ANALYSIS OF THE ROLES OF THE *cwlD* OPERON PRODUCTS DURING SPORULATION IN *BACILLUS SUBTILIS*

Meghan E. Gilmore

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APPROVED

David L. Popham, Chairman

J.S. Chen

A.A. Yousten

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Analysis of the roles of the *cwlD* operon products during sporulation in *Bacillus subtilis*

Meghan E. Gilmore

David L. Popham, Chairman

Department of Biology

(ABSTRACT)

CwlD has sequence similarities to N-acetyl muramoyl-L-alanine amidases, a class of enzymes known to cleave the bond between the peptide side chain and the N-acetyl muramic acid residue in cortex peptidoglycan formation during sporulation. A major difference between vegetative peptidoglycan and spore peptidoglycan is the presence of muramic-δ-lactam (MAL) in spore peptidoglycan. It was previously determined that a *cwlD* null mutant does not contain muramic-δ-lactam in the spore cortex peptidoglycan and the mutant spores were unable to complete germination. Therefore, it is believed that CwlD plays a role in MAL formation during sporulation. However, the specific role of the protein had not been demonstrated. It was also previously found that *cwlD* is in a two-gene operon with *orf1*. Orf1 is produced within the forespore with CwlD. The hypothesized role of Orf1 is to inhibit CwlD activity from within the forespore. Muramoyl-L-alanine amidase activity was demonstrated by CwlD *in vivo*. Therefore, CwlD is carrying out the first step of MAL synthesis, cleaving the peptide side chain while other enzymes are needed to complete MAL formation. Two different forms of CwlD were over-expressed, with and without the protein's signal peptide sequence. Both forms of the protein were purified and in both cases activity was undetectable. Antibodies specific for CwlD were obtained which can be used in future research as a tool to further characterize CwlD activity.

A series of *B. subtilis cwlD* operon mutants were constructed altering the expression patterns of Orf1 and CwlD within the mother cell and forespore compartments. Various resistance properties and the germination ability of the mutant dormant spores were analyzed. It was determined that the absence of just Orf1 or Orf1 and CwlD from within the forespore has no effect on the phenotypes tested.

Peptidoglycan from developing mutant forespores was extracted and analyzed throughout sporulation. Evidence was obtained demonstrating that the role of Orf1 is not to inhibit CwlD from within the forespore as hypothesized.

