primers or SleL-1+pBKJ236SeqB and pBKJ236SeqA+SleL-4 for verification.

Peptidoglycan substrate preparation. A variety of PG substrates were prepared from *B. subtilis* PS832 (wild-type) and DPVB19 (Δ*cwlD*::Sp) spores. *B. subtilis* was cultured in 2×SG medium (48) for 3 days at which point spores were purified by water washing as previously described (69). Decoated spores were prepared as previously described (78) and then stored at 4°C. PG sacculi were purified as previously described (80). When indicated, purified sacculi were partially digested by combining sacculi at an OD 0.2 suspended in 30 mM $NaPO_4$ pH 7.0, 1 mM EDTA, 1 mM DTT and 0.1% Triton with 0.01, 0.05 or 0.25 µg/ml of purified SleB or 0.1 µg/ml of lysozyme (Sigma). To prepare cortex PG fragments, sacculi at an OD 0.5 were resuspended in 500 µl 50 mM Tris-HCl pH 8.0 and physically broken with 250 mg 0.1 mm glass beads using a Wig-L-Bug bead beater. Sacculi were pulsed ten times at 3,800 rpm for 30-second and were incubated on ice for 1 minute between pulses. Proteins were extracted from cortex fragments by mixing the suspension with chloroform and collecting the aqueous phase. Residual chloroform was removed by rinsing the pellets repeatedly with dH_2O. Purified muropeptides were collected by using HPLC as previously described (18).

PG binding assay. SleL binding affinity was analyzed by combining wild-type or *cwlD*$^-$ *B. subtilis* spore cortex PG fragments with the various SleL proteins. Fragmented *B. subtilis* spore PG totaling ~0.3 OD units was centrifuged and resuspended in a buffer solution containing 30 mM $NaPO_4$ pH 7, 1 mM EDTA, 1 mM DTT, 0.1% Triton, and 1.5 µg of each SleL protein. Protein suspensions were allowed to incubate on ice for 10 minutes. Supernatants and pellets were seperated by centrifugation and pellets were resuspended in the buffer solution. Samples were combined with SDS-PAGE sample buffer, boiled, centrifuged briefly and seperated using a 12% SDS-PAGE. The acrylamide gels were stained using Sypro Ruby (Lonza) as per the manufacturer's instructions. Stained proteins were detected using a Typhoon Trio Imager (GE

Healthcare), and banding was quantified using ImageQuant TL software (Amersham Biosciences).

PG hydrolysis assays. Decoated spores, chemically and physically prepared PG fractions, and muropeptides were incubated with 1 μg/ml purified SleL derivatives. Hydrolysis was measured as a decrease in OD. Pellet and soluble samples were separated by centrifugation. DPBa82 and DPBa83 spores at an OD 50 were germinated in a buffered solution of L-alanine and inosine and then digested with 0.5 mg/ml $SleL_{2\text{-}429}$. Samples were analyzed for amino sugar and muropeptide composition as previously described (18). Novel peaks were evaluated by mass spectrometry as previously described (18).

Sporangia and spore fractionation. Sporangia samples were collect between t_0-t_8 and at t_{24} of sporulation. Ten milliliter culture samples were pelleted by centrifugation at 10,000 g for 10 minutes, resuspended in 8 mM $NaHPO_4$, spun at 13,000 g for 1 minute, decanted, and frozen at -80°C. Frozen pellets were lyophilized and then broken using the bead beater as described above at 4,200 rpm for ten, 1 minute intervals. Broken spores were then resuspended in protein sample loading buffer and analyzed by western blotting. Additional 1 ml culture samples were pelleted and resuspended in a lysis solution containing 10 mM glucose, 2 mM EDTA, 5 mM Tris-HCl pH 8.0 and 6 mg/mL lysozyme. After incubating at 37°C for 20 minutes, Sarkosyl was added to a final concentration of 2%, and incubation continued 20 minutes more. Insoluble, forespore-associated material was separated from soluble proteins by centrifugation and then analyzed by western blotting.

Purified spores were further evaluated by physically breaking samples using a bead beater as described above followed by centrifugation at 13,000x*g* for 3 minutes. The insoluble pellet was digested with 6 μg/ml lysozyme at 37°C for 20 minutes. Solublized cortex-associated

proteins were then separated from insoluble coat proteins by centrifugation. All samples were combined with protein sample loading buffer and boiled for 5 minutes before being separated by 12% SDS-PAGE. Proteins were evaluated by western blot analysis using the polyclonal anti-SleL antibody.

