Analysis of Peptidoglycan Structural Changes and Cortex Lytic Enzymes during Germination of *Bacillus anthracis* Spores

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ABSTRACT

Sporulation is a process of differentiation that allows capable cells to go into a dormant and resistant stage of life. To become active again, the spores must germinate into vegetative cells. One key process in spore germination is hydrolysis of the cortex peptidoglycan. This process has been studied in a variety of sporulating species; however, it has not been studied in *Bacillus anthracis*. A clear understanding of cortex degradation may provide information that will allow for better cleanup of spore-contaminated sites.

The structure of cortex peptidoglycan of *Bacillus anthracis* was characterized. The peptidoglycan of the dormant spores was extracted, digested with Mutanolysin, and analyzed using HPLC to determine the structure. The analyses revealed that the cortex peptidoglycan of *B. anthracis* was very similar to other *Bacillus sp.*. Spores were stimulated to germinate and cortex peptidoglycan was extracted and analyzed at various times. *Bacillus anthracis* appeared to hydrolyze its cortex more rapidly than other *Bacillus* species. While the spores of three species release the spore solute dipicolinic acid and resume metabolism at similar rates, the *B. anthracis* spores released 75% their cortex material within 10 minutes while the other species released only 20% in the same

time frame. This suggests that the *B. anthracis* spore coats are more permeable to cortex fragments than those of the other species, or that *B. anthracis* rapidly cleaves the cortex into smaller fragments. Novel cortex fragments analyzed during *B. anthracis* germination were produced by a glucosaminidase; however, additional studies need to be performed for confirmation.

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CHAPTER ONE

Review of the Literature and Research Introduction

Sporulation

Sporulation is a process of differentiation that allows capable cells to go into a dormant and very resistant state of life for what can be extended periods of time. Not all bacteria are able to do this, but two known genera that are capable of sporulation are *Bacillus* and *Clostridium*, both Gram-positive bacteria. The sporulation process is triggered when there is a lack of nutrients present in the environment. This results in the activation of a cascade of genes that will cause the cell to differentiate into two very different compartments, the forespore and the mother cell (30). The cell replicates its entire genome as it would during normal division. However, during sporulation, asymmetric division occurs to produce the two different compartments. The larger compartment, or the mother cell, will engulf the forespore, the smaller compartment, resulting in a free forespore, within the mother cell, surrounded by two separate membranes. The peptidoglycan is then synthesized between these two membranes. The next stages in sporulation are involved in the production of the protein coat layers of the forespore, by the mother cell (26). Additionally, *Bacillus anthracis* has an exosporium, containing collagen-like proteins, outside of the coat layers (35). After it has finished contributing to the production of the spore, the mother cell will lyse and the dormant spore will be released into the environment (30). This free spore, which is metabolically inactive, may lay dormant for years.

Spore Structure and Resistance Properties

The structure of the spore (Figure 1) has similarities to the structure of a vegetative cell; however, there are some slight but very important differences. Unlike a vegetative bacterium, the spore is highly resistant to UV radiation, heat, and a variety of toxic chemicals. The interior of the spore is called the core. It is very dehydrated and is highly concentrated with calcium dipicolinic acid (Ca^{++}-DPA). Additionally, the dehydrated core protects proteins within the cell from becoming denatured while small acid-soluble proteins (SASPs) protect the DNA from damage (13). Surrounding the core is the inner forespore membrane (IFM), followed by the germ cell wall (GCW) and the cortex. The GCW and the cortex are composed of peptidoglycan. These layers are important in maintaining the dehydrated state within the spore. Outside of the cortex is the outer forespore membrane (OFM), followed by a number of spore coats. The spore coat layers, which vary in number between species, provide the spores with protection against damage by harsh chemicals and exclude large molecules, such as harmful enzymes. Lastly, surrounding the coat is the exosporium, whose exact function is still unknown (35).

Peptidoglycan Structure

Peptidoglycan is the molecule that comprises both the germ cell wall and the cortex of the spore, and the cell wall of the vegetative cell (30, 36, 37). Peptidoglycan consists of long glycan strands with repeating disaccharide residues of N-acetyl glucosamine (NAG) and N-acetylmuramic acid (NAM) that are cross-linked by peptide side chains located on the NAM residues (30, 36, 37). In *Bacillus cereus*, a bacterium

Figure 1. Layers of the Spore

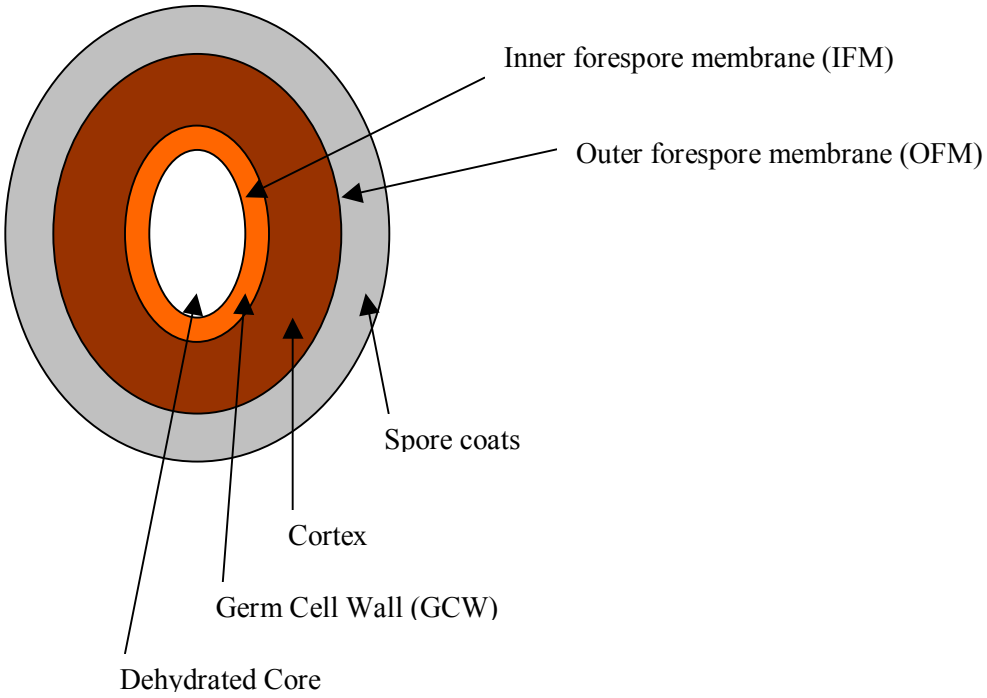


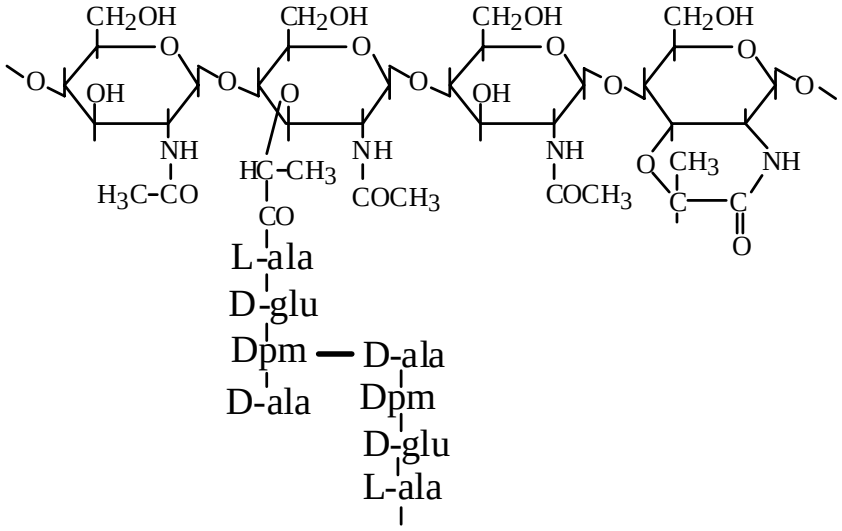
Figure 1. Layers of the Spore. The various layers of the spore provide different levels of protection for the spore while in its dormant state. The spore is able to resist chemicals, UV radiation, and desiccation, along with a variety of other environmental stressors.

closely related to *B. anthracis*, there are some slight changes to the PG backbone structure. Atrih et al in 2001 studied the structure of *B. cereus* spore PG and compared it to other *Bacillus sp*. They found some novel peaks in the HPLC results and did further analysis through mass spectrometry. They determined that these peaks corresponded to fragments, similar in saccharide and peptide length to other known fragments, but that contained de-acetylated amino sugar residues (2). In *Bacillus subtilis*, the peptide side chains, as they extend from the NAM subunit, are L-alanine, D-glutamate, meso-diaminopimelic acid (Dpm), and D-alanine (12, 32). The cross-linking occurs between the meso-diaminopimelic acid of a peptide on one strand and the D-alanine of a tetrapeptide on an adjacent strand (12, 32, Fig. 2). The size of the side chains can vary; some are dipeptides or tripeptides, which therefore are not involved in cross-linking (32). Overall, only 29-33% of the peptide side chains are cross-linked (37). While these characteristics are true of vegetative PG and germ cell wall PG, there are some significant differences in the PG of the spore cortex.

The spore PG consists of two different layers: the germ cell wall and the cortex. (12). The germ cell wall is the inner layer (10-20% of the PG) and has a structure similar to the cell wall of a vegetative cell (12, 30). Most of the peptide side chains are tripeptides and tetrapeptides (5, 14, 20). This layer is used to produce the cell wall of the vegetative cell during spore outgrowth (5). The second layer is the cortex and is much thicker, comprising the other 80-90% of the spore PG (20). The cortex has some chemical modifications when compared to the vegetative PG. One modification is the shortening or complete removal of peptide side chains. Approximately 50% of the

Figure 2. Peptidoglycan Structure of Vegetative Cells and Spores.

a.) Cortex Peptidoglycan



b.) Vegetative Peptidoglycan

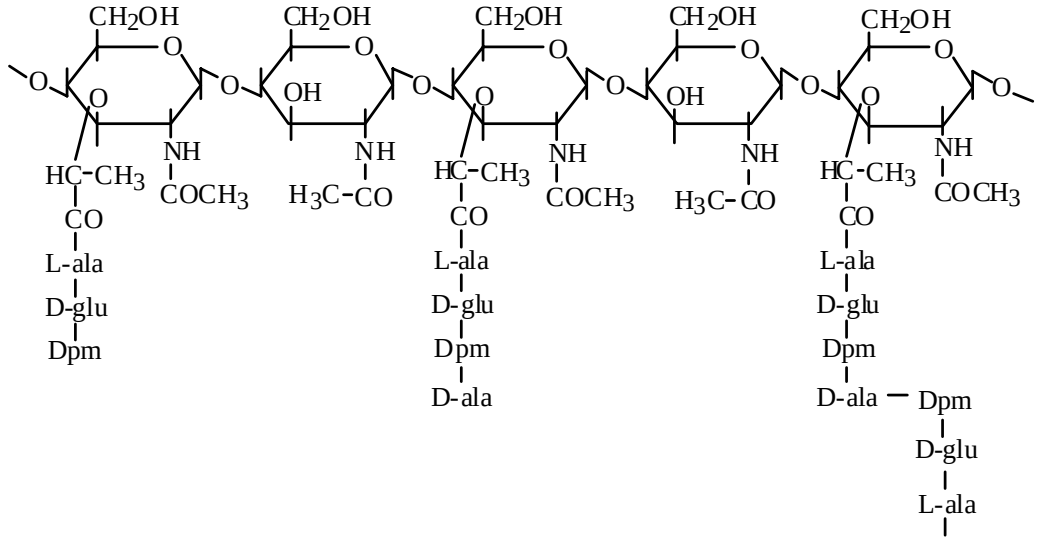


Figure 2. Peptidoglycan Structure of Vegetative Cells and Spores. The differences between the structures of the peptidoglycan during the vegetative and spore stages are vital to the life cycles. The transformation of the N-acetylmuramic acid into muramic δ-lactam, decreases the cross-linking between the peptidoglycan and allows the spore PG to be the substrate for hydrolysis during the germination of the spore.

muramic acid residues (occurs every alternate disaccharide) have their peptide side chains completely removed, and in part by the activities of the CwlD and PdaA proteins (14), these NAM residues are converted to muramic-δ-lactam (5, 13, 30, 32, 36). Some cortical modifications occur in a variety of *Bacillus* species where ~25% of the muramic acid residues are modified to single L-alanine residues and another 25% remain as tetrapeptides (4, 19, 32, 36, Fig. 2). In *Clostridium perfringens,* none of the side chains are shortened to single L-alanine, however, about ~25% are modified to L-alanine-D-glutamic acid dipeptides (19). Both the shortening and removal of peptide side chains cause a decrease in cross-linking between the PG strands (4, 14, 30, 32). The cortex PG has the lowest percentage of cross-linking of all PG, at an average cross-linking of only 3-4% (12, 14). The decrease in cross-links and the production of muramic-δ-lactam are critical to the activity of the lytic enzymes during germination (19). It is because of these differences that, during germination, the cortex is broken down, but the GCW remains intact to be used in the vegetative cell after germination (5, 30). CwlD mutant spores lack muramic-lactam in their cortex, and if a germinant is introduced, these spores are unable to degrade their cortex and cannot completely germinate (4, 14).

Spore Germination

Germination is the process of transitioning from the spore to an active vegetative cell (19). There are many details within the germination process that are still uncertain. For a spore to begin germination, a "germinant" is introduced into the environment (22). Some known nutrient germinants are amino acids such as L-alanine or methionine, and purines such as inosine or adenosine (16), and even non-nutrient

molecules such as DPA (22, 29). These germinants initiate germination by binding to receptors, in the Ger protein family, integrated in the inner forespore membrane (22). There are six tricistronic operons known to encode for Ger sensor proteins in the chromosome of *Bacillus anthracis* (16) and one *ger* operon on the pXO1 plasmid (15). Some of these different receptors are known to bind to different types of germinants and the creation of bacteria that are mutant in these receptors often alters the ability to germinate in the presence of a certain single or combinations of germinants (16, 38). The binding of germinants to one or several of these receptors triggers the two stages of germination to occur (Fig. 3).

In the first stage of germination, the changes within the spore are biophysical (23). The spore becomes partially rehydrated as water enters the spore core, potassium and sodium ions are redistributed, and the Ca^{++}-DPA that was in the core is released into the environment (22). The current theory on the cause of these changes is that the binding of the germinants to the receptors causes an alteration in the permeability of the inner forespore membrane (34). The spore, however, is still limited in its functioning and is not metabolically active (10, 33). The thick spore cortex is still present and prevents full expansion of the spore. To complete the germination process, the spores must go through the second stage, which is more biochemical in nature (22). When the germinant binds, it also triggers the activation of cortex lytic enzymes (22). Many of these enzymes are present in the dormant spore in their mature state and are localized on the edge of the cortex, before the outer forespore membrane (19) and become enzymatically active when a germinant has bound (22). The activated lytic enzymes are