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CHAPTER ONE

Review of the Literature

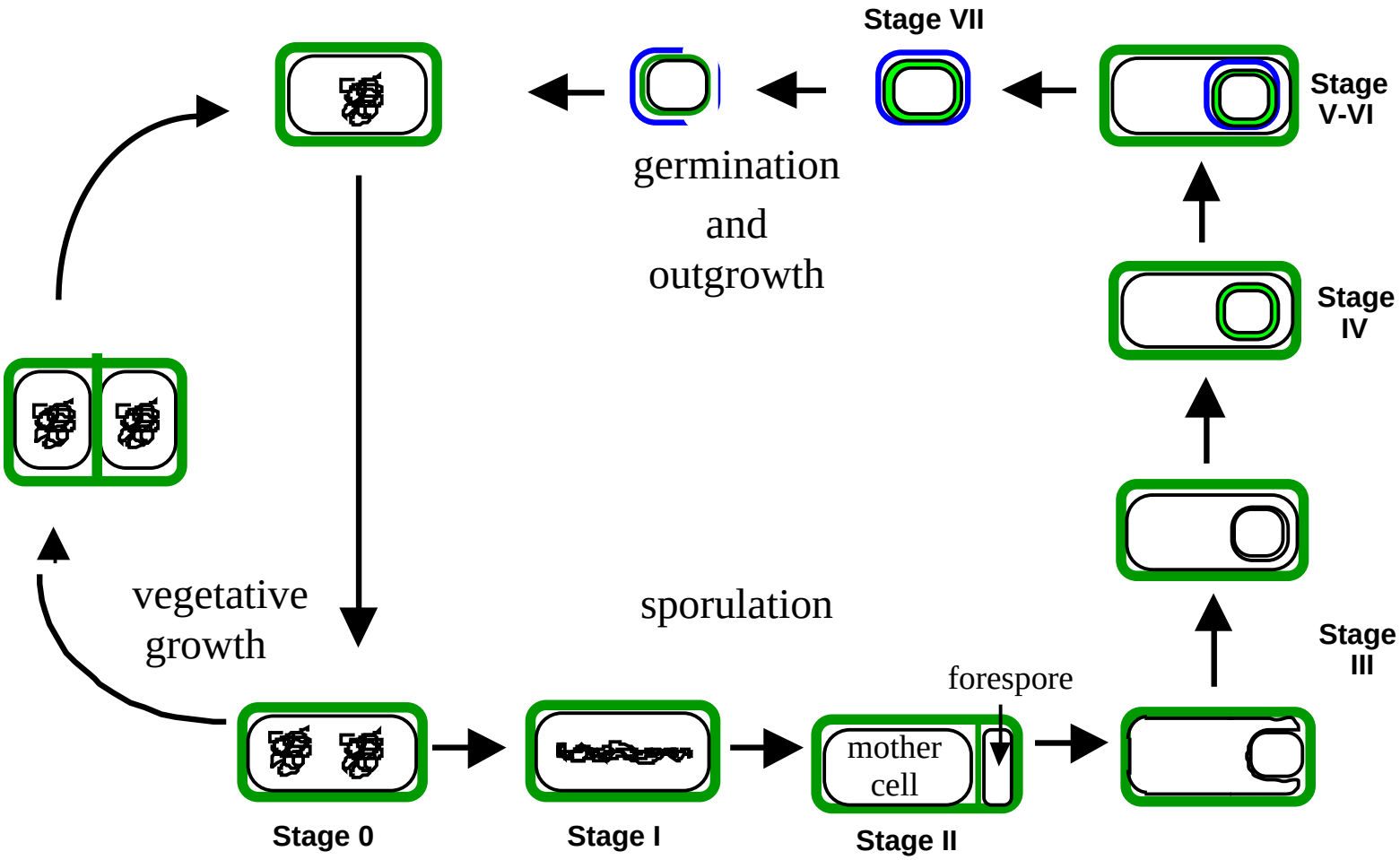
Stages of sporulation

Sporulation is a survival mechanism utilized by certain gram-positive bacteria, such as *Bacillus* and *Clostridium*, when they experience nutrient deprivation. The vegetative cell is able to sense the status of the environment and decide to continue dividing or begin sporulation. The formation of a bacterial endospore enables the organism to enter into a dormant state until its surroundings become favorable to resume vegetative growth. The process of sporulation involves the production of two distinct cells, containing identical genomes, which undergo a number of morphological changes.

The sporulation process has been described as occurring in stages (Fig.1). Stage 0 is the vegetative cell experiencing nutrient deprivation. The cell then enters stage I, which is marked by the DNA condensing into the center of the cell. During stage II, asymmetric septation occurs dividing the cell into two compartments, the mother cell and the forespore, each containing a full copy of the genome. The forespore is then engulfed by the mother cell forming two membranes around the developing spore, termed the inner and outer forespore membranes (stage III). Between these two membranes two distinct peptidoglycan layers are formed (stage IV). First, the thin germ cell wall is produced adjacent to the inner forespore membrane and secondly, the thicker, outer layer is formed known as the cortex. The spore is then surrounded with coat proteins and fully matures (stages V-VI). Finally, the mother cell lyses releasing the dormant spore (stage VII) (5, 8).

The process of sporulation involves a series of developmental stages that are controlled by over 100 different genes. The expression of these genes occurs with the sequential activation of σ factors, subunits of RNA polymerase that direct the protein to transcribe specific sets of genes (12). There are five σ factors involved in the regulation of gene expression during sporulation. The first sigma factor activated is σ^H controlling the genes directing asymmetric septation. After septation, σ^F and σ^E are activated in the forespore and mother cell, respectively, and control genes involved in engulfment, spore maturation, and germination. After engulfment, σ^G becomes active in the forespore while σ^K is activated in the mother cell and both control genes involved in spore maturation and germination (12).

Fig. 1. Stages of the *Bacillus subtilis* life cycle



Resistance properties of bacterial endospores

Bacterial endospores demonstrate high levels of resistance to different treatments such as heat, UV light, desiccation, certain chemicals, and enzymes (3). The spore peptidoglycan has a significant role in achieving these resistant properties and maintaining the metabolically inactive state of the spore (5). First, it has been shown that the presence of the spore peptidoglycan allows the spore core, the cytoplasm of the spore, to maintain a dehydrated state. As a result of this dehydration, the spore is able to remain dormant and exhibit heat resistant properties. Secondly, models have been proposed concerning the mechanism of achievement of dehydration, which utilize the spore peptidoglycan. They suggest that the cortex peptidoglycan is flexible and, therefore, could participate in reducing the spore core volume(13, 28). Reducing the volume could cause expulsion of water and, as a result, a dehydrated state of the spore. Lastly, upon germination, pores in the forespore membranes open and allow water to enter the spore core while releasing spore solutes. At the same time, germination lytic enzymes degrade the spore cortex and, finally, the vegetative cell is able to grow and break out of the spore coat (8).