To evaluate the localization of SleL after germination, purified spores at an OD 0.4 were germinated in either a buffered solution of 8 mM $NaPO_4$ pH 7, 10 mM L-alanine (Fisher) and 1 mM inosine (Sigma) or 50 mM Ca^{2+}-DPA pH 7.5 for 45 minutes. Spore-associated and exudate-associated SleL was separated by centrifugation. Samples were lyophilized, resuspended in sample loading buffer, boiled and analyzed by western blotting using the polyclonal anti-SleL antibody.

RESULTS

SleL domain definition and protein purification. The NCBI Conserved Domains Database (54) was used to predict domains of SleL. Three domains were identified: two N-terminal LysM domains and a C-terminal glycosyl hydrolase family 18 domain (Figure 1A). The secondary structure of SleL was predicted using Protean (Lasergene). Results indicate that there are loop regions at residues 51-53 and 98 of SleL. Therefore, SleL derivatives were designed in a way that the truncations of each LysM domain occurred in these loop regions in order to maintain the integrity of the remaining protein (Figure 4.1A). SleL derivatives were purified using affinity, cation exchange, and gel filtration chromatography. The resulting proteins have predicted molecular masses of 48.2, 43.0, and 37.6 kDa respectively as determined by ExPASy ProtParam. Purified SleL derivatives are shown in figure 4.1B. Gel filtration analysis of SleL derivatives suggest that the purified proteins exist as monomers (data not shown).

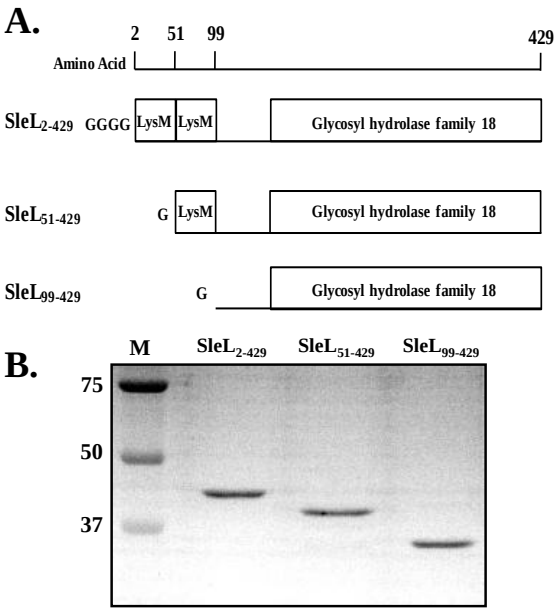

Figure 4.1: Purified SleL proteins. SleL proteins with and without LysM domains were purified using affinity chromotography and gel filtration. A.) Putative SleL domains and derivative constructs. B.) The SDS-PAGE analysis shows M: standard protein marker (KDa), $SleL_{2-429}$, $SleL_{51-429}$, and $SleL_{99-429}$.

SleL is a CFLE that digests partially hydrolyzed cortex PG. SleL was combined with an array of PG constituents in order to determine what types of substrates the protein can recognize and cleave. When combine with decoated *B. anthracis* spores, $SleL_{2\text{-}429}$ was unable to digest the cortical PG since there was no loss in OD (data not shown). To study the enzymatic activity of the SleL derivative on intact, purified cortical PG, *B. subtilis* was chemically and or physically treated to extract the cortex. *B. subtilis* cortical PG has been shown to have a nearly identical structure to *B. anthracis* (18) but it is much easier to purify, thus making it an ideal substrate. $SleL_{2\text{-}429}$ was also unable to digest intact cortical PG isolated from wild-type *B. subtilis* spores (Figure 4.2). Increasing concentrations of purified SCLE SleB were used to partially hydrolyze the cortical sacculi as determined by a loss of OD. The rate of OD loss was dependent on the concentration of SleB. The use of SleB and SleL in combination resulted in a more abrupt loss of OD than when SleB was used alone. This likely indicates that after the cortex PG is digested by SleB, $SleL_{2\text{-}429}$ is able to recognize the substrate and actively digest it.

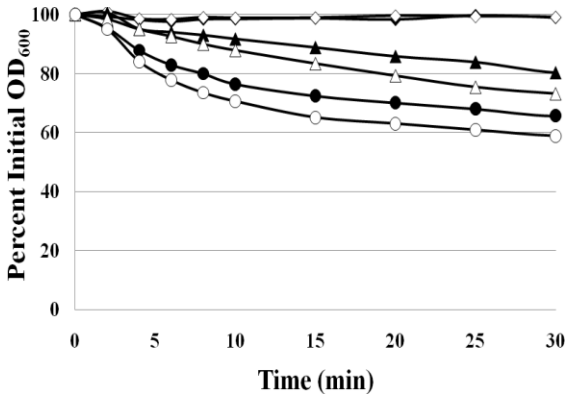

Figure 4.2: $SleL_{2\text{-}429}$ enzymatic activity assay. Wild-type *B. subtilis* PG sacculi were partially digested using various concentrations of SleB (♦ none, ▲ 0.01 µg/ml, or ● 0.05 µg/ml) at 37°C. The rate of PG digestion increased as the concentration of SleB increased. When $SleL_{2\text{-}429}$ was added (open symbols) to each reaction at a final concentration of 1 µg/ml, the rate of break down was even greater. Data shown are averages for three independent activity assays; error bars are omitted for clarity.