Figure 3. Steps of Germination

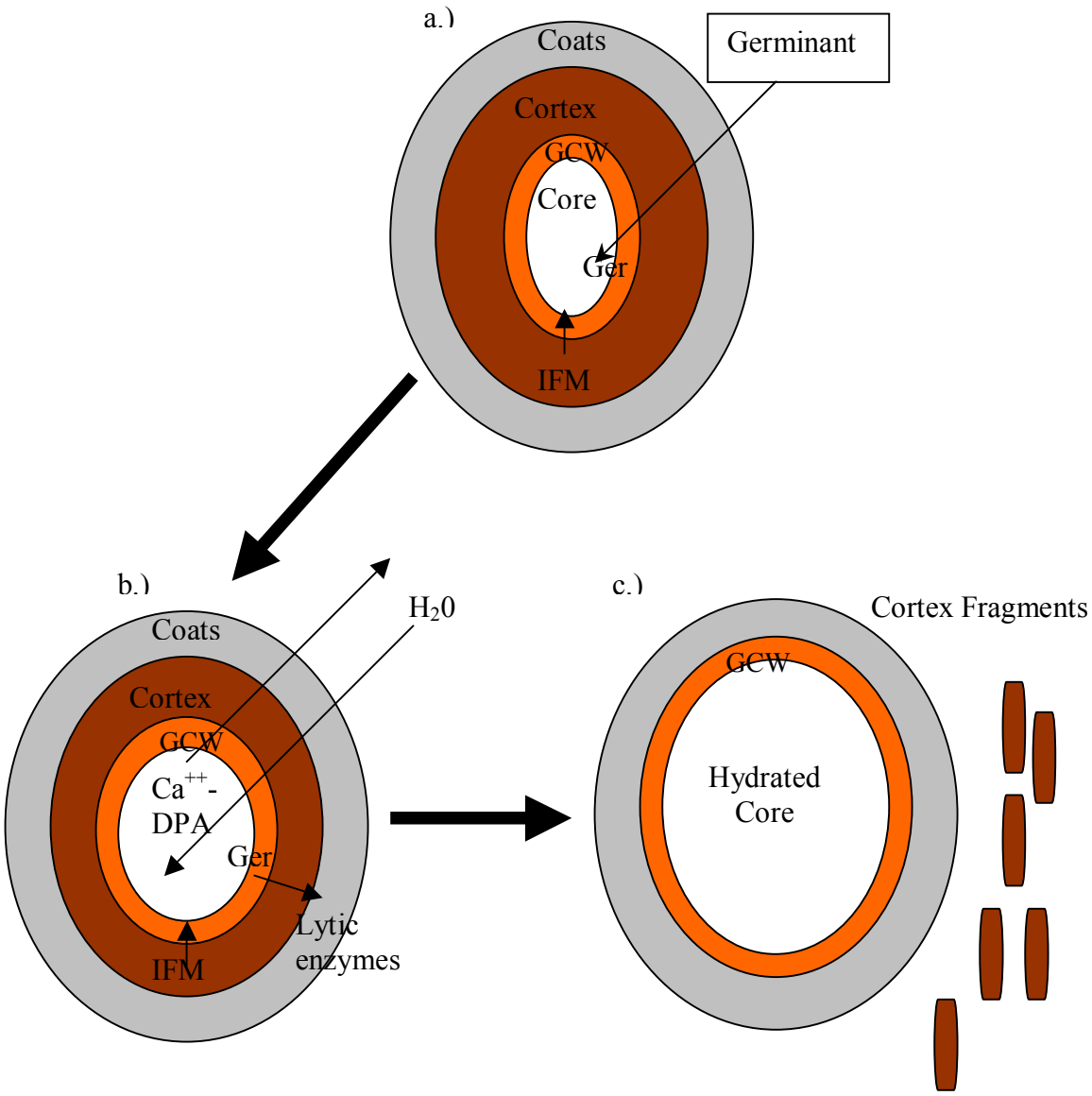


Figure 3. Steps of Germination. (a) Germination occurs when germinants pass through the outer layers of the spore and bind to the receptors, Ger, on the IFM. This triggers the initiation of germination. (b) Water begins to rush into the cell and Ca^{++}-begins to leave the cell. The binding of the germinant will also trigger the activation of lytic enzymes. (c) These lytic enzymes will break down the cortex peptidoglycan and allow for complete hydration of the cell, full metabolic activity, and the release of the cortex fragments.

responsible for the hydrolysis of the spore cortex. The degradation of the PG causes release of muropeptides or cortex fragments to be released into the environment (5); however, the germ cell wall is left intact and is used in cell outgrowth (5). As the cortex is degraded, the cell expands to its full size and regains full metabolic activity (22).

Activity of Different Lytic Enzyme Classes

When the lytic enzymes degrade the cortex, specific types of enzymes will act on certain parts of the PG and will cause various types of muropeptides to be released (19). By characterizing the products created during germination, the type of enzymes that were active in the hydrolysis of the cortex can be deduced.

Endopeptidases of class A cleave the cross-link bond between the two glycan strands, between the D-alanine and the meso-diaminopimelic acid (12). Endopeptidases of class B can cleave within a peptide side chain, between the L-alanine and the D-glutamic acid or between the D-glutamic acid and the meso-diaminopimelic acid (12). Glucosaminidase acts on the bond after the NAG within the backbone of the PG (5). The lytic transglycosylase cuts between the NAM and NAG subunits and produces anhydromuramic acid (5). Amidase has the ability to cleave the peptide side chains off of the glycan strands; it cuts the bond between the NAM and the L-alanine (20, 22). Additionally, it is thought that an epimerase enzyme might also be active during germination. Epimerase has the ability to modify muropeptides, presumably in the muramic-δ-lactam residue, and produce an uncharacterized structure (5). Figure 4 and Figure 5 give pictorial representations of these different enzymatic activities.

In previous studies, if the PG of dormant spores is digested in vitro with a muramidase, the main products found are a tetrasaccharide-tetrapeptide and a tetrasaccharide-alanine (4, 32). Muramidase attacks the bond between the NAM and NAG within the backbone of the peptidoglycan (4, 32, 36, Fig. 4, Fig. 5). However, if the PG of germinating spores is digested with a muramidase, some of these same products will be found along with additional muropeptides resulting from the activity of various cortex lytic enzymes.

Identified Cortex Lytic Enzymes

Cortex lytic enzymes have been studied in several bacterial species. For example, in *Bacillus subtilis*, SleB is a cortex lytic enzyme triggered by L-alanine initiated germination (24). The exact activity of SleB is uncertain, however, it is believed to act as an amidase or a lytic transglycosylase (3, 25). Homologs of SleB exist in *B. cereus* and *B. anthracis* along with some *Clostridium* species (18). CwlJ is another cortex lytic enzyme with its activation tied to the presence of Ca^{++}-DPA, either released from the spore's core or from introduction into the environment (25). The effect that CwlJ has on cortex hydrolysis is unknown, however, it is required for normal germination timing (9). The SleL protein has been found in *B. cereus* and is thought to act as a glucosaminidase or an epimerase (7, 9). Homologs of SleL have been found in other species and in *B. subtilis* this protein is called YaaH (7, 9). Several other lytic enzymes have been identified in *Clostridium perfringens*. *sleC* encodes for a protein with a hypothesized amidase activity and *sleM* encodes for a possible muramidase, however, no homologs of these genes have been found in *Bacillus* species (8, 21).

Figure 4. Spore Peptidoglycan Structure and Location of Enzymatic Activity

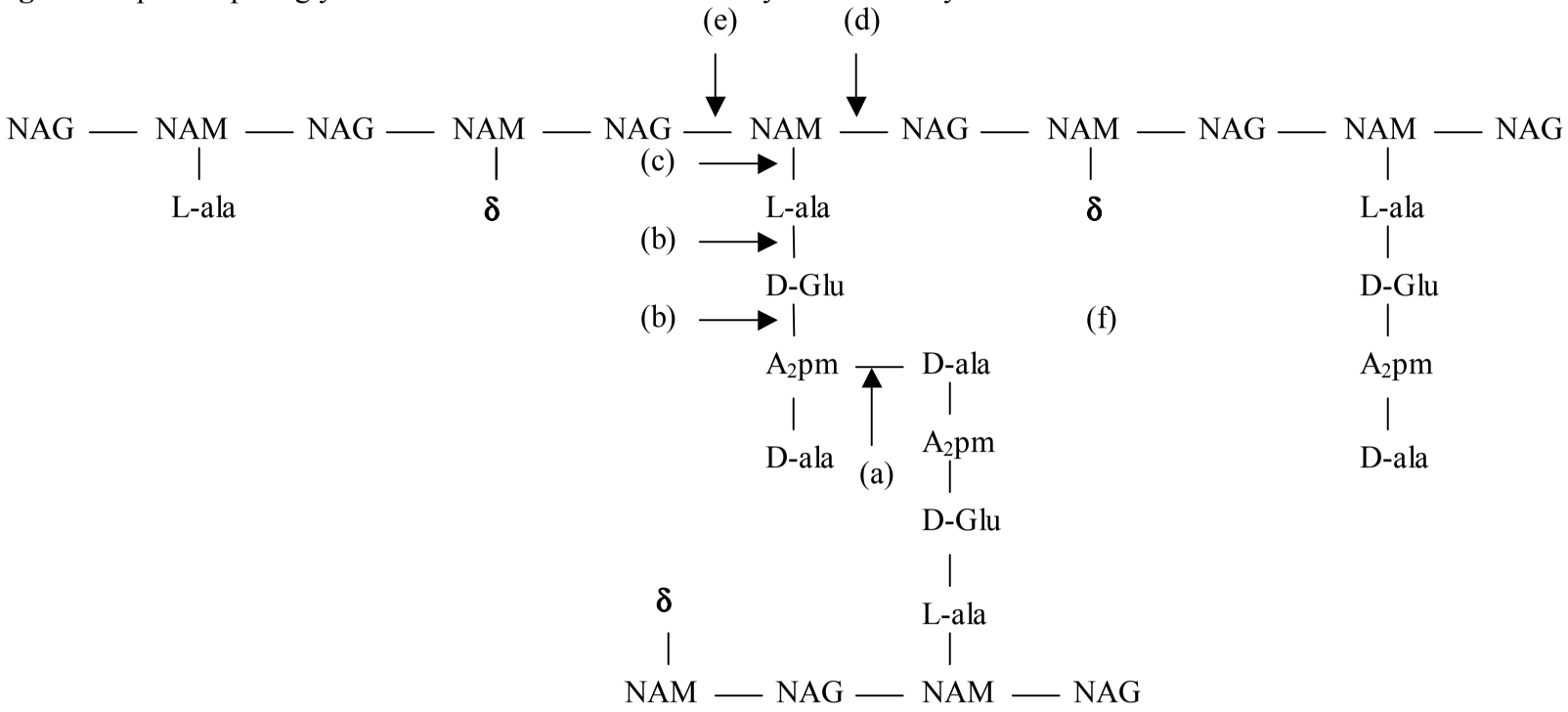


Figure 4. Spore Peptidoglycan Structure and Location of Enzymatic Activity. The spore peptidoglycan differs from the vegetative peptidoglycan. During germination (of *Bacillus subtilis*), various enzymes break down the cortex. These enzymes each attack various bonds with in the PG and an example is noted on the figure above: (a) Endopeptidase A, (b) Endopeptidase B, (c) amidase, (d) lytic transglycosylase, (e) Glucosaminidase, and (f) epimerase. (Abbreviations: NAM- N-acetylmuramic acid, NAG- N-acetylglucosamine, L-ala- L-alanine, D-Glu- D-glutamic acid, A_2pm- meso-diaminopimelic acid, D-ala- D-alanine, δ- muramic δ-lactam)

Figure 5. Hypothetical Muropeptide Formation

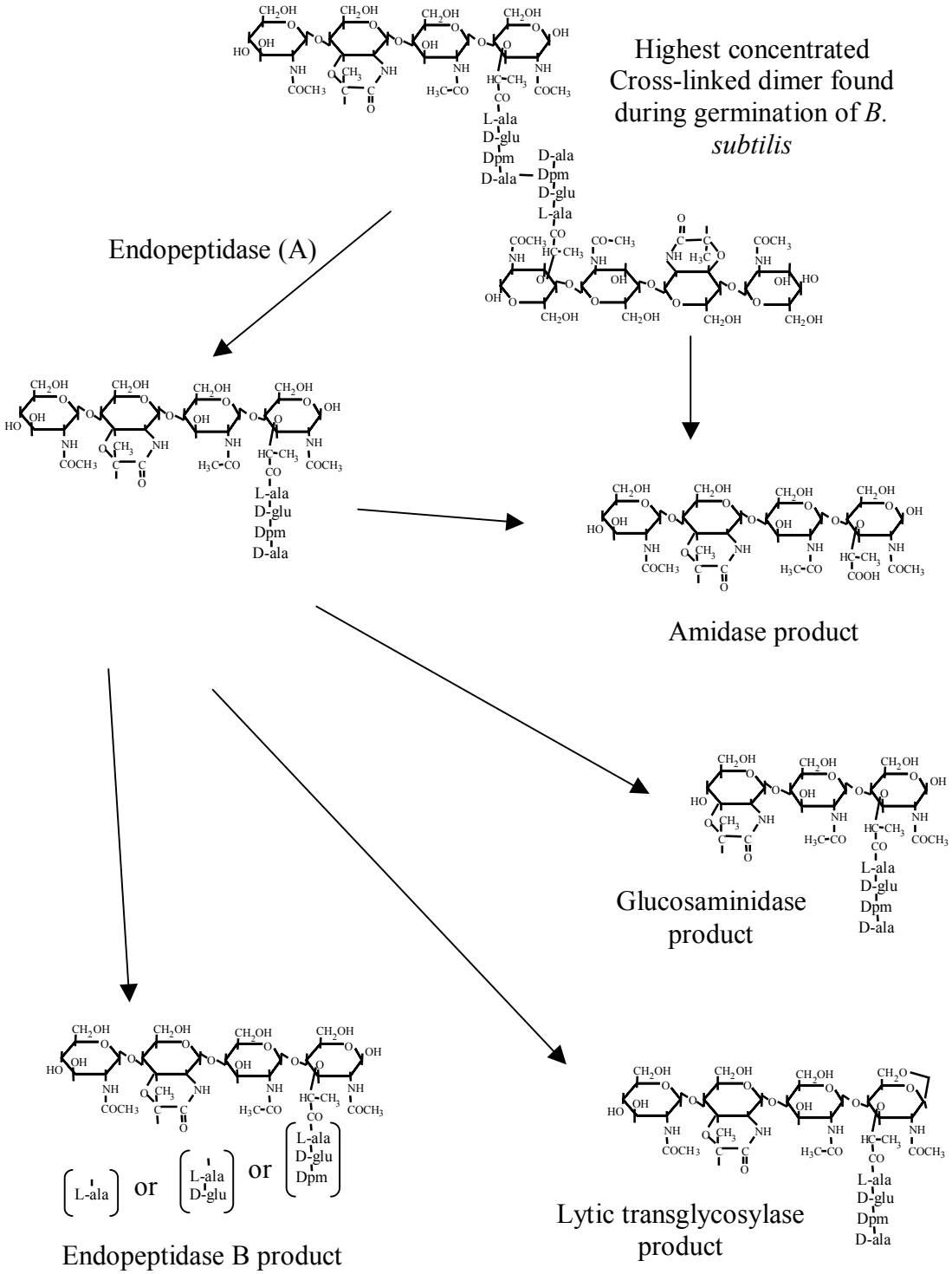


Figure 5. Hypothetical Muropeptide Formation. Purified spore peptidoglycan when digested with a muramidase, reveals two main products, a monomer, and a cross-linked dimer. The products from some possible enzymes active during germination are shown.

There are two other proteins in *Bacillus subtilis* known to have a significant effect on the activity of the CwlJ and SleB proteins. Without GerQ and YpeB, CwlJ and SleB are produced, however, they are not incorporated into the dormant spore, which causes a loss of activity respectively (9).

Cortex Analysis

The cortex of several different sporulating bacterial species has been previously studied in the dormant spore form, in addition to the hydrolysis that occurs during germination. By the use of high-pressure liquid chromatography, or HPLC, and mass spectrometry, the cortex fragments have been analyzed and identified. These analyses provide the information needed to determine the type of enzymatic activity that is occurring in the cortex. For example, in *Clostridium perfringenes*, muramidase and amidase enzymatic products were found (19); in *Bacillus subtilis*, lytic transglycosylase, glucosaminidase, and muramic-δ-lactam epimerase enzymatic products were found (5); in *Bacillus megaterium*, muramic-δ-lactam epimerase enzymatic products were found (5).

HPLC Peak Identities of *Bacillus subtilis*

Previous studies have been performed where the cortex peptidoglycan of *Bacillus subtilis* of both the dormant and germinated states have been analyzed. The HPLC and mass spectrometry results provide information about the identities of the peaks formed in the chromatograms. Figure 6 shows the typical chromatogram produced when dormant *B. subtilis* spore PG was analyzed, as found by Popham et al. (32). The numbered peaks contain the muropeptides and Table 1 gives their identities,

determined through amino acid analysis and MS (32). Additionally, Atrih et al completed studies of the peptidoglycan structural changes that occurred in *Bacillus subtilis* during germination (5). They analyzed the muropeptides of the dormant, germinated spore associated material, along with muramidase-digested and undigested germination exudate. They found 13 novel peaks that occurred in the germinated samples. The novel peaks, from novel fragments, produced during germination were caused by different lytic enzymes active during germination involved in cortex hydrolysis. Additionally, they identified the structure of these peaks by mass spectrometry. From the analysis of the novel muropeptide structures, in *Bacillus subtilis*, the enzymatic activity was hypothesized to be by a lytic transglycosylase, a glucosaminidase and possibly an epimerase (5).

Research Introduction

The breakdown of the cortex during germination of the spore is essential for its transition to the vegetative state. Lytic enzymes that are responsible for this hydrolysis are key to understanding how this step of germination takes place. While previous studies of the cortex hydrolysis and the germination lytic enzymes have been conducted on various *Bacillus* species, such as *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus cereus*, and various *Clostridium* species, this type of analysis has not been carried out for *Bacillus anthracis*.

While understanding the mechanisms behind germination is important for all sporulating bacteria, it has even more significance in *Bacillus anthracis*. *Bacillus anthracis* is a human opportunistic pathogen. In recent current events, *Bacillus*

Figure 6. Muropeptide separation of Dormant *B. subtilis* spores.