Spore peptidoglycan

The structure of the peptidoglycan in the spore cortex differs from that contained within the vegetative cell wall. Vegetative cell wall peptidoglycan consists of glycan strands of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues. Peptide side chains are situated on each NAM residue, of which 40% are involved in cross-linking one glycan strand to another parallel strand. The cortex peptidoglycan within the spore contains two major structural differences when compared to the vegetative cell wall peptidoglycan (Fig. 2) (3, 20, 29-31). It was found that 50% of the NAM residues do not contain side chains and are converted into muramic-δ-lactam (MAL). These MAL residues occur in a regular pattern on every second NAM position within the glycan strand. It was also found that 25% of the NAM residues contain single L-Alanine side chains. The remaining 25% of NAM residues contain peptide side chains of which only 13% are involved in cross-linking. Due to the loss of the peptide side chains during the formation of MAL residues and the occurrence of single L-Alanine side chains the spore cortex peptidoglycan has a low level of cross-linking and, as a result, would exhibit more flexibility than the vegetative cell wall (18). This large range of

Fig. 2. The structure of spore peptidoglycan

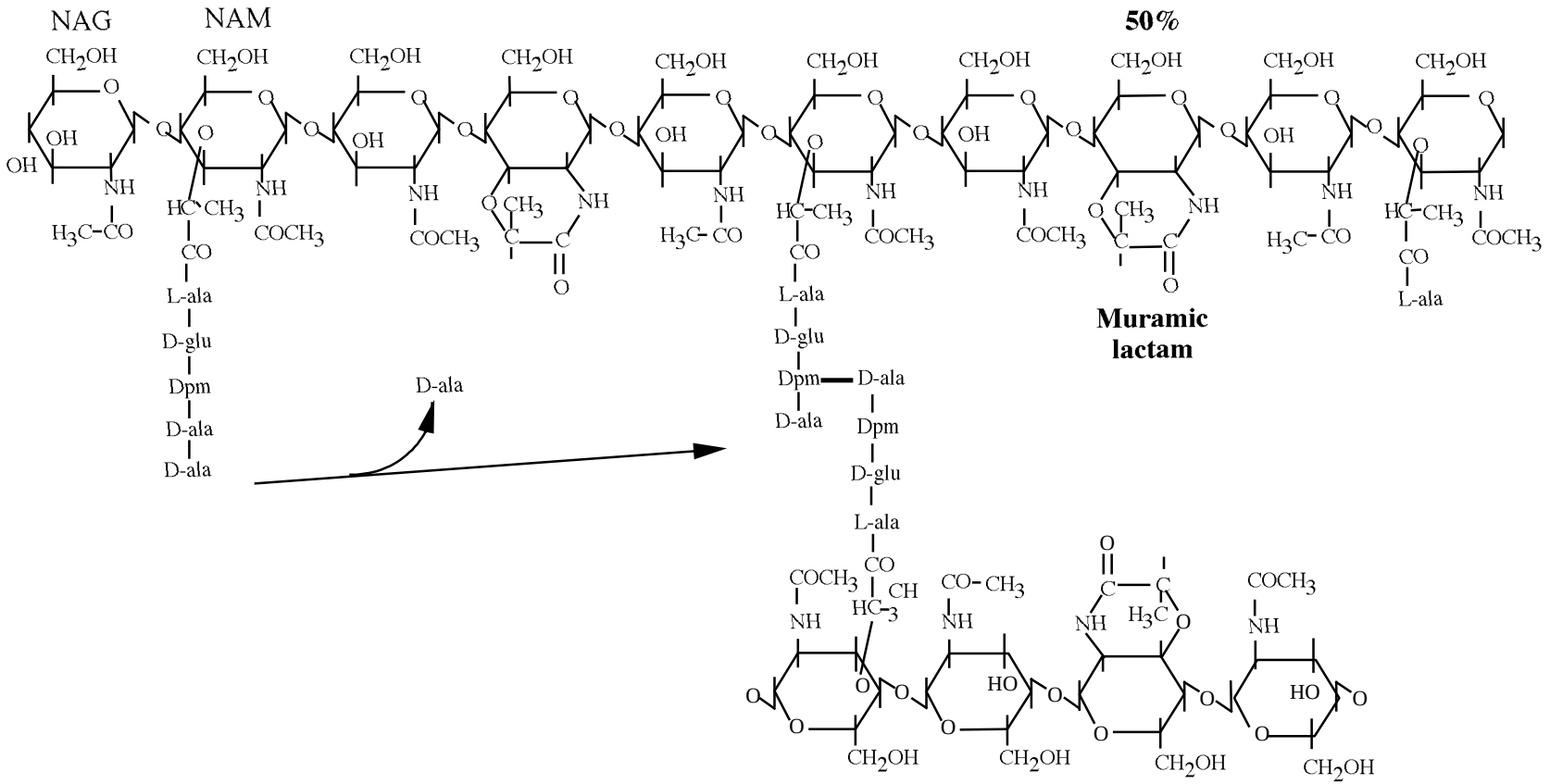


Figure 2. The structure of spore cortex peptidoglycan. Abbreviations: NAG, N-acetyl glucosamine: NAM, N-acetyl muramic acid

motion of the cortex peptidoglycan supports the model proposing that the cortex is able to contract to attain spore core dehydration.

The CwlD protein

While sequencing part of the *Bacillus subtilis* genome, Sekiguchi *et al*. found an open reading frame that appeared to encode a 27 kDa polypeptide. A search of protein databases revealed that the predicted amino acid sequence showed 35% identity over 191 residues to an autolysin produced in *B. subtilis* (25). Autolysins are peptidoglycan lytic enzymes that are found within vegetative cells and spores (21). They are known to play a role in cortex maturation, mother cell lysis, and cortex hydrolysis during germination. There are two major classes of autolysins produced in *B. subitlis*. Class I includes CwlA and CwlL and class II includes CwlB, CwlM, and CwlC (25). "Cwl" stands for cell wall lysis due to the enzyme's activity of cleaving part of the cell wall. Therefore, they called the predicted autolysin CwlD and determined that it had the highest sequence similarity to CwlB. CwlB is an N-acetylmuramoyl-L-alanine amidase (25), a class of enzymes that is found in many bacterial species and is involved in cleaving peptidoglycan. Specifically, these enzymes cleave the amide bond between the L-Alanine of the peptide side chain and the NAM residue to which it is attached (25). The predicted amino acid sequence also revealed that the N-terminal region contains a string of hydrophobic residues that could act as a signal peptide or membrane anchor (25).