After observing a loss in OD over the course of an hour, pellet and exudate samples were collected and analyzed for amino-sugar and muropeptide content. Figure 4.3 shows that digestion using SleL or SleB alone results in the release of little cortical NAM. Digestion with SleL results in 16% of the NAM being released. This may occur if the integrity of the cortical PG is slightly disrupted during purification. Most likely, there are not substantial flaws in the sacculi since there is no loss in OD when SleL is used as the sole lytic enzymes in the activity assay. NAM release also appears to be dependent on the concentration of SleB since a higher percentage of the PG constituent is released upon treatment with increasing concentrations of the enzymes. However, maximum NAM release is not achieved until both proteins are combined. More NAM, nearly 100%, is released to the exudates when SleL and SleB are combine as compared to no protein, SleL, or SleB alone. This release is independent of the SleB concentration.

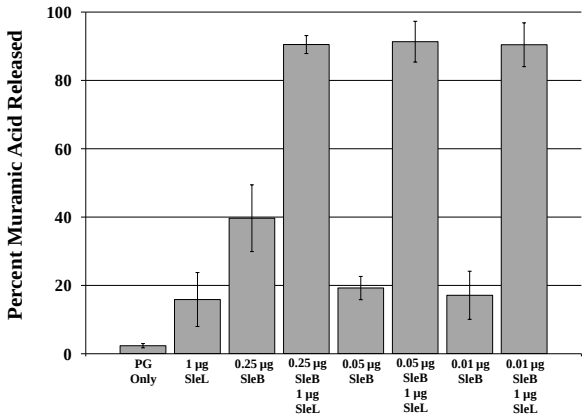

Figure 4.3: NAM release as a result of SleL and/or SleB digestion. After the enzymatic activity assay samples were collected and centrifuged to separate sacculi-associated (pellets) and released (supernatants) NAM. When either $SleL_{2\text{-}429}$ or SleB is used as the sole lytic enzyme little NAM is released. When both proteins cooperatively digest the wild-type *B. subtilis* sacculi nearly all of the cortical NAM is released. Data shown is the average of three independent assays. Error bars represent 1 S.D. of the mean.

These same soluble samples were also analyzed for muropeptide content. HPLC analysis indicates that there are no detectable muropeptides present in the SleB or SleL digestion profiles. Yet, digestion using both proteins results in PG break down and the appearance of peaks G6 and

G7 (Figure 4.4). These muropeptides are tetrasaccharide-tetrapeptide and tetrasaccharide-alanine products, respectively, that result from *N*-acetylglucosaminidase digestion (18, 47).

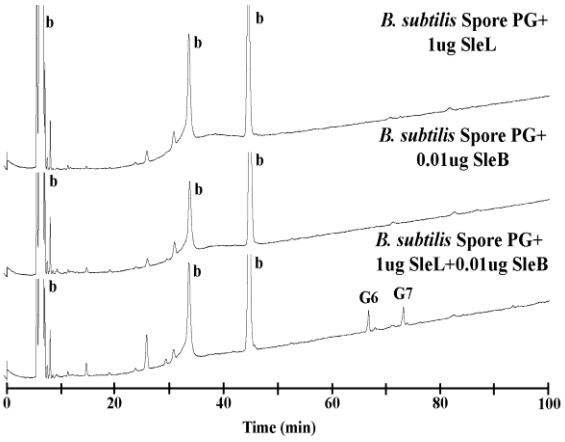

Figure 4.4: RF-HPLC separation of muropeptides resulting from SleL and/or SleB digestion. Wild-type *B. subtilis* spore PG sacculi were digested with 1 µg/ml SleL and/or 0.01 µg/ml SleB. HPLC was used to analyze the resulting muropeptides. When one or the other enzymes were used alone there were no detectable muropeptides produced. However, when PG sacculi were partially broken down with SleB and then further digested with SleL, *N*-acetylglucosaminidase products G6 (tetrasaccharide-tetrapeptide) and G7 (tetrasaccharide-alanine) were evident.