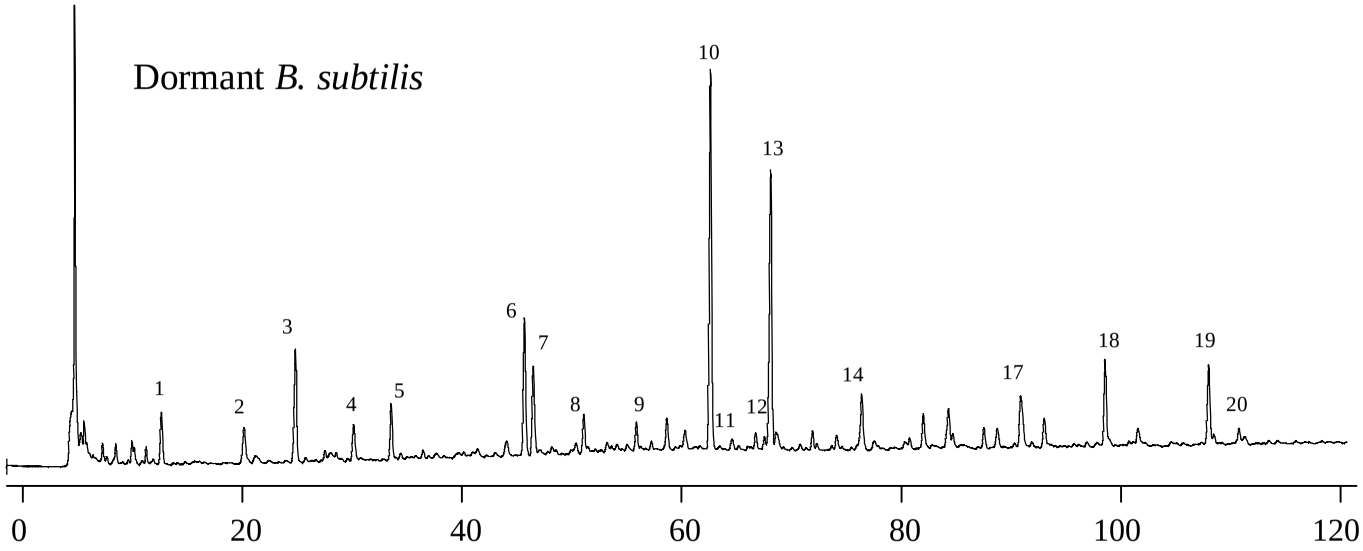


Figure 6. Muropeptide Separation of Dormant *B. subtilis* spores. When the PG of *B. subtilis* spores is separated with RP-HPLC, this chromatogram is produced. The numbers correspond the peaks identified by Popham et al (32). The structures of these peaks were determined by additional analyses.

Table 1. Predicted Structure of Numbered Peaks in *Bacillus subtilis*.

Peak Number	*Predicted Structure in Bacillus subtilis*
1	Disaccharide-tripeptide
2	Disaccharide-Alanine
3	Disaccharide-tetrapeptide
4	Tetrasaccharide-Alanine with open lactam
5	Tetrasaccharide-tetrapeptide with open lactam
6	Tetrasaccharide-tetrapeptide (reduced)
7	Tetrasaccharide-Alanine (reduced)
8	Disaccharide-tripeptide-disaccharide-tetrapeptide
9	Disaccharide-tetrapeptide-disaccharide-tetrapeptide
10	Tetrasaccharide-tetrapeptide
11	Tetrasaccharide-tetrapeptide-tetrapeptide
12	Disaccharide-tetrapeptide-tetrasaccharide-tetrapeptide (reduced)
13	Tetrasaccharide-Alanine
14	Disaccharide-tetrapeptide-tetrasaccharide-tetrapeptide
17	Tetrasaccharide-tetrapeptide-tetrasaccharide-tetrapeptide
18	Hexasaccharide-tetrapeptide
19	Hexasaccharide-Alanine
20	Tetrasaccharide-tetrapeptide-hexasaccharide-tetrapeptide

Table 1. Predicted Structure of Numbered Peaks in *Bacillus subtilis*. The peaks labeled in Figure 6 correspond to the peak numbers listed here. The structure of each peak is listed as found by Popham et al (32) by use of amino acid analysis and mass spectrometry.

anthracis spores have sent through the U.S. postal service, as a form of bio-terrorism, in an attempt to infect people with anthrax. During this event, many buildings were exposed to these spores and needed decontamination. However, due to the dormancy and the resistance properties of the spore, this was a very challenging task. The cortex is responsible for maintaining many of these properties; modification of the cortex causes a loss in both heat resistance and chemical resistance. Additionally, the spore already has mature enzymes that are needed to break down the cortex and that are triggered during germination. Stimulating the spores to germinate would activate these lytic enzymes, cortex hydrolysis would occur, and these spores would then be nearly as susceptible to sterilization methods as vegetative cells. By studying the cortex hydrolysis that occurs during germination and the lytic enzymes that are responsible for this hydrolysis, information can be gained that may be useful in trying to clean up spore-contaminated sites.

Research Objectives

Determine the structure of the cortex peptidoglycan of *Bacillus anthracis* spores through RP-HPLC, mass spectrometry, amino acid analysis, and amino sugar analysis.

Initiate *Bacillus anthracis* spores to germinate at a high efficiency and extract the cortex and cortex fragments.

Determine the lytic enzymatic activity that is occurring during spore germination from analysis of the muropeptides formed throughout cortex hydrolysis, by use of RP-HPLC, mass spectrometry, amino acid and amino sugar analyses.

CHAPTER TWO

Materials and Methods

Bacterial Strain and Growth Medium

All spore samples were grown from *Bacillus anthracis* Sterne Strain 34F2. The bacteria were grown in a modified Schaeffer's (Difco) sporulation medium (26). The following ingredients are used to make a liter of medium: 8g of nutrient broth, 2 ml of 1 M $MgSO_4$, 10.7 ml of 2 M KCl, 500 µL of 1N NaOH, 100 µL of 1 M $MnCl_2$, 3 µL of 0.36 M $FeSO_4$. 250 ml of the medium was put into a 2-liter flask and autoclaved. 20 ml of sterile 50mM $CaNO_2$ was added right before use. The medium was inoculated and grown at 37°C for 3-4 days while shaking, or until approximately 95% of the spores were free from the mother cells, as seen under phase-contrast microscopy. Additionally, *Bacillus subtilis* PS832 and *Bacillus sphaericus* strain 9602 spores were used for the comparative analyses.

Preparation of Clean Spore Samples

The spores were centrifuged at 8,000 rpm for 10 minutes at 4°C (Beckman JA-14). The supernatant was removed and the spores were resuspended in ~200 ml of 0.1% Triton X-100 (Fisher), 0.1 M NaCl. The spores were centrifuged again and these washing steps were repeated several times, until the preps appeared to be ~90% spores when viewed under a phase-contrast microscope. The spores were often stored at 4°C after several washings and were further washed at a later date if needed.

Metrizoic Acid Gradient Spore Purification

Once the spore preps appear to be ~90% clean, they were further cleaned by running them over a metrizoic acid gradient. This helped to remove any vegetative cell debris or mother cell remnants that may have been in the sample but that were not visible under the microscope. The spore preps were centrifuged at 8,000 rpm for 10 minutes at 4°C (Beckman JA-14 rotor), and resuspended in 12 ml of cold, sterile water. 8 ml of 50% metrizoic acid (Sigma) were added and allowed to come to room temperature. 12 ml of 50% metrizoic acid were transferred into each of eight glass Corex tubes. 2.5 ml of the spore suspension were aliquoted on top of the metrizoic acid carefully to maintain the separation between layers. The spore suspensions were centrifuged for 30 minutes at 11,000 rpm at room temperature, with no brake (Beckman JA-20). The supernatant was removed by aspiration, and the pellet was resuspended in 5 ml of cold sterile water. The samples were recombined in a 250 ml centrifuge bottle and washed with cold water 5 times to remove any residual metrizoic acid (8000 rpm, 4°C, 10 minutes). The spores were stored in 0.1% Triton X-100, 0.1 M NaCl with gentle shaking at 4°C until needed for analysis.

Dormant Spore PG Preparation

To determine the structure of the dormant cortex peptidoglycan, the peptidoglycan was extracted and analyzed. For this analysis, 60 optical density (OD) units (Spectronic Genesys 5, A_{600nm}) were removed from the spore sample. The spores were centrifuged (13,000 g for 45 seconds) and resuspended in 1 ml 50mM Tris-HCl

pH 7.5, 1% sodium dodecyl sulfate (SDS) (Sigma), 50 mM dithiothreitol (DTT) (Labscientific, Inc.) and boiled for 20 minutes.

The spores were centrifuged, 13,000 g for 2 minutes, and the supernatant was removed and the pellet was resuspended in water. The pellet was then resuspended in 1 ml of sterile water and these washing steps were repeated three times. The pellet was then resuspended in 5% trichloroacetic acid and heated to 95°C for six minutes. The sample was cooled and centrifuged at 13,000 g for 2 minutes. The supernatant was removed, and the pellet was resuspended in 1 ml of 1 M Tric-HCl pH=8.0. The pellet was centrifuged at 13,000 g for 2 minutes, and the supernatant was removed. This was repeated until all the SDS was removed, generally 4 additional washes with water. The pellet was then resuspended in 20 mM Tris-HCl pH= 8.0, 10 mM $CaCl_2$ and digested with 0.1 mg/ml trypsin (Worthington TRTPCK). The spores were incubated at 37°C for approximately 16 hours.

After the incubation, 50 µl of 20% SDS was added and the samples were boiled for 15 minutes. Centrifugation at 13,000 g for 2 minutes pelleted the spores, and the supernatant was removed. The pellet was resuspended in water and the washing steps were repeated until the SDS is completely removed, usually approximately 7-10 washes. Once the washes were completed, the pellet was resuspended in 250 µl 12.5 mM $NaPO_4$ pH= 5.5 and digested with 125 units of Mutanolysin (Sigma) and incubated at 37°C for 16 hours.

After the Mutanolysin digest, the insoluble material was removed by a 15 minute centrifugation at 13,000 g. The supernatant, containing the muropeptides, was

transferred to a new tube. The supernatant was lyophilized (Savant Speedvac) and stored at –20°C until ready to be analyzed on the HPLC.

Germinating Spore PG Preparation

To study the hydrolysis of the cortex during germination, the spores were initiated to germinate and samples were taken at various time points for analysis. To begin the germination procedure, 200 OD units of the clean spore prep were obtained. The spores were heat activated for 30 minutes at 70°C. This treatment killed any vegetative cells that may have been in the sample, and also activated the spores to germinate synchronously. The spores were put on ice for 10 minutes. The spores were then added to a 125 ml flask with 0.8 ml of 0.5 M $NaPO_4$ pH=7.0, and the volume was brought up to 9 ml with water. The flask was shaken in a 37°C water bath for five minutes, before the germinant was added. The optical density of the spore suspension was taken before the germinant was added to obtain a baseline value for germination analysis. To initiate germination, 1.0 ml of 1 M L-alanine, 10 mM Inosine was added to the spore suspension. The optical density (Spectronic Genesys 5, A600nm) of the suspension was monitored, with readings taken at 1, 4, 10, 14, 20, 29, 45, and 60 minutes. Initially, readings were taken at 0, 1, 5, 10, 14, 20, 30, 44, 60, 90, and 119 minutes, however, the germination experiment was shortened and the reading times were modified after initial results were obtained. Samples for PG extraction were taken at 5, 15, and 30 minutes; they were taken at 15, 45, and 120 minutes before the protocol modification. 3 ml were taken (split between two microfuge tubes) for each PG sample

and immediately centrifuged (13,000 g for 45 seconds), and the exudate was removed and stored at –80°C until further analysis.

The pellet was resuspended in 1 ml 50 mM Tris-HCl pH 7.5, 1% SDS, 50 mM DTT, and boiled for 30 minutes. The samples were centrifuged (13,000 g for 2 minutes) and the supernatant was removed. The pellets were then resuspended in 1 ml of sterile water and these washing steps were repeated three times. The pellet was then resuspended in 5% trichloroacetic acid and heated to 95°C for six minutes. The pellet was cooled and centrifuged at 13,000 g for 2 minutes. The supernatant was removed, and the pellet was resuspended in 1 ml of 1 M Tric-HCl pH=8.0. The samples were centrifuged at 13,000 g for 2 minutes, and the supernatant was removed. The pellets were washed two more times with water before the like samples were combined together. The sample was then washed until the SDS was completely removed. The pellet was resuspended in 20 mM Tris-HCl pH= 8.0, 10 mM $CaCl_2$ and digested with 0.1 mg/ml trypsin (Worthington TRTPCK) at 37°C for approximately 16 hours.

After the incubation, 50 µl of 20% SDS were added and the samples were boiled for 15 minutes. Centrifugation at 13,000 g for 2 minutes pelleted the spores, and the supernatant was removed. The pellet was resuspended in water and the washing steps were repeated until the SDS was completely removed, usually approximately 7-10 washes. Once the washes were complete, the pellet was resuspended in 250 µl 12.5 mM $NaPO_4$ pH= 5.5 and was digested with 125 units of Mutanolysin (Sigma) and incubated at 37°C for 16 hours. After the Mutanolysin digest, the insoluble material was removed by a 15 minute centrifugation at 13,000 g. The supernatant was

transferred to a new tube and contained the muropeptides. The supernatant was lyophilized (Savant Speedvac) and stored at –20°C until ready to be analyzed on the HPLC.

Germination Exudate PG Preparation

The exudate samples that were removed at the three time intervals during germination were initially stored at –80°C until needed for analysis. The exudates were removed from the freezer and lyophilized for 8 hours (Savant Speedvac). The dried samples were resuspended in 100 µl of sterile water and the like samples were combined together. The samples were briefly vortexed and split between two microfuge tubes. One set of exudates was stored at –20°C, and the other set was resuspended in the Mutanolysin digest, total volume of 250 µl 12.5 mM $NaPO_4$ pH=5.5 with 125 units of Mutanolysin. This exudate set was incubated for 16 hours at 37°C.

After incubation, both sets of exudate (with and without Mutanolysin digest) were lyophilized (Savant Speedvac) for ~ 3 hours. The dried samples were then stored at –20°C until needed for HPLC analysis.

Reduction of the Muropeptides for HPLC preparation

To prepare the muropeptides of all four samples, the terminal sugars were reduced. The dried muropeptide samples were first resuspended in 100 µl 0.25 M Na_2B4O_7 pH= 9.0 and the pH of the samples was adjusted to 9.0 by adding 1 N NaOH. The terminal sugar residues were reduced to the alcohol by adding 25 µl of 25 mg/ml $NaBH_4$ in 0.25 M Na_2B4O_7 pH = 9.0. The reduction was run for ten minutes and

vortexed frequently. The reaction was terminated by adding 3.5 µl (4.0 µl for the exudate) of H_3PO_4. The pH was then adjusted to 2.0 with H_3PO_4. The samples were centrifuged for 5 minutes at 13,000 g to pellet any remaining insoluble material, and 50 µl of sample were transferred to autosampler vials for analysis with the HPLC.

Muropeptide Separation by Reverse-Phase HPLC

Once the samples had been reduced, the desired amount was transferred to the autosampler vials and run through the HPLC for analysis. The column used was an ODS Hypersil, 250x4.6 mm, 3 um (Keystone Scientific) column with a flow rate of 0.5 ml/min. A linear gradient of the buffer was used, starting with 100% Buffer A and increased from 0-100% of Buffer B over 120 minutes. A methanol gradient was used initially; Buffer A was 50 mM sodium phosphate pH=4.31 and Buffer B was 50 mM sodium phosphate 20% methanol pH=4.95. The second buffer system used was the acetonitrile gradient, Buffer A was 0.1% trifluoroacetic acid and Buffer B was 0.1% trifluoracetic acid 20% acetonitrile. The second buffer system was used to ensure that each peak produced on the methanol gradient was only one muropeptide fragment, and that there was no co-elution of different fragments.

Peak Analysis and Identification

To identify the muropeptides that produce each peak, the fractions were collected from the HPLC as they eluted off the column. The samples were then put through amino acid analysis, amino sugar analysis and MALDI-TOF MS. This procedure was described previously by Popham et al (32). These methods are used to determine the exact saccharide and peptide composition of each fragment. However,

these more advanced methods are used for peak identities that could not be confirmed by other methods, such as co-chromatography.

Dipicolinic Acid Release Analysis

A good indicator that germination is actually occurring is the release of dipicolinic acid. As the spore germinates, the first stage involves biophysical changes, including the release of calcium dipicolinic acid from the core of the spore, and uptake of water into the core. Therefore, the change in dipicolinic acid amounts in the spore compared to the change of dipicolinic acid amounts in the environment is a commonly used indicator of germination. To determine these concentrations, 100 µl samples were taken from the germinating spore preps at 0, 1, 10, 25, and 40 minutes. The samples were immediately centrifuged for 45 seconds at 13,000 g. The supernatant was transferred to a new tube, and both samples were stored at –20°C until needed for analysis.