Phenotypic characteristics of the *cwlD* mutant

A *cwlD* null mutant was constructed and it was found to have normal cell growth, cell separation, motility, and produced refractile spores equal in amount to wild type (21, 25). The mutant spores can carry out the first steps of germination such as rehydration of the spore core and the release of the spore solute, dipicolinic acid (DPA) equal to wild type, therefore, causing a decrease in optical density. However, the germination lytic enzymes are unable to degrade the cortex peptidoglycan and, as a result, the spore can not complete outgrowth to form colonies (21, 25). In order to determine if the alteration in the spore peptidoglycan structure of the *cwlD* mutant affected the heat resistance of the mutant spore, the block in outgrowth was overcome. First, the spore coat proteins were chemically permeabilized and the spores were treated briefly with lysozyme in an isotonic solution (21). It was found that the *cwlD* mutant spores

demonstrated heat resistance equal to that of wild type (21). The *cwlD* mutant dormant spores and spores in the first stages of germination were compared to wild type through the use of transmission electron microscopy. The staining of the dormant mutant spore was similar to the wild type spore. Upon germination, the wild type spore showed changes in the staining of the cortex and protoplasm while showing an increase in size due to the intake of water into the spore core. However, the *cwlD* mutant spore showed no significant changes in staining or size (21). Therefore, it is evident that germination is blocked when *cwlD* is not expressed.

When examining the structure of the mutant spore peptidoglycan through the use of reversed phase-high performance liquid chromatography (RP-HPLC), Popham *et al.* and Atrih *et al.* discovered the *cwlD* mutant spores did not contain MAL in the cortex peptidoglycan (3, 21). There was also a two-fold increase in the number of peptide side chains involved in cross-linking glycan strands which would lead to less flexibility of the cortex. The mutant spores also showed little change in spore core dehydration as compared to wild type (21, 23). Since the spores showed relatively normal spore core dehydration even though there was an increase in cross-linking, it is possible that flexibility of the cortex may not be important in achievement of dehydration (23). The spore peptidoglycan of mutants lacking other autolysins such as CwlA, CwlB, CwlC, CwlG was analyzed. There were no structural changes seen in the spore peptidoglycan and the spores demonstrated wild type heat resistance (21).