To characterize the role of LysM domains in SleL PG degradation, spore PG was again partially hydrolyzed with 0.05 µg/mL of SleB and further digested using 1 µg/ml of $SleL_{2\text{-}429,}$ $SleL_{51\text{-}429,}$ or $SleL_{99\text{-}429.}$ The average of three independent assays is shown in figure 4.5. Digestion of intact wild-type *B. subtilis* sacculi by SleB resulted in a ~24% loss of the initial OD. When sacculi were digested with a combination SleB and $SleL_{2\text{-}429,}$ $SleL_{51\text{-}429,}$ or $SleL_{99\text{-}429}$ the SCLE digested the cortex sufficiently so that each SleL derivative recognized the substrate and digested it, thus increasing the rate of OD loss as compared to digestion with SleB alone. However, in the absence of one or both LysM domains the rate of OD loss is slightly slower than when both domains are intact (Figure 4.5A).

To evalulate the enzymatic activity of SleL on cortical peptidoglycan lacking muramic-δ-lactam, a substrate recognition determinant, protein derivatives were combine with purified

cwlD^{-} *B. subtilis* sacculi that had been partially digested with a limiting concentration of lysozyme. Lysozyme was used to partially digest the sacculi since SleB is unable to recognize the substrate in the absence of the recognition determinant (unpublished data). Although lysozyme loosens the cortical PG resulting in a loss of OD, there is no difference in digestion of the muramic-δ-lactam-deficient PG in the presence of any SleL derivative (Figure 4.5B). A combination of lysozyme and $SleL_{2-429}$ was able to digest wild-type *B. subtilis* sacculi (data not shown).

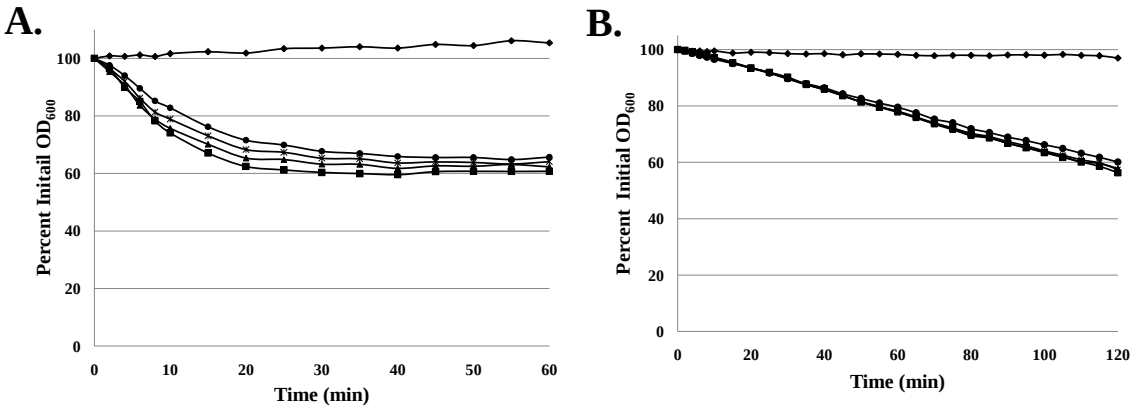

Figure 4.5: Digestion of wild-type and *cwlD*$^{-}$ *B. subtilis* spore PG with SleL proteins. PG from wild-type (A.) or *cwlD*$^{-}$ (B.) *B. subtilis* spores was digested with 0.05 µg/ml SleB (A.) or 0.1 µg/ml lysozyme (B.) (●), and 1 µg/ml $SleL_{2-429}$ (∗), $SleL_{51-429}$ (▲), or $SleL_{99-429}$ (■). Undigested PG was used as a control (♦). The rate of digestion of wild-type *B. subtilis* spore PG is dependent on the presence and amount of LysM domains. No matter the construct of SleL, it is unable to digest *cwlD*$^{-}$ *B. subtilis* spore PG.

LysM domains are involved in substrate recognition and bind cortical PG. To test the hypothesis that LysM domains are involved in substrate recognition and binding we analyzed the percentage of SleL derivatives that associated with purified extracted spore PG. Data analysis of SleL in the bound and unbound fractions of purified, extracted, wild-type *B. subtilis* cortical PG shows that the highest percentage (~77%) of SleL binds its substrate when both LysM domains are intact (Figure 4.6). There is a substantial decrease in the amount of SleL associated with the bound fraction when one or both LysM domains are removed. Only ~35% of $SleL_{51-429}$ and ~8%

of $SleL_{99-429}$ associates with the wild-type substrate. The presence or absence of SleL LysM domains does not affect the ability of the derivatives to associate equally with *B. subtilis cwlD*$^-$ PG lacking muramic-δ-lactam. However, substantially less $SleL_{2-429}$ and $SleL_{51-429}$ bind cortical PG lacking the recognition determinant as compared to wild-type PG. Conversely, when both SleL LysM domains are removed, more $SleL_{99-429}$ bind the muramic-δ-lactam deficient cortical PG that binds the wild-type PG.