The pellet samples were resuspended in 1 ml of 10 mM Tris-HCl pH=8.0 and boiled for 20 minutes, then put on ice for 15 minutes. 900 µl of sterile water were added to the exudate samples, and both the exudate and pellet samples were centrifuged at 13,000 g for 2 minutes. 800 µl of the supernatant from each sample were transferred to a new tube and 200 µl of the DPA assay reagent were added. The reagent consisted of 25 mg of L-Cysteine, 170 mg of $FeSO_4$, and 80 mg of $(NH_4)_2SO_4$ dissolved into 25 ml of 50 mM sodium acetate pH = 4.6. The samples were vortexed and centrifuged for 2 minutes at 13,000 g. A set of standards with known DPA concentrations was made and the A_{440nm} was determined. The A_{440nm} of the samples was measured and the

concentration of released and retained dipicolinic acid was determined for each time point. Additionally, the spectrophotometer was zeroed with the prepared standard with no DPA for the pellet samples and the rest of the standards; however, a modified blank was used to zero the spectrophotometer for the exudate sample. Due to the exudate samples containing other materials present in the germination reaction, the standard needed to be modified to accurately measure the DPA and not other chemicals that may react with the regent. The modified blank consisted of 982 µl of sterile water, 8 µl of 0.5 M $NaPO_4$ pH = 7.0 and 10 µl of 1 M L-alanine, 10 mM Inosine.

Hexosamine Analysis

The progression of the cortex hydrolysis during germination can be analyzed by determining the concentrations of various cortical components relative to their location. The diaminopimelic acid (Dpm) is an amino acid specific to peptidoglycan and therefore the accumulation of Dpm in the exudate is related directly to the breakdown of cortex. Additionally, the NAM sugar residues also provide information on the cortex hydrolysis. To complete this analysis, 10 µl aliquots were taken from the germinating spore prep at 0, 1, 10, 25, 40, and 60 minutes and added to 90 µl of water. The samples were immediately centrifuged, and the exudate was transferred to a new microfuge tube. The samples were stored at –20°C until needed for analysis.

The samples, both pellet and exudate, were dried in the speedvac. The dried samples were resuspended in 20 µl of 6N HCl and hydrolyzed for 4 hours at 95°C. The samples were dried again in the speedvac. 25 µl of 2:2:1 Methanol:dH_2O:triethylamine

(TEA) were added and the samples were dried again in the speedvac. The samples were resuspended in 25 µl of 7:1:1:1 methanol:dH_2O:TEA:phenylisothiocyanate(PITC) and incubated at room temperature for 20 minutes. The samples were dried again and resuspended in 50 µl of HPLC buffer (0.14 M Sodium acetate pH = 6.2 0.05% TEA). The samples were centrifuged for 4 minutes at 13,000 g and then 40 µl were transferred to autosampler vials for HPLC analysis.

CHAPTER THREE

Analysis of wild-type *Bacillus anthracis* spores

Germination

The analysis of germinated spores was performed after the addition of a germinant to the spore samples. However, before the cortex hydrolysis could be studied, efficient germination had to be controlled and initiated. During the germination procedure, the optical density (A_{600nm}) of the sample was measured at various times to determine the efficiency of the germination. As the spores germinate into vegetative cells, they degrade a significant amount of cell mass (cortex), release solutes, and lose refractivity. This causes less light to be scattered and a corresponding decrease in the optical decrease to occur. Loss of OD during germination should be around 60% to be considered efficient. Initially, the OD drop was not comparable to this and review of the spores under the microscope did not show germination. However, after some procedural changes, and modifications to the germinant composition and concentrations, an efficient method to stimulate germination was obtained. Initially L-alanine was the sole germinant used, at various concentrations between 1 mM and 100 mM, but germination was not occurring. Mixtures of L-alanine and Inosine at various concentrations between 1 mM and 100 mM were used next. Finally, 100 mM L-alanine, 1 mM Inosine was found as the successful germinant composition, and Figure 7 shows the typical OD changes of all the germination assays completed. This germinant concentration was used for the PG analysis results discussed later. Figure 7 shows the percentage drop of optical density from the initial OD as the germination procedure

Figure 7. OD and DPA analysis during *Bacillus anthracis* germination

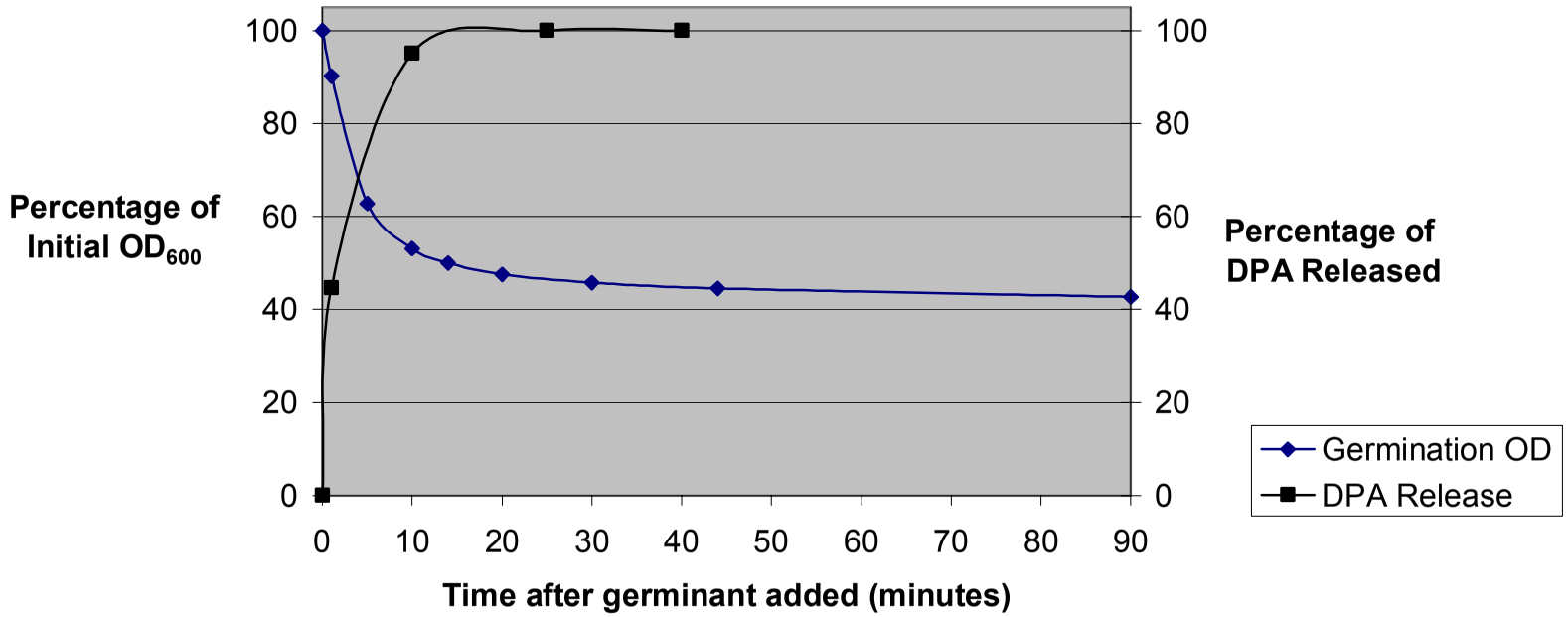


Figure 7. OD and DPA analysis during *Bacillus anthracis* germination. When the *Bacillus anthracis* spores were initiated to germinate by 100 mM L-alanine, 1 mM Inosine, a decrease in OD and an increase in DPA release occurred over time. Overall, the OD dropped 60% and 100% of the DPA was released by the end of the germination period. These both give sufficient evidence to prove that germination was occurring.

progressed. The decrease, of about 60%, that was observed is typical of an efficiently germinating sample. This measurement confirmed that germination was initiated and that the biophysical stage of germination was occurring. However, this was only a measure of refractivity loss and it did not directly reflect the cortex hydrolysis.

DPA Release Assay

To provide further evidence that the germination was occurring in the spores, as seen in the optical densities changes, the release of DPA was also measured. The accumulation of DPA outside of the spore, along with the decrease in the DPA concentration within the spore, confirms that the biophysical germination stage was occurring. The samples for DPA analysis were taken at various time points during germination along with a baseline sample before the germinant was added. The samples were centrifuged and the exudate was analyzed to determine the amount of DPA released from the spore, and the pellet was analyzed to determine the amount of DPA retained in the spore, at the various times during germination. These values were compared to confirm that the biophysical stage was initiated in the germination process. Figure 7 shows the typical results obtained from the DPA release analyses preformed during the germination experiments. When the spore germinates, it releases the DPA from the core into the environment. So, over time, the amount of DPA retained in the spore should decrease and the amount of DPA in the supernatant should increase. Ninety-eight percent of the initial DPA in the spores was released after only 10 minutes, and 100% was released after 25 minutes. These results confirmed that the germinant

had sufficiently initiated the spores to germinate and they quickly progressed through the biophysical stage of germination.

Hexosamine Analysis

To confirm that germination was occurring, not only did the biophysical characteristics need to be analyzed, but the biochemical changes needed to be studied as well. In addition to the HPLC analysis of the PG, the hexosamine analysis allowed for the determination of the concentration of various PG components based on their location. The samples were taken at various time points during germination and immediately centrifuged. The exudate was analyzed to determine the amount of PG material that had been degraded and had been released from the spore, and the pellet was used to determine the amount of PG material that was still associated with the spore. The samples were prepared and analyzed on RP-HPLC (Spherisorb II, 150x4mm, 5um, Keystone Scientific), along with Dpm and NAM standards. NAM and Dpm are known to produce characteristic peaks when run on this column and these peaks were found in the exudate and pellet germination samples. The NAM peak is known to elute around 4.1 minutes, and the Dpm peak is known to elute around 18.8 minutes. The size of the peak is relative to the amount of the PG fraction present in the sample.

The results found were consistent between repeated *B. anthracis* trials. The results showed that the amounts of Dpm and NAM increased in the supernatant samples and decreased in the pellet samples over time. Additionally, the most significant change in amounts occurred during the first ten minutes of germination. Approximately

75% of the total NAM and 50% of the total Dpm in the spore was released 10 minutes after germination was initiated. After 60 minutes of germination, the total amount of Dpm released was about 65% and the total amount of NAM released was about 80% (Figure 8). Previous experiments have shown that ~80% of the spore PG is the cortex, with the remaining 20% being germ cell wall is not broken down during germination, so some NAM and Dpm was expected to stay associated with the spore. Additionally, the degree of cross-linking in the GCW is higher then the cortex, so the release of Dpm should be less because it is not equally distributed between the different types of PG. This partially explains why the Dpm release was less then the NAM release. This shows that the majority of the cortex hydrolysis occurred during the first ten minutes, proving that the biochemical stage of germination in *Bacillus anthracis* occurred very rapidly.

Comparative analysis with other *Bacillus sp.*

To confirm the rapidity of the cortex breakdown that occurs in *B. anthracis*, this analysis was performed on *B. subtilis* and *B. sphaericus*, to provide a comparison. The samples were prepared in the same manner that the *B. anthracis* samples were prepared. The samples were germinated with the same concentration of L-alanine/Inosine, and samples were taken at the same time points for analysis. The OD of the germinating cultures was monitored to determine the efficiency of germination and is shown in Figure 8. The DPA release was monitored and hexosamine assay was performed on these two different bacilli (Figure 8). The release of NAM and Dpm were analyzed (Figure 8).

Figure 8a. OD_{600} Drop During germination

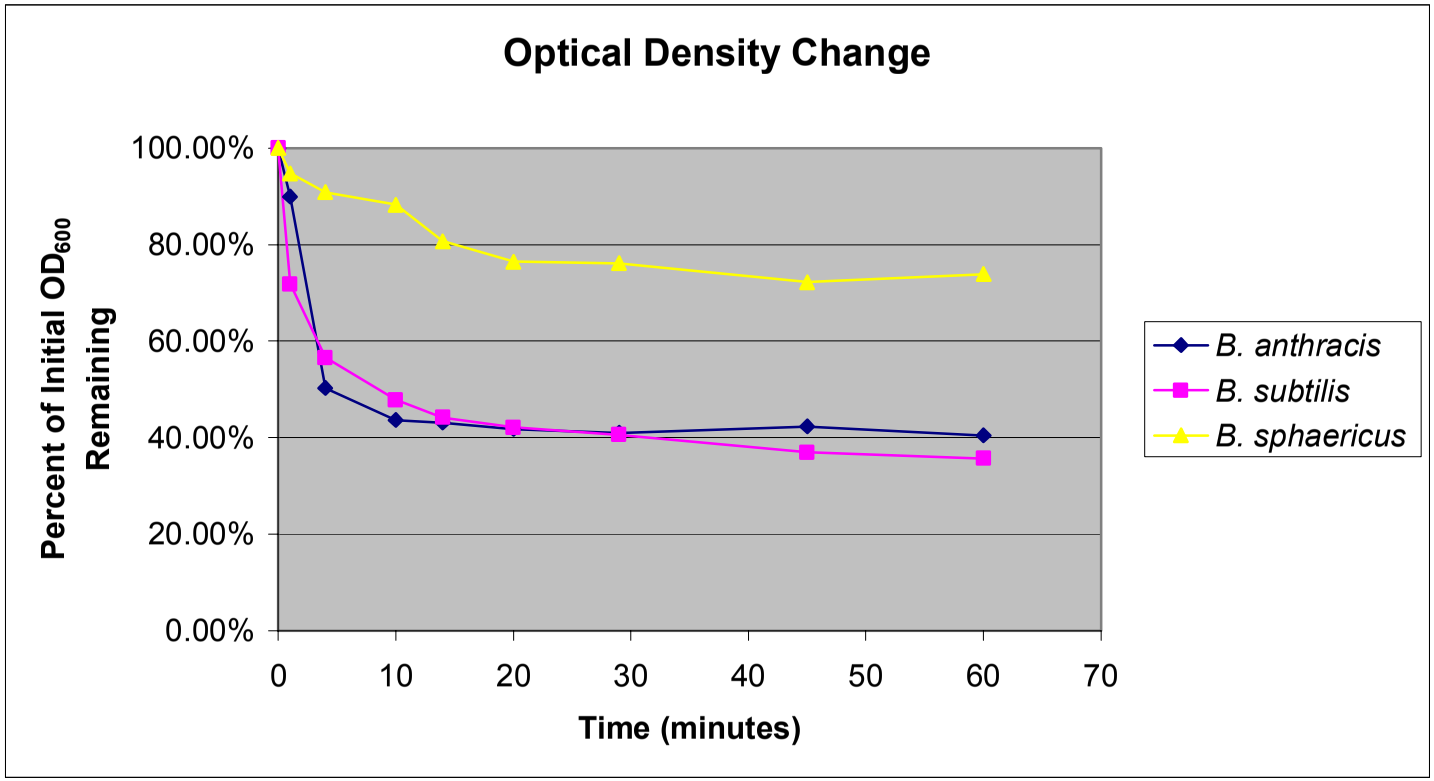


Figure 8a. OD_{600} Drop During Germination. All three species of sporulating bacilli had germination induced by the introduction of the same germinant. The drop in OD was monitored from t_0 to t_{60} and the percentage of that was lost over the time period was diagramed. 60% loss of initial OD is enough to infer that germination has been initiated.