The fact that a *cwlD* null mutant produces spores without MAL in the cortex peptidoglycan and these spores are unable to complete germination suggests that MAL is a specificity factor for germination lytic enzymes (21). This conclusion would support the idea that the reason the germ cell wall is not degraded by germination lytic enzymes is due to the fact that it does not contain MAL. Therefore, the germ cell wall remains intact during germination and can act as a template for the synthesis of the vegetative cell wall, while the cortex is rapidly degraded. Further evidence supporting the theory of MAL acting as a specificity factor is the observation that some purified spore lytic enzymes are capable of degrading wild-type spore peptidoglycan, but are unable to degrade *cwlD* spore peptidoglycan that lacks MAL (6).

Synthesis of muramic-δ-lactam

Muramic-δ-lactam is a common element found in the peptidoglycan of spores from many bacterial species (21). It is believed that CwlD plays a role in formation of MAL from NAM

during spore peptidoglycan synthesis. However, the specific substrate used and final product produced by CwlD in this pathway is unknown. A proposed pathway of synthesis of MAL from NAM begins with a muramoyl-L-alanine amidase cleaving the amide bond between the peptide side chain and the NAM residue (Fig. 3). Next, the acetyl group must be removed in order for lactam cyclization to occur. It is possible that these steps can occur at the same time by one enzyme or in a sequential manner utilizing different enzymes. Does CwlD carry out the entire process of MAL formation from NAM? Or does this protein produce an intermediate in the pathway while other enzymes are needed to complete MAL formation?

The *cwlD* operon

While sequencing part of the *B. subtilis* genome, Sekiguchi *et al*. also found a second open reading frame upstream of *cwlD* termed *orf1* (25). Through northern blot analysis of the RNA transcripts containing *cwlD,* they determined the gene is in a two-gene operon with *orf1* and the operon contains two promoters. They found *cwlD* was transcribed alone in the mother cell approximately three hours after t_0 (estimated beginning of sporulation) from a σ^E-dependant promoter. Approximately 4.5 hours after t_0, they saw *cwlD* and *orf1* transcribed together in the forespore from a σ^G-dependant promoter (Fig. 4). A search of protein databases showed that the predicted amino acid sequence for *orf1* did not show any sequence homology to other proteins. Unlike CwlD, Orf1 did not contain a signal sequence; however, it did contain a region with a potential helix-turn-helix structure which could be required for protein-DNA interactions (25).

Since CwlD contains a signal peptide it is possible that the protein is transported across a membrane. Therefore, CwlD produced in the mother cell could be transported across the outer forespore membrane and CwlD produced in the forespore could be transported across the inner forespore membrane into the intermembrane space to act on forming peptidoglycan. This hypothesis seems logical for CwlD produced in the mother cell for two reasons. First, it is known that cortex peptidoglycan is made from precursors that are produced in the mother cell and transported into the intermembrane space (27) and, second, the cortex does contain MAL (21). However, why is CwlD produced within the forespore? If the forespore-produced CwlD is transported across the inner forespore membrane it would most likely act on the first layers of peptidoglycan produced, the germ cell wall (15). However, the germ cell wall does not contain MAL (3, 31). Therefore, it is our hypothesis that the role of Orf1 is to somehow inhibit CwlD