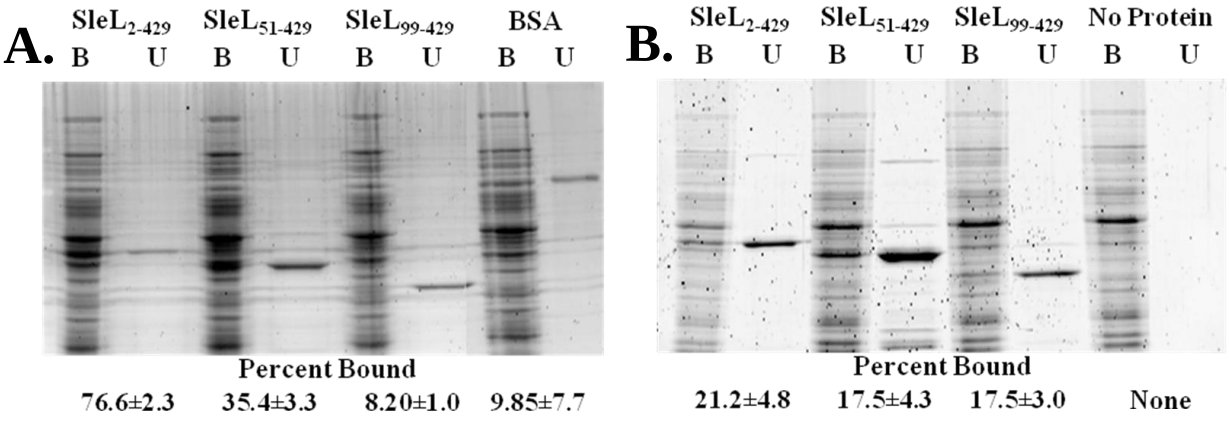

Figure 4.6: SleL peptidoglycan binding assay. Purified, extracted wild-type (A.) or *cwlD*$^-$ (B.) *B. subtilis* cortical PG was combine with 5 μg of $SleL_{2-429}$, $SleL_{51-429}$, or $SleL_{99-429}$. Proteins bound (B) to the cortical PG were separated from unbound (U) protein and analyzed by SDS-PAGE and Sypro Ruby staining to detect proteins. Bovine serum albumin (BSA) was used as a negative control.

LysM domains are involved in directing protein localization. A second predicted function of SleL's LysM domains it to direct the protein to the developing forespore during sporulation. Therefore, sporangia samples were collected from wild-type and complemented *B. anthracis* strains throughout sporulation. Whole sporangia and forespore-associated proteins were separated and analyzed by western blotting using the anti-SleL antibody (Figure 4.7). Full-length SleL from wild-type or DPBa96 ($SleL_{2-429}$) spores is first identified at T_{1-3} of sporulation. The protein appears in the forespore fraction as early as T_5. Each full-length protein in found in purified spores. Both $SleL_{51-429}$ and $SleL_{99-429}$ are detectible by T_3 but the amount of proteins

gradually decreases and is barely detectable by T_7. Neither derivative is found in purified forespores or free spores.

Mature wild-type and DPBa96 ($SleL_{2\text{-}429}$) spores were also fractionated in an effort to characterize the final location of SleL. Upon physically breaking spores and detection of SleL by western blotting using the anti-SleL antibody, ~10% of SleL was associated with the soluble exudate fractions of each strain. After lysozyme digestion ~40% of the spores wild-type or $SleL_{2\text{-}429}$ cache is associated with the solubilized cortex. However, the remaining 50% of the protein remained associated with the insoluble coat fraction (Figure 4.7).