Figure 8b. DPA Release Assay

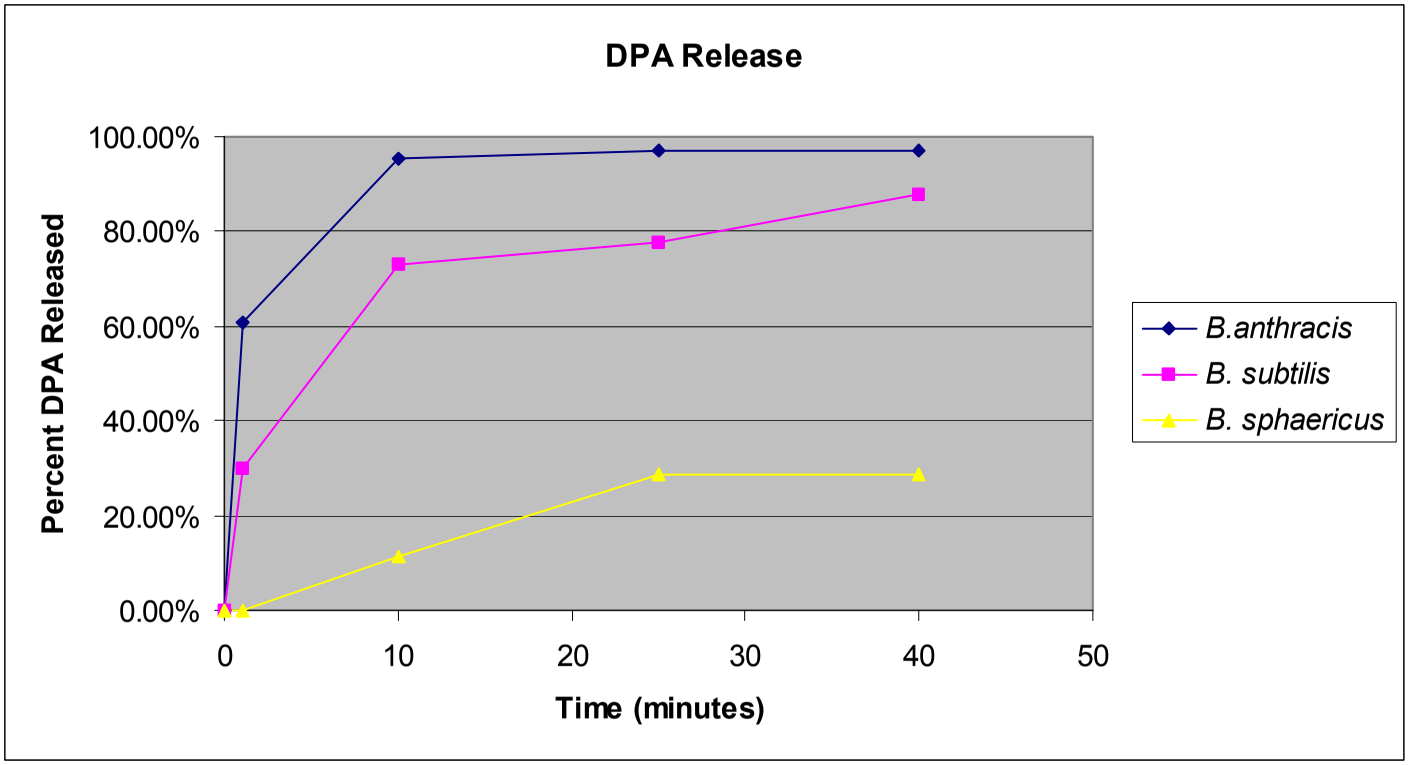


Figure 8b. DPA Release Assay. In addition to the loss of OD that occurs during germination, a significant change in the location of the DPA is an indicator of germination. An increase of DPA in the environment is an indication that the DPA is being released from the core of the spore, the first stages of germination. The DPA release was measured for all three *Bacillus sp*. during germination and compared.

Figure 8c. Dpm Release Analysis

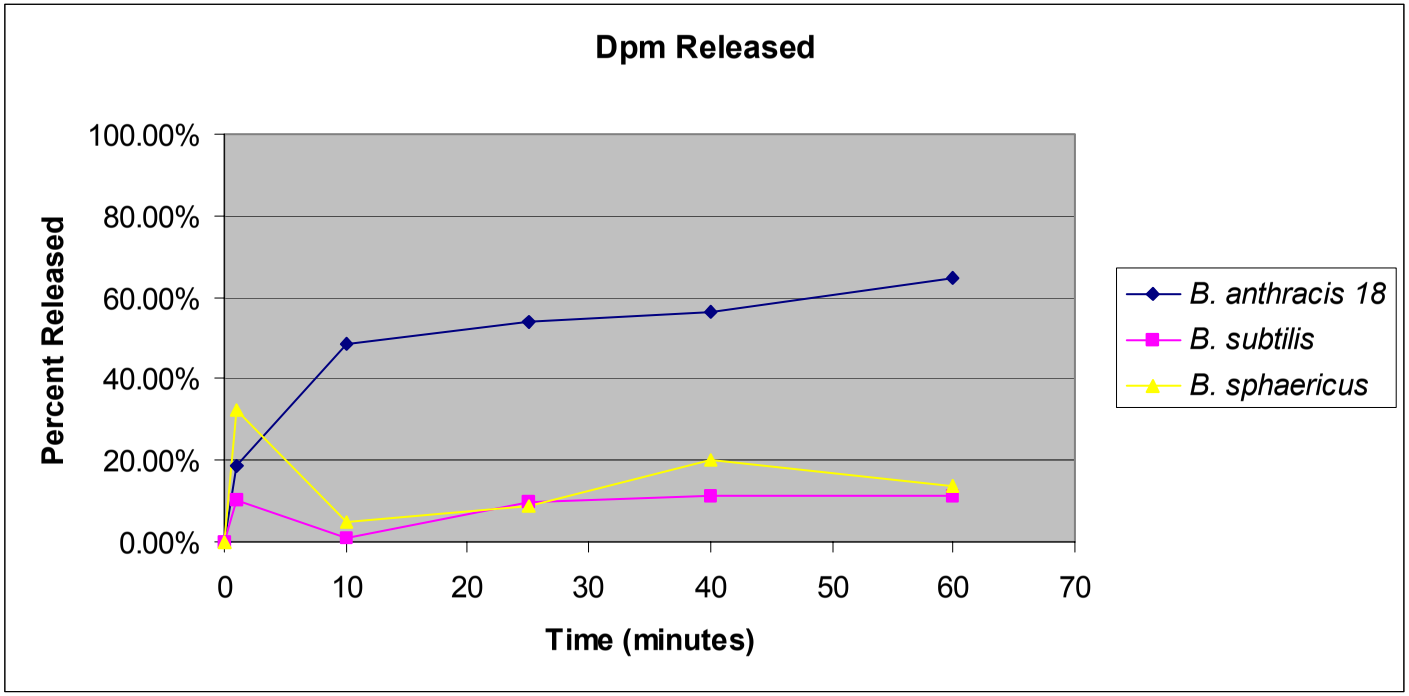


Figure 8c. Dpm Release Analysis. To determine if the cortex is being hydrolyzed, the amount of Dpm was measured in relation to its location. As the cortex is degraded, there is a decrease of Dpm in the spore, and a corresponding increase of Dpm in the environment. The percentage of DPA released from the spore was measured for each germinating *Bacillus sp.*.

Figure 8d. NAM Release Analysis

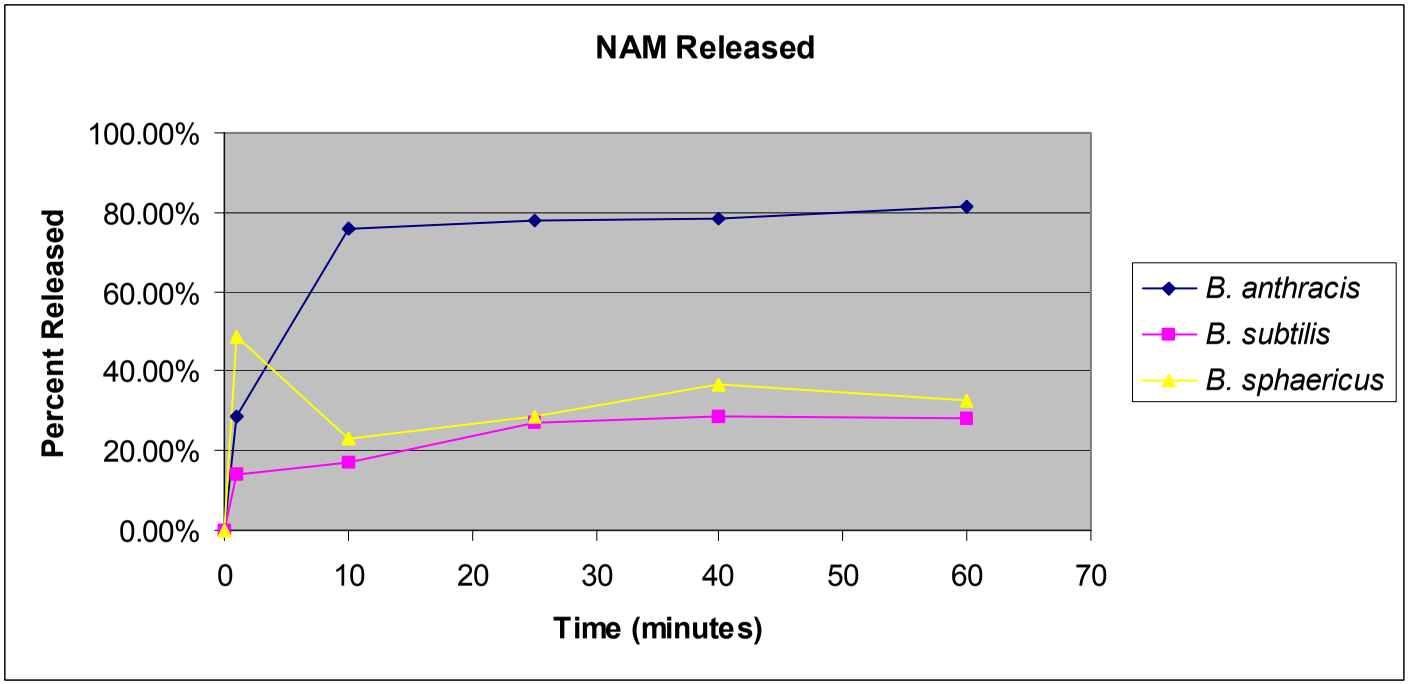


Figure 8d NAM Release Analysis. Another useful PG component that is released from the spore during germination is NAM. As the cortex is broken down, there is an accumulation of NAM outside of the spore. The percentage of the total NAM that was released from the various spores during germination was measured and compared.

In *B. subtilis*, the OD dropped significantly, indicating that efficient germination was occurring in the spore sample. There was approximately a 50% drop in OD in the first 10 minutes, and a 60% drop in OD over the hour germination experiment. The DPA release was monitored and the *B. subtilis* spores appeared to release their DPA at about the about the same rate as *B. anthracis*. After 10 minutes, 75% of the total DPA was released, and after 40 minutes, 90% of the total DPA was released. This varies only slightly when compared to *B. anthracis*. These analyses both only represent the first stage of germination and not directly to cortex hydrolysis, therefore, the hexosamine assay was performed to measure this. Figures 8c and 8d show what percentage of the total amount of Dpm and NAM from the spore had been released due to hydrolysis. It appeared that after 10 minutes, at most 10% of the Dpm was released and 20% of the NAM. After the entire 60-minute assay, only 12% of the total spore Dpm was released, and 30% of the total spore NAM was released.

The *Bacillus sphaericus* samples did not produce a similar drop in the OD after the germinant was added. There was only a drop of about 30% of the initial OD, which means that the germination initiation rate was not very high. If *B. sphearicus* had an OD decrease in each germinating spore similar to that of *B. subtilis* and *B. anthracis*, this would suggest that approximately 50% of the *B. sphearicus* spores germinated in the experiment. The DPA release assay mirrored these results with only about 30% of the total DPA content being released after 40 minutes, and only 10% after the first ten minutes. After 10 minutes it appeared that only 20% of the total NAM content was released, and about 10% of the total Dpm content was released. If only 50% of the

spores had initiated germination, this would correspond to 40% NAM and 20% Dpm release. The first sample after germination initiation, taken after 1 minute, appeared to have a significant measured amount of PG components released, however, the measured amount of released PG components significantly drops at ten minutes. If this was actually caused by the release of PG components, there would have been a similar increase in these components in the exudate, and this did not occur. Therefore, this data entry was most likely due to error caused by sample loss. After the entire sixty minutes, the accumulated amounts were only slightly higher, with Dpm at 15% and NAM at 30%. These results could possibly be caused by the L-alanine Inosine germinant combination not being sufficient to stimulate *B. sphaericus* spores to germinate.

Comparing the results of the *B. anthracis* and *B. subtilis* samples (Figure 8a and 8b) showed that the germinant was able to initiate both species to efficiently germinate, as seen by the OD decrease and the DPA release. However, even though they both entered germination at the same rate, they did not complete germination at the same rate. Complete cortex hydrolysis is necessary for the spores to transition into a metabolically active cell. *Bacillus anthracis* spores clearly break down their cortex much quicker, meaning they are able to complete germination much quicker. Previous studies of *B. subtilis* have quantified cortex hydrolysis and found that only 37% of the PG is broken down and released within 90 minutes of germination (33). This experiment showed results similar to that, but also showed that *B. anthracis* released comparable amounts in slightly over a minute. Similar comparisons could be made between *B. anthracis* and *B. sphaericus*.

Dormant Spore PG Structure

Determining the structure of the dormant spore PG was essential before the germination structural changes could be studied. The dormant spore samples were obtained from the clean spore preps, without a germinant being added. The PG was extracted, digested with Mutanolysin, a muramidase that cuts between the NAM and NAG units, reduced and run on the RP-HPLC. Figure 9 shows the peaks produced by the various fractions that eluted off the column using the methanol gradient. Based on co-chromatography, the identities of the majority of the peaks were believed to be identical to peaks identified in previous experiments performed on *B. subtilis* (5, 32). These peaks eluted off at the same time as *Bacillus subtilis* on both buffer systems and Table 2 gives the proposed identities of these identical peaks. Since the elution times were identical on both buffer systems, this is enough to verify the identity and no further analysis of these peaks was needed. However, the fractions that were not immediately identifiable were collected as they eluted off the column and then further analyzed. Twelve substantial peaks were found in the dormant *B. anthracis* spore HPLC fractions that were not seen in *B. subtilis* analysis. However, some of the peaks appeared to elute at the same time as *B. cereus* peaks, but these elution times were only available on one buffer system. These peaks were further analyzed to determine their identity because their structure could not be immediately verified based on co-chromatography. The hypothesized identities of these peaks based on amino acid analysis are listed in Table 2, and these predicted structures are waiting confirmation by mass spectrometry.

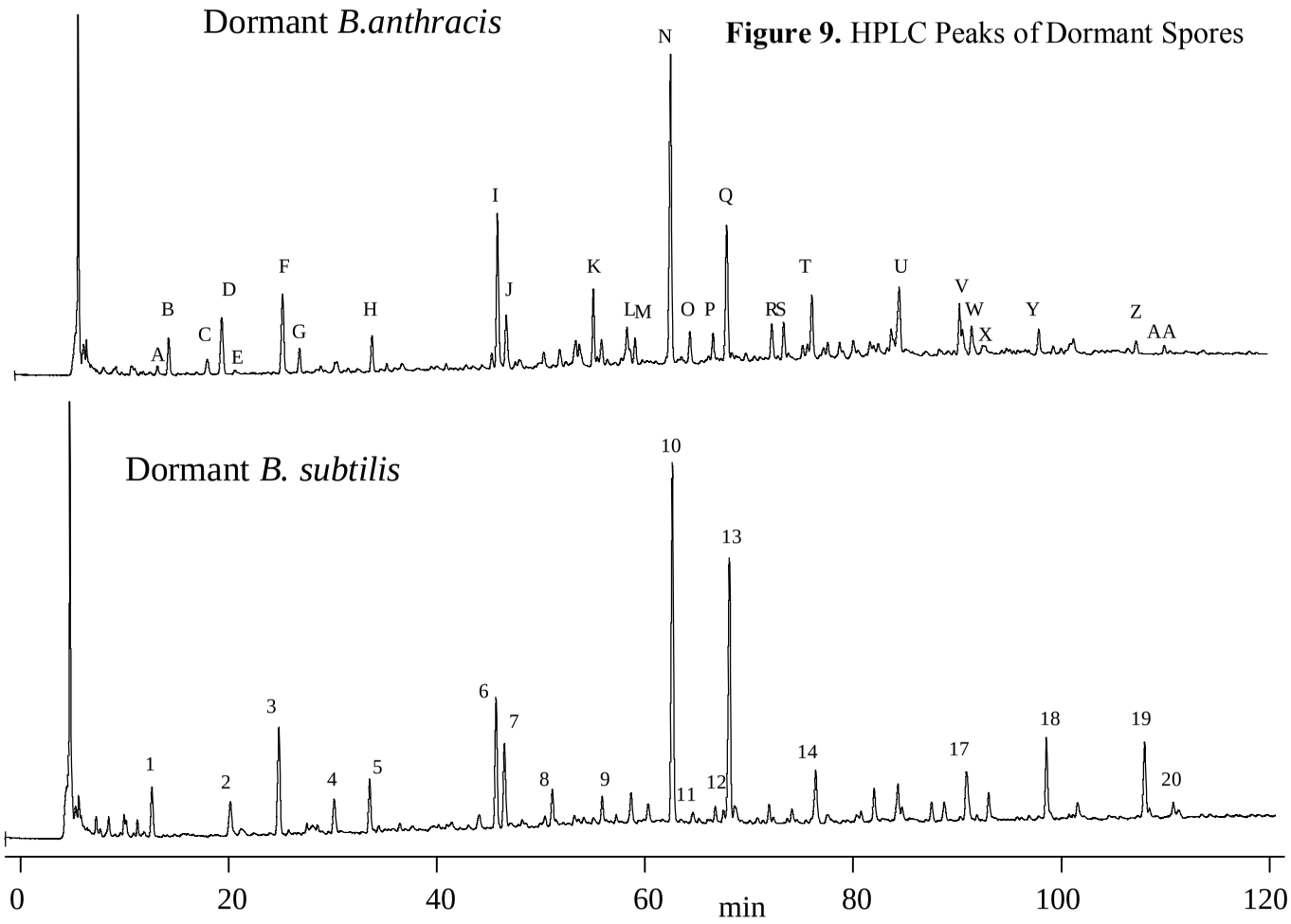


Figure 9. HPLC Peaks of Dormant Spores

Figure 9. HPLC peaks of Dormant Spores. The chromatograms above provide comparison of the muropeptide separation in *B. anthracis* and *B. subtilis* dormant spores. The co-chromatography that occurs between peaks allows for inference of *B. anthracis* muropeptide structure based on known *B. subtilis* structures.