Fig. 3. The predicted steps in the synthesis of muramic-δ-lactam

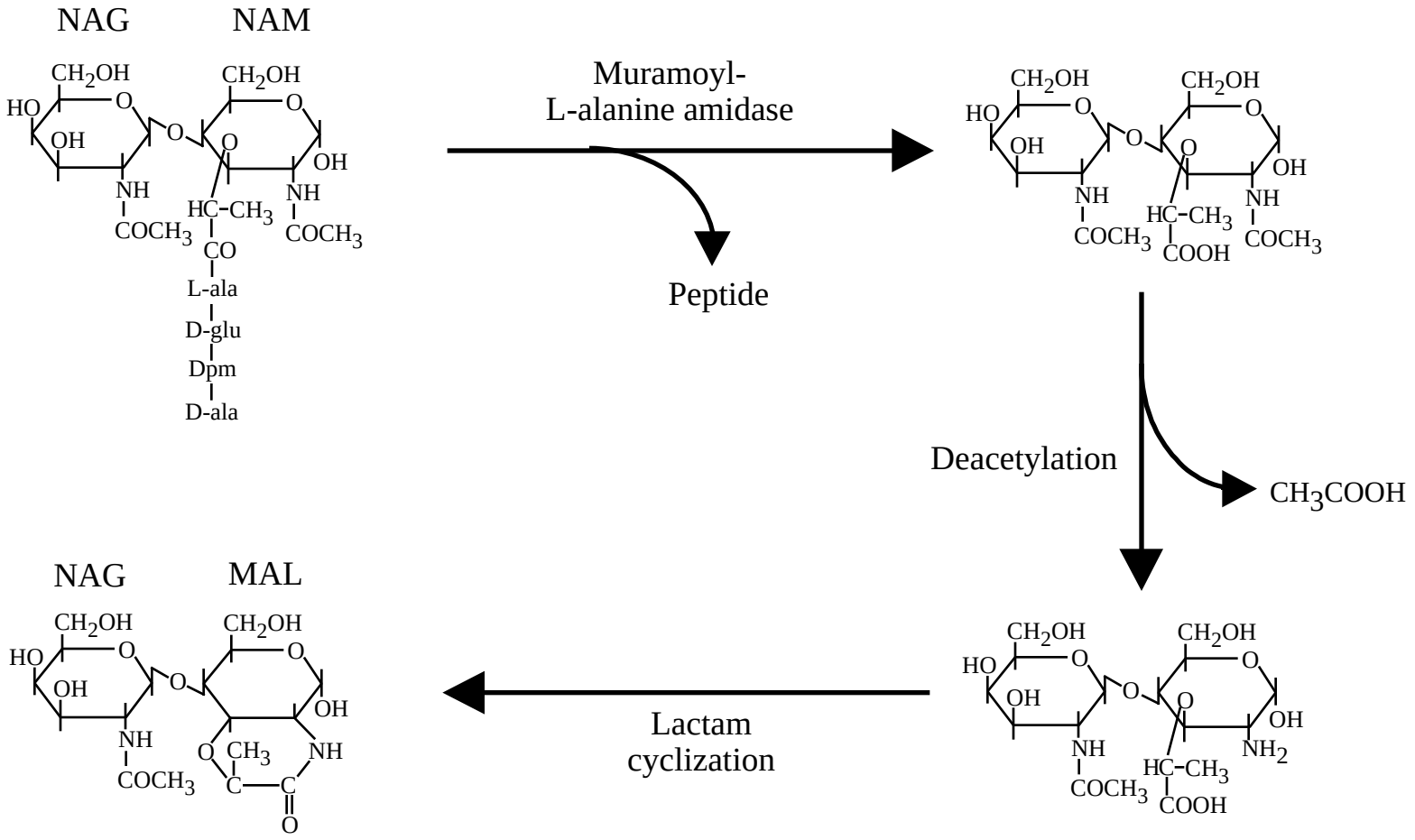


Figure 3. The predicted steps in the synthesis of muramic-δ-lactam from muramic acid during spore peptidoglycan synthesis. Abbreviations: NAG, N-acetyl glucosamine; NAM, N-acetyl muramic acid; MAL, muramic-δ-lactam.

activity from within the forespore. As a result, the forespore-produced CwlD would be unable to act on the first layers of peptidoglycan synthesized resulting in the absence of MAL in the germ cell wall.