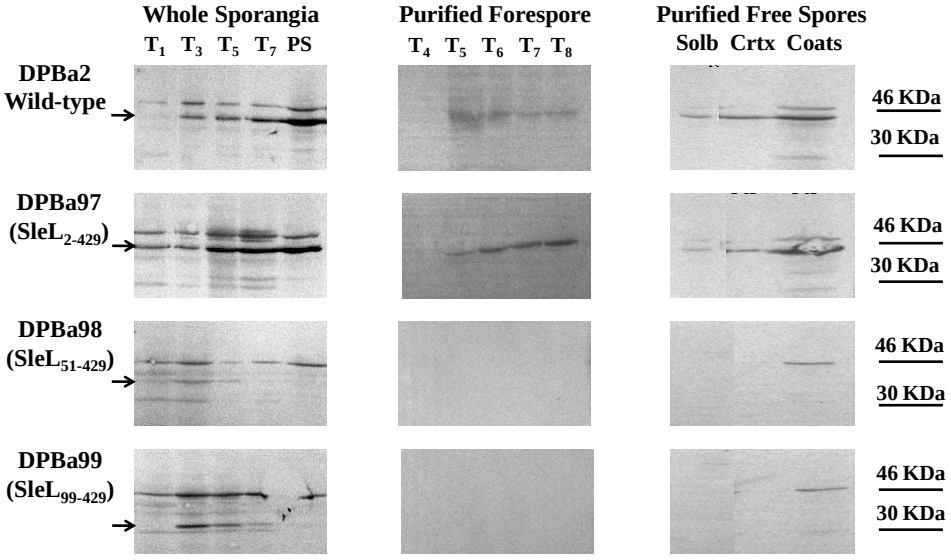

Figure 4.7: SleL's LysM domains are required for protein localization. Samples of wild-type and complemented *B. anthracis* strains were collected throughout sporulation. Whole sporangia, purified forespores, and purified free spores were evaluated for the presence of SleL proteins. Arrows indicate the position where SleL derivatives should be present. Values on the far right are representative of molecular weight markers. PS: purified spores, Solb: soluble, Crtx: cortex, KDa: kilodalton.

Finally, the location of SleL after treatment with nutrient and non-nutrient germinants was assessed (data not shown). Wild-type, DPBa35 (Δ*sleL*), DPBa74 (Δ*cwlJ1* Δ*cwlJ2*) and

DPBa85 (Δ*cwlJ1* Δ*cwlJ2* Δ*sleB*) spores were germinated and then pellet and exudate samples were evaluated for the presence of SleL. When germination was triggered with L-alanine and inosine only 2-5% of SleL was released from wild-type, DPBa74 and DPBa85 spores. However, when spores were triggered to germinate using Ca^{2+}DPA there was no SleL released from the spores. Spores that were physically disrupted, digested with lysozyme, and then treated with Ca^{2+}-DPA did not release any SleL from the coat proteins.

DISCUSSION

The *B. anthracis* SleL protein which is thought to be an *N*-acetylglucosaminidase involved in spore cortex hydrolysis (47) was characterized *in vitro* and *in vivo* in order to substantiate its enzymatic activity, characterize its substrate preference, and evaluate the role(s) of its LysM domains in PG binding and/or protein localization. Purified *B. anthracis* SleL is incapable of digesting intact cortical PG in the form of wild-type decoated *B. anthracis* spores or purified *B. subtilis* sacculi. However, all three SleL derivatives are capable of digesting *B. subtilis* cortical PG that has been partially hydrolyzed by SleB or lysozyme. The resulting muropeptides, G6 and G7, have been shown to be *N*-acetylglucosaminidase products (18, 47). This supports previously published data that suggests the protein is a CFLE that recognizes partially degraded cortical PG as a substrate (11).

During *in vivo* germination of DPBa83 (*ΔcwlJ1 ΔcwlJ2 ΔsleL*) spores SleB plays a dominant role in initiating cortex degradation thus producing an identifible substrate for SleL. We suspect that the anhydro-muropeptides that result from SleB lytic transglycosidase activity (34) are further digested by SleL *in vitro*. The novel muropeptides that are produced are likely anhydro-tetra or anhydro-trisaccharides that result when SleL cleaves SleB products between NAG and muramic-δ-lactam. Digestion of large SleB products results in high amounts of G6 and G7.

LysM domains have been identified in more than 4000 prokaryotic and eukaryotic proteins. Recognizing and non-covalently binding N-acetyl-glucosamine moieties of cell wall PG is one characteristic of the LysM domains (8). Proteins containing LysM domains have been shown to favor an optimum number of the domains for maximum PG binding and biological function. When LysM domains are added or removed by manipulating the genetic sequence of a

bacterium, protein binding and activity decreases (92, 93). For these reasons we chose to evalute the defects resulting from loss of LysM domains. Loss of one or both LysM domains decreases the rate of PG hydrolysis as compared to $SleL_{2-429}$. This difference indicates that LysM domains are less likely to be directly involved in cortex hydrolysis. Instead, the difference in digestion is more likely an indirect affect resulting from a decrease in the ability of the protein derivatives to bind the substrate efficently. In bacterial hydrolases, the LysM domain may be required to properly position the active site of the catalytic domain towards it substrate (93). Thus in the absence of one or both LysM domains digestion could still occur but would be dependent on substrate binding by the enzymatic active site.