Table 2. Proposed Structure of Dormant PG Peaks from Co-Chromatography

Co-Chromatography		
***B. subtilis* peak**	***B. anthracis* peak**	**Identity**
1	A	DS-TriP
2	E	DS-ala
3	F	DS-TP
5	H	TS-TP
6	I	TS_{red}-TP
7	J	TS_{red}-ala
10	N	TS-TP
11	O	TS-TP-TP
12	P	DS-TP-TS_{red}-TP
13	Q	TS-ala
14	T	DS-TP-TS-TP
17	V	TS-TP-TS-TP
18	Y	HS-TP
19	Z	HS-ala
20	AA	TS-TP-TP-HS

Abbreviations: DS- disaccharide, TriP- tripeptide, ala- L-alanine, TP- tetrapeptide, TS- tetrasaccharide, TS_{red}- tetrasaccharide with a reduced muramic lactam, HS- hexasaccharide.

Table 2. Proposed Structure of Dormant PG peaks from co-chromatography. The identities of the peaks found in the chromatograms shown in Figure 9 are listed. The *B. subtilis* structured have been previously determined, and the *B. anthracis* structure have been identified based on the co-chromatography of the peaks using two different buffer systems with RP-HPLC.

Table 3. Hypothetical Structures of Dormant Spore Novel Peaks.

***B. anthracis* Peak**	**Proposed Identity**
B	DS-TriP de-acetylated
C	Unknown
D	DS-ala de-acetylated
G	DS-TP deacetylated*
K	TS-ala de-acetylated*
L	TS-TP de-acetylated*
M	Unknown
R	DS-TP-TP-TS de-acetylated
S	DS-TP-TP-TS de-acetylated
U	HS-TP de-acetylated*
W	HS-ala de-acetylated
X	HS-ala de-acetylated

*These structures come from the co-chromatography of these peaks with fragments found in *B. cereus* or *B. megaterium* spores (1).

Table 3. Hypothetical Structures of Dormant Spore Novel Peaks. Some of the peaks produced during muropeptide separation were not identical to peaks found in *B. subtilis*. Some of these other peaks were identified as peaks found in *B. cereus* and *B. megaterium*. Other peak identities were hypothesized from peak placement and from the other known peaks present.

The major peaks produced, or the cortex fragments present in the highest concentration, were identical to *B. subtilis*. The peaks were the tetrasaccharide tetrapeptide (Peak N), and tetrasaccharide-alanine (Peak Q) fragments along with their further reduced forms (Peaks I and J). The peaks that appeared to correspond with the *B. cereus* structure and not to the *B. subtilis* structure were caused by de-acetylation of the amino sugar residues. *B. subtilis* does not have any de-acetylated sugars, so these peaks were not present, however, *B. cereus* is known to have these modifications. *B. cereus* is more closely related to *B. anthracis*, so it is likely that their PG structures are more similar then *B. subtilis*. From these preliminary results, of the twelve peaks not found in *B. subtilis*, three of them appeared to be found in *B. cereus*, and one was found in *Bacillus megaterium* (1); all of which are commonly found structures, but with amino sugar de-acetylation. Of these ten remaining peaks, it was hypothesized that most of them were other various fragments with some form of de-acetylation on the amino sugar residues. Since the peaks that appeared to be similar to *B. cereus* and *B. megaterium* were a DS-TP de-acetylated, TS-ala de-acetylated, TS-TP de-acetylated, and HS-TP de-acetylated, there was reason to believe that fragments such as the DS-ala, DS-TP-TP-TS, and HS-ala may also have had de-acetylated counterparts. Since de-acetylation can occur on one or multiple sugar residues in a fragment, some of these structures may elute in different places based on which sugar residue was de-acetylated or how many sugar residues were de-acetylated. Additionally, Peak M was highly variable in its size and appearance between samples. Through further analysis of vegetative cell wall, Peak M was determined to be the major peak formed in the HPLC

results. The size of the peak in the spore samples corresponded to the amount of vegetative contamination in that particular spore preparation.

Amino acid and amino sugar analysis was performed on many of the peaks formed in the dormant samples. Table 4 shows the results that confirmed the predicted identity of many of the fragments. The molar ratio of the individual PG components for each fragment was determined and is shown in parenthesis. The structures that appeared to be lacking any NAM residues were believed to have the further reduced lactam structures because these sugars are not detectable in this type of analysis.

By determining the identity of these novel muropeptides, this proved that the dormant structure of *Bacillus anthracis* spore PG was slightly different from *Bacillus subtilis*. The main structural components were similar, however, the de-acetylated sugar residues made the PG structure more similar to *B. cereus*.

Cortex Hydrolysis during Germination

To determine the enzymatic activity related to cortex hydrolysis occurring during germination, spore samples were germinated and the exudate and spore material was separated. The pellet was treated and the spore-associated PG was extracted and analyzed with the HPLC. These analyses produced very little data, and it appeared that there was a significant amount of sample loss occurring during the spore processing steps. However, further analysis of the spore exudate samples, showed there was a substantial amount of PG in the exudate samples at the earliest analysis time of 15 minutes. This led to the conclusion that there was little PG being extracted from the spore-associated samples because the cortex PG was hydrolyzed so quickly. There

Table 4. Amino Acid Analysis of Dormant Spore Muropeptides.

NAME	Predicted Structure	NAM	NAG	GOH	MOH	Glu	Dpm	Ala
A	DS-TriP		1.1 (1)		1.0	0.8 (1)	1.4 (1)	0.9 (1)
B	DS-TriP deacetylated		1.2 (1)		1.0	0.9 (1)	1.2 (1)	0.8 (1)
C	DS-TriP ?		1.2 (1)		1.0	0.7 (1)	0.6 (1)	0.7 (1)
D	DS-TriP deacetylated		1.4 (1)		1.0	1.0 (1)	1.4 (1)	0.9 (1)
F	DS-TP		1.4 (1)		1.0	0.9 (1)	1.0 (1)	1.7 (2)
G	DS-TP deacetylated		0.9 (1)		1.0	0.8 (1)	1.0 (1)	1.6 (2)
H	TS-TP open lactam	1.2 (1)	2.4 (2)		1.0	0.8 (1)	1.2 (1)	1.6 (2)
I	TS-TP reduced lactam		2.3 (2)		1.0	0.8 (1)	1.4 (1)	1.6 (2)
J	TS-Ala reduced lactam		2.2 (2)		1.0			0.7 (1)
K	DS-TriP-TP-DS ?		2.6 (2)		2.0	1.8 (2)	2.4 (2)	2.0 (2)
L	TS-TP deacetylated	1.2 (1)	1.7 (2)		1.0	1.0 (1)	1.2 (1)	1.9 (2)
N	TS-TP	1.4 (1)	2.1 (2)		1.0	0.9 (1)	1.0 (1)	1.7 (2)
O	TS-TP-TP	0.9 (1)	2.2 (2)		1.0	1.6 (2)	1.8 (2)	3.3 (3)
P	DS-TP-TP-TSreduced		2.8 (3)		2.0	2.0 (2)	2.0 (2)	3.2 (3)
Q	TS-Ala	1.1 (1)	1.9 (2)		1.0			1.0 (1)
T	DS-TP-TP-TS?	0.0	1.4 (1)		2.0	2.0 (2)	0.8 (1)	2.6 (3)
U	HS-TP deac.	1.5 (2)	4.3 (4)		1.0	0.8 (1)	1.2 (1)	1.5 (2)
V	TS-TP-TP-TS	1.6 (2)	4.3 (4)		2.0	1.6 (2)	1.6 (2)	2.2 (2)

Table 4. Amino Acid Analysis of Dormant Spore Muropeptides. The results from the hydrolyzation of the spore fragments are shown above. The individual components of each fragment were determined and the structure was predicted based on the fragment composition. The molar ratios are shown in parenthesis. The values shown in red contained values that did not exactly match with the predicted structures and they require further analysis.

actually was very little PG still associated with the spores and majority of the PG had already been released into the exudate. These results lead to a slight procedural change regarding when during the germination samples were taken for analysis. This activity corresponded to the results found in the hexosamine assay; the majority of the cortex was hydrolyzed within 15 minutes of adding the germinant

Figure 10 shows the HPLC results of the germination samples at five and thirty minutes, including the spore-associated material, the exudate digested with Mutanolysin, and the undigested exudate. The chromatograms produced at 15 and 30 minutes looked very similar, with only a slight change in peak size, meaning there was not much change in the amount of the fragments because there was relatively little cortex hydrolysis occurring in the last fifteen minutes of the experiment. However, even though slight, the changes produced in the last 15 minutes of germination helped to make the most dramatic visual chromatogram differences occur between the five and thirty minute samples.

After five minutes, there was a substantial amount of PG already in the exudate, which was evident by the large size peaks seen in the chromatograms, and reinforced by the relatively small peaks found in the spore-associated material chromatograms. When comparing the peak sizes of the 5 and 30-minute samples, there was a change in the size of the peaks, corresponding to a change in the amount of that specific muropeptide. However, since there was not a significant change in the size of the peaks between the 15 and 30-minute samples, there was little to no hydrolysis occurring. The only substantial difference in peak size was seen when the 5 and 15 minute samples were