Fig. 4. The *cwlD* Operon

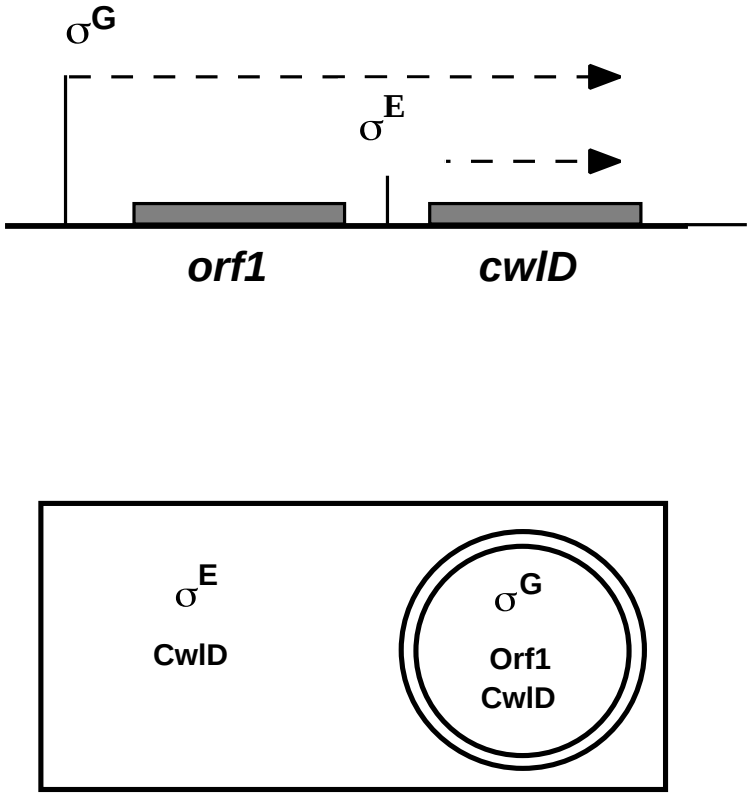


Figure 4. The *cwlD* operon (top) and expression patterns of *orf1* and *cwlD* within the mother cell and the developing forespore (bottom).

CHAPTER TWO

The Biochemical Analysis of CwlD

The first goal of the biochemical analysis of CwlD was over-expression in *E.coli* and purification of the protein. The second goal was to use the purified protein to demonstrate muramoyl-L-alanine amidase activity by CwlD on purified peptidoglycan. Finally, through the analysis of the peptidoglycan structure the enzyme's substrate specificity and the structure of the final product would be determined.

Materials and Methods

Construction of strains and plasmids

Restriction enzymes were purchased from Promega and digestion was carried out for 1 hour at 37^0C. DNA was purified from low melt agarose gel slices with Wizard PCR Preps DNA purification system. DNA was ethanol precipitated with 1/10 volume of 3 M sodium acetate pH 5.2 and 3 volumes of ethanol. Ligations were carried out at 25^0C with T4 DNA ligase (Promega). Plasmid DNA was transformed into *E.coli* competent cells, strain JM109, through heat shock at 42^0C and gentle agitation in rich medium for 1 hour at 37^0C.

Plasmid DNA was extracted with the plasmid miniprep procedure from 3 ml of turbid culture. First, the cells were pelleted, resuspended in 100 µl GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl pH 8.0), and 200 µl of lysis solution (0.2 N NaOH, 1% sodium dodecyl sulfate (SDS) (Sigma)) was then added. Next, the suspension was mixed with 150 µl 3 M cold potassium acetate, 2 N glacial acetic acid and placed on ice for 5 minutes. Samples were centrifuged 15,000 x *g* for 10 minutes at 4^0C and the supernatant was transferred to a new tube. RNase A (50 µg) (Sigma) was added and samples were placed at 37^0C for 30 minutes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and samples were mixed and centrifuged (15,000 x *g* for 3 minutes). The top layer was then transferred to a new tube, 1 ml cold ethanol was added to the aqueous phase and left at 25^0C for 10 minutes. The samples were centrifuged (15,000 x *g,*10 minutes), the pellet was vacuum dried, and resuspended in water.

***E.coli* strain construction and growth media**

All *E.coli* strains were grown in 2xYT with the appropriate drug additions (24). *E.coli* strain, DPVE16, over-expresses CwlD without the signal peptide region and with a N-terminal 6x His affinity tag. First, plasmid pDPV28 was constructed with the PCR fragment obtained from CwlD primers 3 and 4 (Table 1). CwlD primer 3 begins after the signal peptide region, therefore, excluding the first 21 codons of the *cwlD* gene and adds on a *Sal*I site. CwlD primer 4 includes the stop codon of *cwlD* and adds on *Bam*HI and *Hin*dIII restriction sites. The PCR product was cloned into pUC19 at the *Hin*cII restriction site. Next, plasmid pDPV28 was cut with *Bam*HI and *Sal*I to obtain a 650 bp fragment carrying *cwlD* without the signal peptide region. The over-expression vector, pET15b (Novagen), was cut with *Xho*I and *Bam*HI and the *cwlD* fragment was inserted to produce plasmid pDPV32. This plasmid was then transformed into the over-expression host, DPVE13 (Novagen induction host BL21 λDE3 pLysS), an *E.coli* strain containing a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. Therefore, when IPTG is added to a growing culture, T7 RNA polymerase is expressed, binds the T7 promoter, and transcribes *cwlD*. This over-expression strain also carries a pLysS plasmid that produces a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase. Normally the cell can tolerate the T7 lysozyme; however, when the cell membrane is disrupted through freeze thawing, the lysozyme is able to penetrate the inner membrane and causes the cells to lyse.