GSLEs are thought to recognize muramic-δ-lactam residues that are found in cortical PG but are absent in germ cell wall PG. This allows the enzymes to selectively digest the cortex without compromising the integity of the germ cell wall. Our data suggests that when SleL derivatives are incubated with purified spore PG that lacks the muramic-δ-lactam recognition determinant the proteins are able to bind the PG but unlike they bind wild-type PG. This variation is independent of the presence of SleL LysM domains suggesting that binding may be the result of another region of the protein. The $SleL_{99-429}$ derivative may be able to bind the muramic-δ-lactam-deficient PG better because there is less steric henrince in the absence of the recognition determinant allowing the protein to interact with the substrate. Although SleL proteins are unable to bind muramic-δ-lactam-deficent PG they are unable to digest the substrate.

LysM domains are involved in spore development. When the signal sequence of β-lactamase was replaced with two LysM domains from the *B. subtilis* SleL homolog, YaaH, the fusion protein was detectable by t_2 of sporulation, and it localized to the surface of the developing forespore (43). Fusion proteins lacking the YaaH LysM domains but under the

control of the *yaaH* promoter were also detectable from t_2, but the protein was gradually reduced from t_5 and was undetectible in protein preparations of purified spores. For these reasons LysM domains have been identified as forespore localization signals (8). Like YaaH LysM domains, loss of *B. anthracis* SleL LysM domains adversely effects protein localization. Wild-type and DPBa96 spores containing $SleL_{2\text{-}429}$ have detectable levels of the protein in whole sporangia extracts. The protein is evident in forespore fractions at t_5. The native protein is most likely expressed in the mother cell and then directed to the developing forspore. We suspect that without both LysM domains the $SleL_{51\text{-}429}$ and $SleL_{99\text{-}429}$ proteins are expressed in the mother cell but are probably degraded since the derivatives disappear and never localize to the forespore. Degradation could be the result of an improperly folded protein being more suseptible to proteases, or if the protein cannot localize because it cannot interact with its substrates when its LysM domains are removed, the cell will eventually degrade it and recycle the amino acids.

Another spore-associated protein that contains LysM domains is SafA. SafA is targeted to the developing forespore where it interacts with SpoVID at the cortex-coat interface via a protein-protein interaction. The two proteins promote attachment of the spore coat to the cortical PG and likely interact with other coat proteins (15). Previous studies indicate that in *B. subtilis*, like SafA and SpoVID, SleL and YaaH are localized towards the periphery of the spore (11). In *B. anthracis*, when SleL localizes to the spore it is associated with both the cortex and coat fractions of disrupted purified spores. The LysM domains of SleL may be associating with the cortical PG while other regions of the lytic enzyme are involved in protein-protein interactions in the coats of the spore. This localization pattern would require SleL to transverse the outer forespore membrane. Since SleL does not contain a signal sequence it is unclear how the protein crosses the membrane. However, other *N*-acetylglucosaminidases and PG anchoring proteins

including *B. thuringiensis* Mgb, *B. subtilis* CwlC, *B. licheniformis* CwlM and *B. halodurans* Endo-BH, BL and LP all lack signal sequences but are transported across the parent cell membrane (92).

The data presented here have given us insights into how SleL is assembled into a spore, and how it actively degrades cortical peptidoglycan during germination. Further analysis of SleL localization and possible protein-protein interaction studies may reveal why the protein is associated with not only the cortex but also the coat proteins. This information will be useful in designing methods to externally activate GSLEs in an effort to initiate germination. This strategy will be helpful in developing simpler spore decontamination methods.

ACKNOWLEDGEMENTS

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Chapter 5:

Final Discussion

In recent years *B. anthracis* has been used as a biological weapon. In 2001 a number of letters containing *B. anthracis* spores were sent through the United States Postal Service to several media and government agencies. Carriers and recipients of the letters contracted anthrax, and some individuals died. Decontaminating post offices and other buildings was a long process that required the use of harsh chemicals. The procedure was not only time consuming but also very expensive. For these reasons, research has been focused on the development of modified decontamination methods that are not only less expensive and faster but that also eradicate a higher percentage of spores in the environment.

The purpose if this research was to evaluate spore germination in an effort to deduce a method for enhanced *B. anthracis* decontamination. The focus of the study was directed toward germination-specific cortex-lytic enzymes that are involved in breaking down the protective layer of spore cortex peptidoglycan. One protein in particular, SleL, was thoroughly characterized. The activity of this protein was also studied in the context of its cooperative lytic enzymes; CwlJ1, CwlJ2, and SleB. The rationale behind this investigation centers around the fact that vegetative cells are much more suseptible to eradication methods than spores. Therefore, by jump starting germination it may be easier to clean up contaminated sites. Because spore-forming bacteria cause not only anthrax but other illnesses, the information from this study will be relevent to understanding germination in not only *B. anthracis* but other *Bacilli* and *Clostridia*.