Figure 10. HPLC Results of Germination Samples

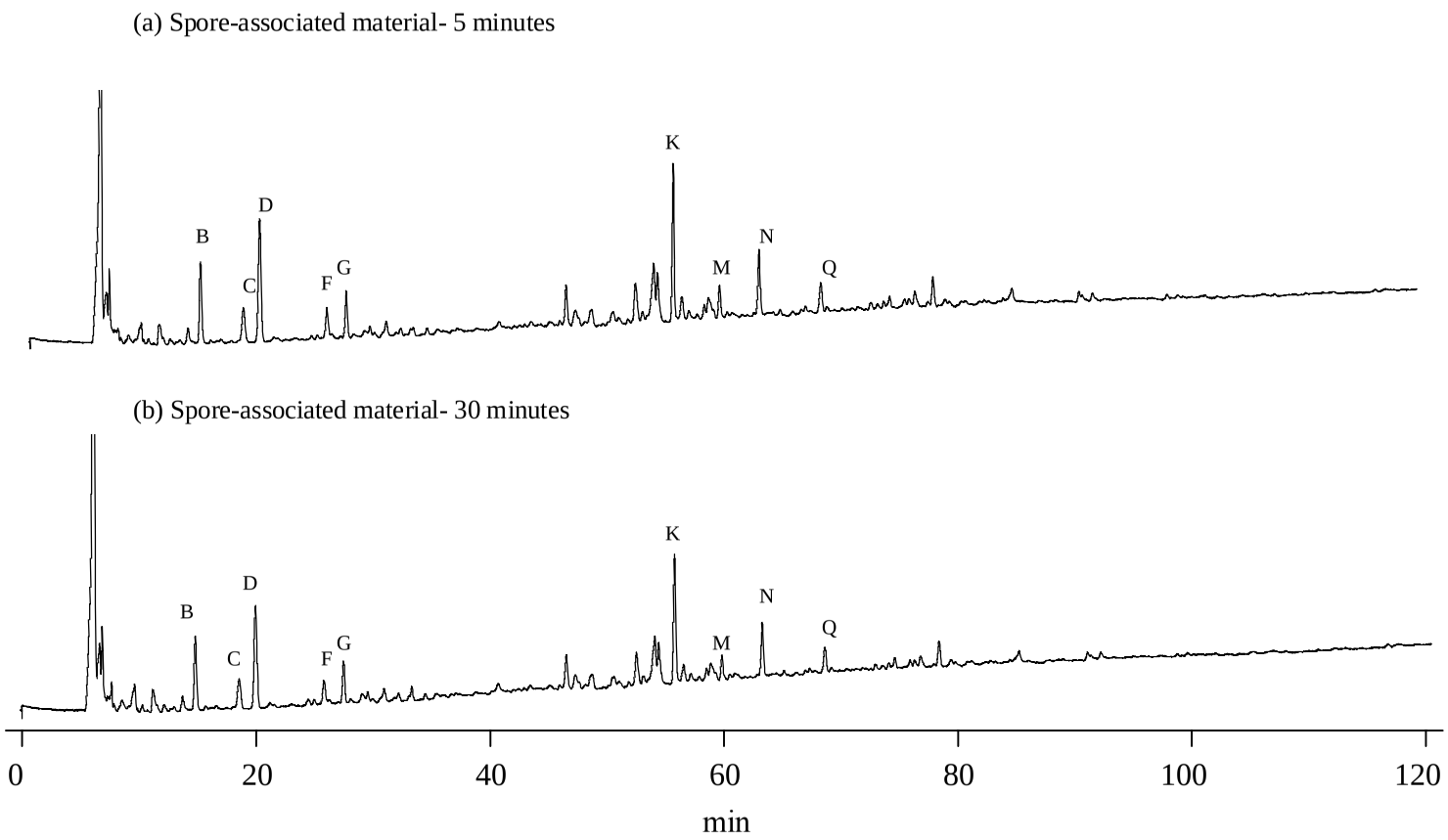


Figure 10.HPLC Results of Germination Samples

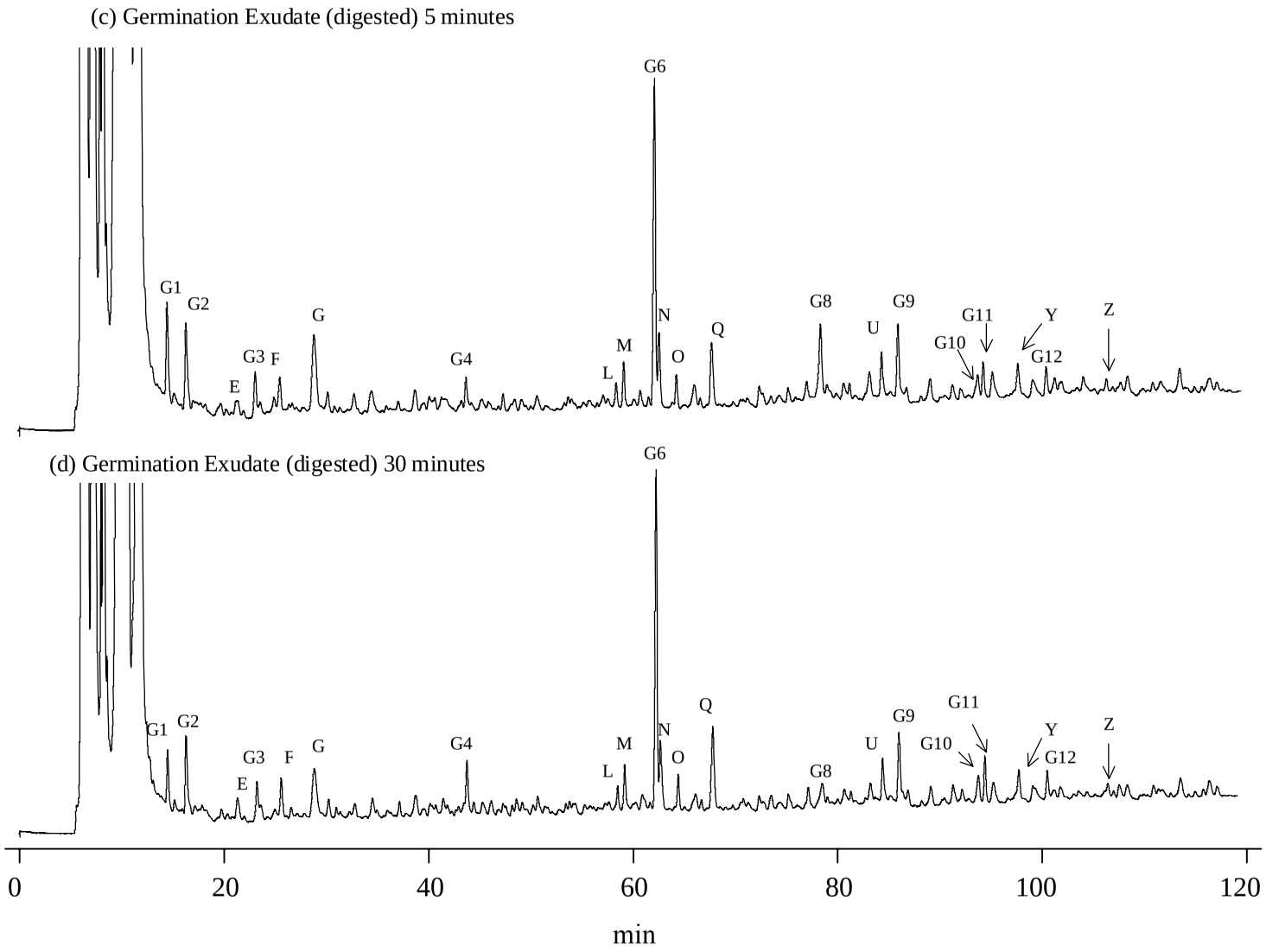
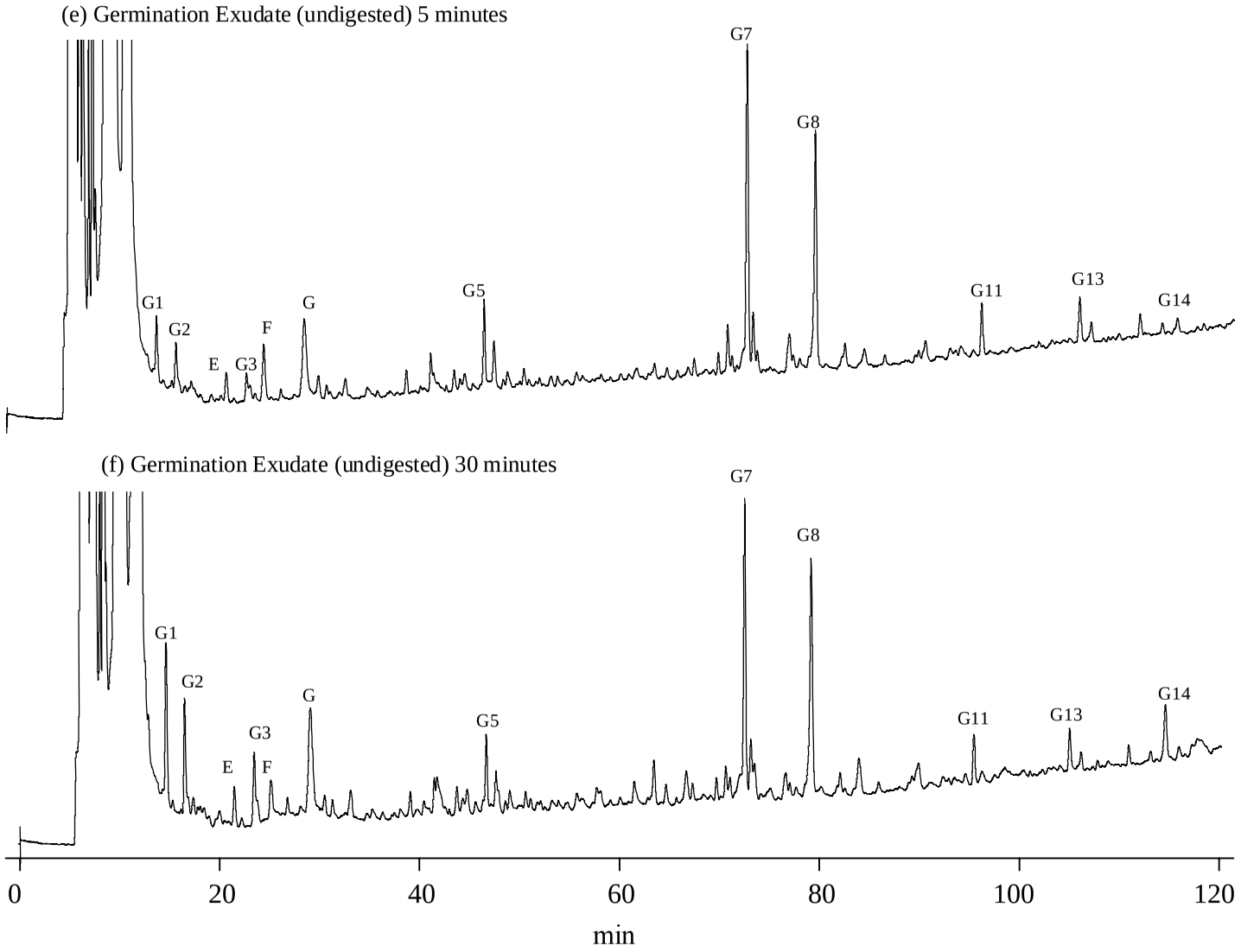


Figure 10 (continued) HPLC Results of Germination Samples



compared, meaning that the majority of the cortex hydrolysis took place during this time frame. Additionally, this coincided with the results from the hexosamine assay, where the majority of the PG components were found in the exudate within 15 minutes of germination.

Muropeptide Structure Identification and Analysis

The analysis of the germination muropeptides, however, was very informative. All of the muropeptides in the spore-associated samples were found in the dormant spore PG samples; no novel muropeptides were found. The 9 significant peaks were determined to be produced from the digestion of the germ cell wall during the Mutanolysin digest. The major spore peaks essentially disappeared; therefore, these samples did not provide significant information about the enzymatic activity. The germination experiment produced 14 novel germination muropeptides, labeled G1-G14 in Figure 10, that were found only in the exudate samples. Several of the novel muropeptide peaks were found in both types of exudate samples, with and without the Mutanolysin digestion; however, some were seen only in one type of exudate sample. While the exudate samples produced these novel peaks, they also produced a few of the same peaks found in the dormant samples.

Most of the peaks that appeared to be the same as the dormant sample peaks were identified based on co-chromatography along with amino acid analysis or by co-chromatography in both buffer systems. Peaks E, F, G, L, M, N, O, Q, U, Y, and Z all appeared in the germination exudate samples. These peaks that were identified from co-chromatography based on *B. subtilis*, and were also confirmed by amino acid

analysis. Mass spectrometry was attempted on these samples, however, for many of the muropeptides there was not enough material present to get useful data, therefore, the collection procedure has been repeated using more starting sample, and the results are pending. The mass spectrometry data will provide identity confirmation and will clear up any discrepancies that appeared between the co-chromatography and the amino acid analysis.

Determining the identity of these novel muropeptides was done in several ways. Some of these peaks were identical to peaks found in the previous Atrih et al study (5) on *B. subtilis*. However, knowing that there are some differences between the dormant PG of *B. subtilis* and *B. anthracis*, it was expected that many of these peaks would be different. Of the fourteen novel peaks found, seven appeared to be similar to peaks found in *B. subtilis* by Atrih et al (5). Of these seven, three had been confirmed by amino acid analysis along with mass spectrometry, and appeared to have the structure that was predicted. The other four structures were predicted by co-chromatography, have been confirmed by amino acid analysis, however, they need verification by mass spectrometry. The predicted structures of these fourteen muropeptides are listed in Table 5 and the amino acid results for eleven muropeptides are listed in Table 6. The seven completely new peaks also need to be analyzed by mass spectrometry. The initial mass spectrometry analysis of these fourteen peaks did not produce a useable mass for eleven of the peaks. This was due to a low amount of sample provided for analysis. Larger amounts of the samples have been recollected and the results are pending.

Possible Lytic Enzyme Activity

The presence of PG in the exudate was a result of the cortex hydrolysis occurring during germination. By analyzing the structure of the muropeptides, the type of lytic activity that was occurring during germination could be speculated. The fragments produced in the dormant sample were caused by Mutanolysin enzymatically working on the PG in the spore. So, comparison of the exudate samples that had been digested with the Mutanolysin to the samples provided that had not been digested provided insight to method of cortex degradation.

In the exudate sample without muramidase digestion, two major peaks were produced. As seen in Figure 10, G7 and G8 were the most abundant muropeptides. The structure of these fragments was determined and verified to be a TS-TP and a TS-ala. Peaks with these identical structures were present in the dormant sample; however, the elution times were quite different indicating there was some kind of structural difference in the fragments causing the shift. Since these fragments occurred in the samples without the Mutanolysin digestion, it was determined that there was some kind of lytic activity occurring in the saccharide chains of the cortex to produce these fragments with a tetrasaccharide backbone. The two types of enzymes that could have possibly been active were a muramidase or a glucosaminidase, or possibly both. The products that would be formed from each digestion are illustrated in Figure 11.
The other peaks found in the undigested germination exudate samples, G1, G2, G3, G11, G13, and G14 supported the idea that the cortex hydrolysis was due to an

Table 5. Identity of Novel Germination Muropeptides.

Peak Number	Hypothetical Structure	Location	
		Exudate w/ Mutanolysin	Exudate w/o Mutanolysin
G1	MS-DP[3]	+	+
G2	MS-DP[3]	+	+
G3	DS-TriP[3]	+	+
G4	TriS-TP reduced[3]	+	-
G5	TS-TP reduced, NAG terminated[1,2,3]	-	+
G6	TriS-TP[1,3]	+	-
G7	TS-TP NAG terminated[2,3]	-	+
G8	TS-Ala NAG terminated[1,2,3]	-	+
G9	Anhydro TS[1,3]	+	-
G10	AnhydroTS[1]	+	-
G11	TS-TP[3]	+	+
G12	HS-TP NAG terminated[1,3]	+	-
G13	Unknown	-	+
G14	Unknown	-	+

[1], Predicted structure based on co-chromatography with *B. subtilis* (2)

[2], Predicated structure based on co-chromatography with *B. subtilis* (2) and verified using mass spectrometry.

[3], Predicted structure based on amino acid analysis

Table 5. Identity of Novel Germination Muropeptides. The listed peaks were novel in the germination exudate samples. Some of the peaks were produced in both the digested and undigested exudate samples. Some of the structures have been confirmed by mass spectrometry, and co-chromatography. Other structures have been hypothesized from the dormant spore structure or other peaks present.

Table 6. Amino Acid Analysis of Novel Germination Muropeptides.

NAME	Predicted Structure	NAM	NAG	GOH	MOH	Glu	Dpm	Ala
G1	? MS-DiP				1.0	1.3 (1)		1.3 (1)
G2	? MS-DiP				1.0	2.2 (2)		2.6 (3)
G3	DS-TriP		0.8 (1)		1.0	1.0 (1)	0.6 (1)	1.2 (1)
G4	TriS-TP reduced lactam		1.0 (1)		1.0	0.7 (1)	1.0 (1)	1.6 (2)
G5	TS-TP reduced lactam (NAGterminated)		0.8 (1)	1.0		1.3 (1)	1.4 (1)	2.4 (2)
G6	TriS-TP	1.1 (1)	1.3 (1)		1.0	0.7 (1)	0.8 (1)	1.5 (2)
G7	TS-TP (NAGterminated)	2.1 (2)	1.3 (1)	1.0		0.9 (1)	1.2 (1)	1.8 (2)
G8	TS-Ala (NAGterminated)	1.6 (2)	1.1 (1)	1.0				0.7 (1)
G9	AnhydroTS-TP ?	0.9 (1)	1.8 (2)		1.0	0.8 (1)	1.2 (1)	1.6 (2)
G 11	TS-TP ?	0.8 (1)	1.4 (1)		1.0	0.7 (1)	1.0 (1)	1.6 (2)
G 12	HS-TP ?	2.0 (2)	2.8 (3)		1.0	1.1 (1)	1.2 (1)	1.5 (2)

Table 6. Amino Acid Analysis of Novel Germination Muropeptides. The analysis confirmed the predicted structure of many of the novel germination peaks. Additionally, it provided information about the identity of the sugar residue on the reducing end of the sugar residue.

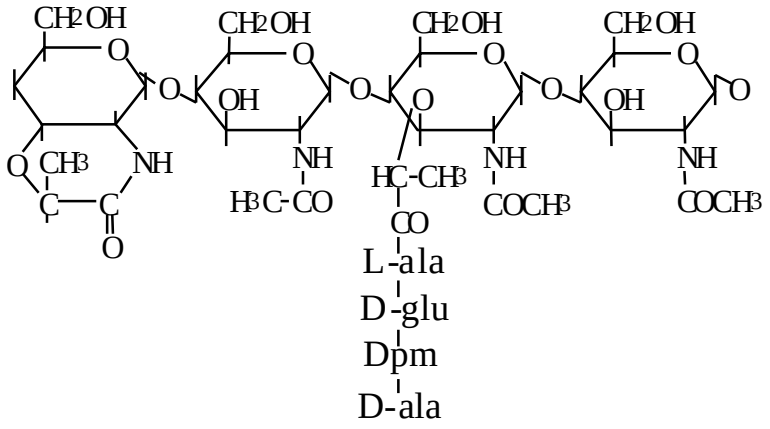
exoenzyme. An exoenzyme would act on the ends of the glycan strand, cutting off fragments and then continue along the rest of the fragment. This kind of activity would produce various types of smaller fragments, such as disaccharides and tetrasaccharides. Many of the muropeptide fragments found in the undigested germination exudate samples were hypothesized to have structures similar to that.

The presence of peaks that contained an anhydromuramic acid, such as peaks G9, G10, suggested that there was lytic transglycosylase activity occurring during germination as well. However, since these peaks were not the major peaks produced in the results, the lytic transglycosylase did not appear to be the major enzymatic activity contributing to the cortex hydrolysis.

Analysis of the major peaks found in the digested germination exudate also gave some insight into the lytic enzyme activity. Two important observations were made from these results. The first important observation was the disappearance of the two major peaks found in the undigested samples, G7 and G8, in the digested sample. There are two possible reasons as to why these peaks were not present in the digested sample. The first hypothesis was that these peaks were muramidase sensitive. The peaks were produced during germination as tetrapeptides, and when the samples were treated with Mutanolysin, they were broken down. However, this theory does not completely hold true to previous research. The fragments found by Atrih et al (5) that corresponded with the G7 and G8 fragments found here, were not muramidase sensitive. They did not disappear when muramidase was added to the exudate, and were found in both types of exudate samples. The second possible reason why these peaks disappeared was due to

Figure 11. Structure of Enzymatic Products.

a.) Glucosamine Product.



B.) Muramidase Product

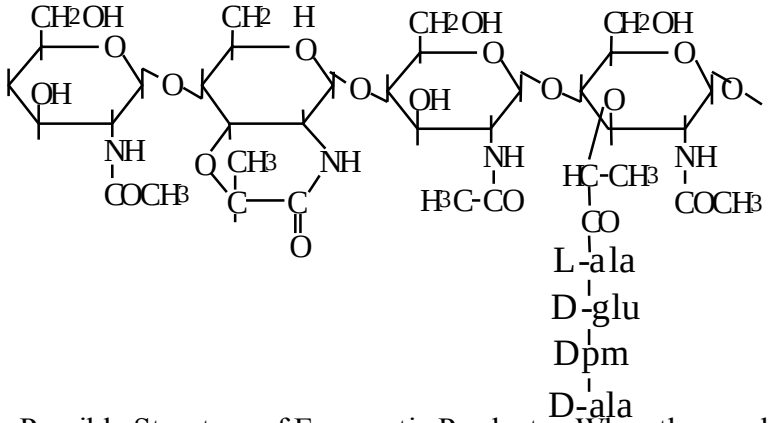


Figure 11. Possible Structure of Enzymatic Products. When the saccharide backbone was digested during germination, tetrasaccharides were the major product. The fragments could have been produced by glucosaminidase (a) or muramidase (b) activity. The resulting fragments are shown above.

another enzyme active in the exudate. When the exudate samples were processed, the digested samples were incubated overnight to allow for the muramidase digestion, however, it is possible that other enzymes in the exudate also acted on the muropeptides during this time. The undigested samples were never exposed to this incubation, and therefore, were not able to exhibit this type of enzymatic activity. The exudate samples were boiled immediately after they were removed from the spores during germination, however, this may not have denatured all of the lytic enzymes present in the exudate. Incubating the undigested sample in an identical manner as the digested sample could solve this problem. The disappearance of these peaks after this incubation would confirm that there was continuing lytic activity occurring in the samples after they were removed from the germination. The results of this experiment indicated that peaks G7 and G8 were not altered by simple incubation, indicating that their disappearance during Mutanolysin digestion was due to muramidase sensitivity rather than to the presence of a lytic enzyme in the exudate. Seeing that the peaks were Mutanolysin sensitive, the bond broken by a muramidase was therefore still present, and the production of these two tetrasaccharide fragments had to be from a different type of enzyme. The simplest conclusion is that these peaks are tetrasaccharides that were produced by a glucosaminidase, with glucosamine at their reducing ends. It is uncertain why Atrih et all found these muropeptides to be muramidase insensitive.

The second important observation, that confirms that glucosaminidase was the major enzyme acting on the cortex during germination, was that the major peak produced in the digested sample, G6, was not found in the undigested sample. This

meant that the formation of the peak was a result of the Mutanolysin digestion and not from enzymatic activity produced by the spores. The TriS-TP fragment was not produced by the spore, but rather the Mutanolysin digestion step, and the enzyme simply further broke down a fragment that was already present. This coincided with the idea that the major peaks in the undigested samples were Mutanolysin sensitive. Therefore, addition of Mutanolysin would result in the production of large amounts of trisaccharide fragments because these tetrasaccharide fragments produced by the spore would have muramidase sensitive bond. This bond was cleaved and resulted in a trisaccharide and a monosaccharide.

Further analysis of the G7 and G8 fragments were performed to determine their exact structure and the enzymatic activity that occurred to produce them. The fragments were collected as they eluted off the HPLC column and analyzed with amino acid and amino sugar analysis. The samples were run with NAM, NAG, Alanine, Glutamate, and Dpm standards. Additionally, reduced NAM and NAG, muramitol and glucosaminitol, were generated and run as standards as well. The amino acid analysis results of these fragments revealed that in the G7 and G8 fragments, a glucosaminitol was present and the muramitol was absent. In fragments produced by a muramidase, the reducing end contains a muramic acid, and the reduction for HPLC analysis causes the muramic acid to be reduced to muramitol. The absence of the muramitol in the G7 and G8 peaks, and the presence of the glucosaminitol proved that the reducing end of the fragment was not a muramic acid, but rather a glucosamine. This confirms the conclusion that the fragments were the result of a glucosaminidase.

A second confirmation test was also preformed. The G7 and G8 fragments were collected from the HPLC analysis. The fragments were then digested with Mutanolysin, reduced, and run on the HPLC. The results proved that the fragments were Mutanolysin sensitive because the peaks corresponding to the originally isolated fragments were no longer present. Additionally, in the G7 sample, not only did the G7 peak disappear, but also the G6 peak appeared. This confirmed that the major peaks in the undigested samples were Mutanolysin sensitive and that G6 in the digested samples was produced as a direct result from the digestion of the TS-TP (G7) fragment (Figure 12).

Final Discussion and Conclusions

Overall, many major accomplishments occurred in protocol development. *Bacillus anthracis* does not seem to sporulate as efficiently as some other sporulating bacterium, so obtaining a clean spore preparation for analysis was challenging. The metrizoic acid gradient procedure appeared to be the most effective way to clean the spores of all other debris. Additionally, a germination procedure was developed that could artificially stimulate the spores to germination at a very high rate. Additionally, procedures were adapted from *B. subtilis* experiments for PG extraction and analysis (15).

The *B. anthracis* spores did germinate at the same rate as *B. subtilis* when the OD change and the DPA release analyses were observed. Spores of both species have membrane modifications that allow for the release of DPA and the uptake of water at

Figure 12. Digestion of Major Germination Muropeptides.

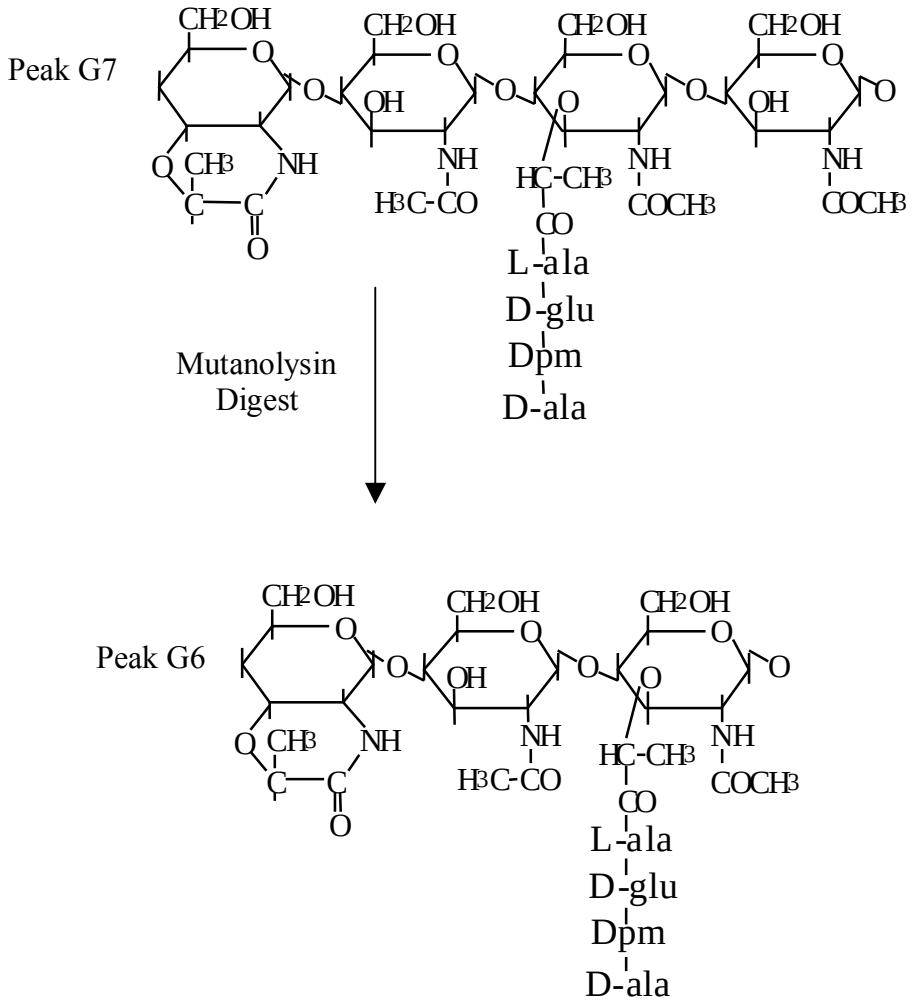


Figure 12. Digestion of Major Germination Muropeptides. The major peaks from the undigested exudate samples were collected, digested with Mutanolysin, reduced, and analyzed on the HPLC. The results showed that the digestion of the fragment found in peak G7 caused a shift in the peak, to the position of peak G6. This shift was caused by the structural changes shown in the figure.

the same rate. However, the results from the hexosamine and PG analyses showed that *B. anthracis* is much more rapid at breaking down the cortex then *B. subtilis*. The implications of this finding are yet to be determined, however, since *B. anthracis* is a pathogen, it may be beneficial for the spore to transition quickly from one state to the other.

The analysis of the dormant spore PG proved that *B. anthracis* did not have a significantly different structure from other studied *Bacillus sp.*. The backbone consists of alternating NAM and NAG sugar residues and the cortex had muramic-lactam modifications. From the amino acid analyses done on the fragments, it appeared that the peptide side chains were the same as *B. subtilis*, consisting of L-alanine, D-glutamate, meso-diaminopemelic acid, D-alanine. The presence of de-acetylated sugar residues on some fragments made the cortex structure more similar to *B. cereus* then to *B. subtilis*. There are no known advantages to having de-acetylated sugars in the cortex PG. However, de-acetylation of vegetative cell wall PG is known to make the PG insensitive to lysozyme digestion (1).

The germination lytic activity that occurred in *B. anthracis* spores was determined. Preliminarily, it appeared that there was glucosaminidase activity, and possibly muramidase activity. Peaks G7 and G8 were determined to be muramidase sensitive tetrasaccharides produced by the activity of a glucosaminidase. The structure of the tetrasaccharide produced was Muramic-lactam-NAG-NAM-NAG. The formation of the trisaccharide fragment in the Mutanolysin digested exudate samples was caused by further digestion of the G7 peak due to the presence of the NAM-NAG

bond. Additionally, the presence of fragments containing the anhydromuramic acid residues proved that lytic transglycosylase activity was occurring. However, this was not the major activity occurring.

Since the majority of this study was preliminary data, there is plenty of future work that can be performed involving these results. Initially, obtainment of mass spectrometry verified structures for all of the significant identified peaks is essential. This information will allow for a more complete understanding of the cortex structure and hydrolysis. A more clear determination of the lytic enzymes active during germination should also be completed. The creation of genetic mutations in the genes homologous to the known cortex lytic enzymes should be completed. The affect these mutations have on cortex hydrolysis will provide critical information. From there, germination lytic enzymes could be extracted from the exudate of germinating spores and used to treat spore PG to determine the exact function.

REFERENCES

1. **Atrih, A., G. Bacher, R. Korner, G. Allmaier, and S. J. Foster**. 1999. Structural analysis of Bacillus megaterium KM spore peptidoglycan and its dynamics during germination. Microbiology. **145**:1033-1041.
2. **Atrih, A. and S. J. Foster**. 2001. Analysis of the role of bacterial endospore cortex structure in resistance properties and demonstration of its conservation amongst species. J Appl Microbiol **91**:364-372.
3. **Atrih, A. and S. J. Foster**. 2001. In vivo roles of the germination-specific lytic enzymes of *Bacillus subtilis* 168. Microbiology **147**:2925-2932.
4. **Atrih, A., P. Zollner, G. Allmaier, and S. J. Foster**. 1996. Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. J Bacteriol **178**:6173-6183.
5. **Atrih, A., P. Zollner, G. Allmaier, M.P. Williamson, S. J. Foster**. 1998. Peptidoglycan structural dynamics during germination of *Bacillus subtilis* 168 endospores. J Bacteriol **180**:4603-4612.
6. **Boland, F. M., A. Atrih, H. Chirakkal, S. J. Foster, and A. Moir**. 2000. Complete spore-cortex hydrolysis during germination of *Bacillus subtilis* 168 requires SleB and YpeB. Microbiology **146**:57-64.
7. **Chen, Y, S. Fukuoka, and S. Makino**. 2000. A Novel Spore Peptidoglycan Hydrolase of *Bacillus cereus*: Biochemical Characterization and Nucleotide Sequence of the Corresponding Gene, *sleL*. J Bacteriol **182**:1499-1506.

8. **Chen, Y., S. Miyata, S. Makino, and R. Moriyama**. 1997. Molecular Characterization of a Germination-Specific Muramidase from *Clostridium perfringenes* S40 Spores and Nucleotide Sequence of the Corresponding Gene. J Bacteriol **179**:3181-3187.
9. **Chirakkal, H., M. O'Rourke, A. Atrih, S. J. Foster, and A. Moir**. 2002. Analysis of cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. Microbiology **148**:2383-2392.
10. **Cowan, A. E., D.E. Koppel, B. Setlow, and P. Setlow**. 2003. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. Proc Natl Acad Sci USA **100**:4209-4214.
11. **Foster, S. J., and K. Johnstone**. 1987. Purification and properties of a germination-specific cortex-lytic enzyme from spores of *Bacillus megaterium* KM. Biochem. J. **242**:573-579.
12. **Foster, S. J., and D. L. Popham**. 2002. Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers, and capsules, p. 21-41. *In* A.L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and Its Close Relatives: From Genes to Cells. American Society for Microbiology, Washington, D.C.
13. **Frenkiel-Krispin, D., R. Sack, J. Englander, E. Shimoni, M. Eisenstein, E. Bullitt, R. Horowitz-Scherer, C. S. Hayes, P. Setlow, A. Minsky, and S. G. Wolf**. 2004. Structure of the DNA-SspC Complex: Implications for DNA

packaging, Protection, and Repair in Bacterial Spores. J Bacteriol. **186**:3525-3530.

14. **Gilmore, M. E., D. Bandyopadhyay, A. M. Dean, S. D. Linnstaedt, and D. L. Popham.** 2003. Production of Muramic δ-Lactam in *Bacillus subtilis* Spore Peptidoglycan. J Bacteriol **186**:80-89.
15. **Guidi-Rontani, C., Y. Pereira, S. Ruffie, J. C. Sirard, M. Weber-Levy, and M. Mock**. 1999. Identification and characterization of a germination operon on the virulence plasmid pXO1 of *Bacillus anthracis*. Mol Microbiol. **33**:407-414.
16. **Ireland, J. A., and P. C. Hanna.** 2002. Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* DeltaSterne endospores: *gerS* mediates responses to aromatic ring structures. J Bacteriol **184**:1296-1303.
17. **Ishikawa, S., K. Yamane, and J. Sekiguchi**. 1998. Regulation and Characterization of a Newly Deduced Cell Wall Hydrolase gene (*cwlJ*) Which Affects Germination of *Bacillus subtilis* Spores. J Bacteriol **180**:1375-1380.
18. **Makino, S., N. Ito, T. Inoue, S. Miyata, and R. Moriyama**. 1994. A spore-lytic enzyme released from *Bacillus cereus* spores during germination. Microbiology **140**:1403-1410.
19. **Makino, S., and R. Moriyama.** 2002. Hydrolysis of cortex peptidoglycan during bacterial spore germination. Med Sci Monit **8**:RA119-127.
20. **Meador-Parton, J., and D. L. Popham**. 2000. Structural analysis of *Bacillus subtilis* spore peptidoglycan during sporulation. J Bacteriol **182**:4491-4499.

21. **Miyata, S., R. Moriyama, N. Miyahara, and S. Makino**. 1995. A gene (*sleC*) encoding a spore-cortex-lytic enzyme from *Clostridium perfringenes* S40 spores; cloning, sequence analysis and molecular characterization. Microbiology **141**:2643-2650.

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

23. **Moir, A. and D. A. Smith**. 1990. The genetics of bacterial spore germination. Annu Rev Microbiol. **44**:531-553.

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

25. **Moriyama, R., S. Kudoh, S. Miyata, S. Nonobe, A. Hattori, and S. Makino**. 1996. A Germination-Specific Spore Cortex-Lytic Enzyme from *Bacillus cereus* Spores: Cloning and Sequencing of the Gene and Molecular Characterization of the Enzyme. J Bacteriol **178**:5330-5332.

26. **Nicholson, W. L., and P. Setlow**. 1990. Sporulation, germination, and outgrowth. p. 391-450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, England.

27. **Paidhungat, M., K. Ragkousi, and P. Setlow**. 2001. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca(2+)-dipcolinate. J Bacteriol. **183**:4886-4893.

28. **Paidhungat, M., and P. Setlow**. 1999. Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. J Bacteriol **181**:3341-3350.

29. **Paidhungat, M. and P. Setlow**. 2000. Role of Ger Proteins in Nutrient and Non-nutrient Triggering of Spore Germination in *Bacillus subtilis*. J Bacteriol **182**:2513-2519.

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

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

32. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow**. 1996. Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. J Bacteriol **178**:6451-6458.

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

34. **Stewart G. S. A. B., M. W. Eaton, K. Johnstone, M. D. Barratt, and E. J. Ellar**. 1979. An investigation of membrane fluidity changes during sporulation and germination of *Bacillus megaterium* KM measured by electron spin and nuclear magnetic resonance spectroscopy. Biochem Biophys Acta **600**:270-290.

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

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

37. **Warth, A. D., and J. L. Strominger.** 1971. Structure of the peptidoglycan from vegetative cell walls of *Bacillus subtilis*. Biochemistry **10**:4349-4358.

38. **Weiner, M. A., T. D. Read, and P. C. Hanna**. 2003. Identification and characterization of the *gerH* operon of *Bacillus anthracis* endospores: a differential role for purine nucleosides in germination. J Bacteriol. **185**:1462-1464.

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- Graduate Teaching Assistant Workshop August 2004
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Presentations:

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Service:

- Lab Tour for the Microbiology Club October 2004

Awards and Honors:

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- Graduate Teaching Assistantship January 2004-May 2004
- Phi Beta Kappa Honor Fraternity May 2004- present
- Phi Kappa Phi Honor Society-Spring 2003-present
- College of Arts and Sciences Dean's Roundtable Scholar Fall 2002
- The National Dean's List Fall 2002
- College of Science Dean's List-Fall 2000-Spring 2004
- Virginia Tech Honors Program Fall 2000-Spring 2004

National Organizations:

- American Society of Microbiology- March 2005-present

Activities:

- American Society of Microbiology, Virginia Branch October 2004-present
- RHF Hall Council Representative September 2001-May 2002
- Alpha Epsilon Delta Sept 2001-May 2002
- Basketball Pep Band January 2001-May 2002, November 2004-March 2005)
- Symphony Band Spring 2001
- Marching Virginians August 2000-January 2004 (Rank Captain August 2001-present)
- Campus Crusade for Christ October 2000-December 2001
- NLCF October 2000-December 2001

Poster Presentations:

- "Cortex Peptidoglycan Analysis of Dormant and Germinating *Bacillus anthracis* spores" American Society of Microbiology, National Meeting, Atlanta, GA. June 8, 2005.
- "Cortex Peptidoglycan Analysis of Dormant and Germinating *Bacillus anthracis* Spores" Anthrax: The Biotechnology of Biodefense Symposium. Center for Structural Biology. Wake Forest University, Winston-Salem, NC. May 26, 2005.
- "Cortex Peptidoglycan Analysis of Dormant and Germinating *Bacillus anthracis* Spores" Department of Biology Research Day. Virginia Tech, Blacksburg, VA. March 19, 2005
- "Cortex Peptidoglycan Analysis of Dormant and Germinating *Bacillus anthracis* spores" American Society of Microbiology, Virginia Branch Annual Meeting, Bridgewater College, Bridgewater, VA. November 5, 2004