Chapter 2 describes genetic analysis of the *sleL* gene. This gene had only been vaguely studied in *B. anthracis* prior to this research (6, 51). We found that *sleL* is highly expressed at $t_{2\text{-}3}$ of sporulation, and the gene is under the control of σ^E, likely in the mother cell. The loss of *sleL* did not affect vegetative growth, spore viability, or the initial stages of germination. However,

later stages of germination including cortex degradation were affected by the loss of SleL. Athough there was not a dramatic difference in the loss of optical density during germination as compare to wild-type *B. anthracis* there was a striking variation in the release of muropeptide fragments from the spore. A large amount of cortical PG components were retained in *sleL* mutant *B. anthracis* spores suggesting that only the SCLEs were active. Dowd et al (18) had previously reported that the major enzymatic activity responsible for the release of muropeptide fragments from *B. anthracis* spores was that of an *N*-acetylglucosaminidase. Our study revealed that *sleL* encodes the protein responsible for this lytic activity, and the enzyme is likely a CFLE. In the absence of SleL, spores were capable of completing germination which suggests that the SCLEs CwlJ1, CwlJ2 and SleB hydrolyze the cortex sufficently to allow full rehydration of the spore core, allowing for the resumption of metabolism.

To futher understand the cooperation between the four *B. anthracis* GSLEs, the second objective of the study was to compile a library of multideletion strains lacking one, two, three, or all four cortex lytic enzymes. Chapter 3 presents data showing that each lytic enzyme plays a unique role in the digestion of the cortical PG. SleB had been shown to be a lytic transglycosylase (35), and our data support not only the predicted enzymatic activity but also indicate that the SCLE plays a dominant role in initiating the break down of the cortex. The majority of the muropeptide fragments that result from SleB digestion are retained within the spore, but some are small enough to be released without the help of SleL.

Determining the enzymatic activity of the other SCLEs, CwlJ1 and CwlJ2, has proven problematic. Our data suggests that the enzymes may be either muramidases or enzymes that cleave very infrequently. In any case, the proteins likely have similar if not the same enzymatic activity. Both are required for a full response to Ca^{2+}DPA-triggered germination. Despite the

similarities between CwlJ1 and CwlJ2 the proteins are not identical. This is evident in the fact that CwlJ1 can independently hydrolyze the cortex PG sufficently to allow for spore outgrowth while CwlJ2 cannot. This difference may be because of variations in the expression levels of the two genes (35).

We predict that, based on the suggested localization of CwlJ1, CwlJ2, and SleB and the fact that the CwlJ proteins respond to Ca^{2+}DPA, CwlJ1 and CwlJ2 may initiate cortex degradation from the more highly cross-linked outer layers of the cortical PG while SleB cleaves from the inner layers. Once the cortex in loosened, SleL is then able to futher degrade the PG resulting in a very efficent and fast germination response. The loss of multiple GSLEs has an additive inhibitory effect on the germination response.

Our final study of SleL involved analyzing the protein *in vitro* and *in vivo* in an effort to not only demonstrate the enzymatic activity of the pure protein but also to evaluate the roles of the LysM domains. We were able to show that purified SleL derivatives do have *N*-acetylglucosaminidase activity that only recognizes fragmented cortical PG. We demonstrated that SleL can act on SleB digestion products resulting in previously identified (18, 35) and novel muropeptides. The data in Chapter 4 indicates that SleL requires both LysM domains to efficently bind its substrate which would probably allows for the most efficent PG digestion. The LysM domains likely orient the protein properly so that the catalytic glycosyl hydrolase family 18 domain can cleave the bond between NAG and muramic-δ-lactam (47). However, the LysM domains do not appear to be required for binding to muramic-δ-lactam-deficent PG. We predict that the active site of the SleL protein is responsible for identifying this substrate.

We hope that the results from the studies presented here will promote an effort to develop more effective decontamination method. Future research will involve a closer look at each

GSLE. SleB and CwlJ1 will be randomly mutagenized in order to determine specific regions of the proteins that are involved in substrate binding and enzymatic digestion. Studies are also underway to determine other factors that may be involved in keeping CwlJ1 and SleB inactive in the dormant spores. Analysis of GerQ, YpeB and YlaJ may help us understand how the GSLEs are properly localized. Using fluorescent microscopic experiments using labeled GSLEs will shed light on the actual location of each enzyme in the spore. This information will be helpful in further understanding the intriguing process of germination.

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