E.coli strain, DPVE14, over-expresses CwlD with the signal peptide region and containing a C-terminal 6x His affinity tag. First, plasmid pDPV25 was constructed with the PCR product from CwlD primers 1 and 6 (Table 1). CwlD primer 1 begins at the second codon of the *cwlD* gene and adds on a *Bam*HI restriction site and CwlD primer 6 starting at the end of the *cwlD* gene adds on a *Xho*I restriction site. The PCR fragment was cloned into the *Hin*cII site of pUC19 to produce pDPV25. Plasmid pDPV25 was cut with *Bam*HI and *Xho*I to obtain the full-length *cwlD* gene. This 720 bp fragment was cloned into the *Bam*HI and *Xho*I site of pET21a over-expression vector (Novagen) to produce plasmid pDPV29. This vector system over-expresses *cwlD* in the same manner as explained above when transformed into the over-expression host DPVE13.

Table 1. Primer sequences used to construct strains.

Name	**Primer sequence**	**Restriction enzyme site added**	**Placement in chromosome**
CwlD 1	5’ CGG GAT CCA GGA AAA AGC TTA AAT GG	*Bam*HI	2nd codon in *cwlD* N-terminus, signal peptide
CwlD 3	5’ GTC GAC AAG TAT CAG TTC AGC AAT	*Sal*I	22nd codon in *cwlD* N-terminus, no signal peptide
CwlD 4	5’ GGA TCC AAG CTT ACT CCG GAG GGT CTC C	*Bam*HI, *Hin*dIII	C-terminus of *cwlD*, stop codon
CwlD 6	5’ CTC GAG CTC CGG AGG GTC TCC TTT	*Xho*I	C-terminus of *cwlD*, no stop codon
Cwl I	5’ CGG GAT CCT TAG AAG ACC AAA TCA AAG GCC	*Bam*HI	270 bp upstream of *orf1*
Cwl III	5’ GCG CGC ATG CGG GTG TCT TAT GCT TAG AACC	*Sph*I	18 bp downstream of *cwlD*

Induction of CwlD

Induction of CwlD over-expression strains, DPVE16 and DPVE14, was carried out in 2xYT media at 37°C. Cells were grown to an optical density of approximately 0.5 (Spectronic Genesys 5, A_{600}) and IPTG was added to a final concentration of 1mM to induce expression of T7 RNA polymerase from the *lacUV5* promoter. DPVE16 and DPVE14 cultures were then shifted to 30°C for 2 hours and for 1 hour, respectively. Cells were harvested with centrifugation (9,800 x g, 10 minutes, 4 °C) and were stored at -80 °C. Induction of the protein was verified using SDS-PAGE with cell lysates from induced and non-induced cultures.

Separation of protein fractions

The soluble, insoluble and membrane protein fractions were separated with centrifugation. First, cell pellets from 300 ml of culture were resuspended in 30 ml buffer A (0.1 M Tris HCl pH 8, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol (Sigma), 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) (Jersey Lab Supply)) and sonicated. The broken cells were then centrifuged at 7700 x g (Beckman J2-21 centrifuge) for 15 minutes at 4°C. The pellet contains the insoluble fraction. The supernatant was then centrifuged at 48,000 x g (Beckman L8-M ultracentrifuge) for 1 hour at 4°C. The supernatant is the soluble fraction and the remaining pellet was resuspended in 30 ml Buffer B (50 mM Tris HCl pH 8, 1 mM β-mercaptoethanol, 0.1 mM PMSF) and centrifuged at 48,000 x g for 1 hour at 4°C. The pellet was again resuspended in 30 ml buffer B and centrifuged at 3,000 x g for 5 minutes at 4°C. Any remaining pellet is insoluble material, which was discarded, and the supernatant is the membrane fraction.

Purification of CwlD with metal affinity chromatography

The *E.coli* strain DPVE16 was induced for over-expression of CwlD without the signal peptide region and containing a N-terminal 6x His tag. The soluble fraction from the induced cells was obtained and used in the purification process. To isolate CwlD, Talon metal affinity resin (Clontech) was used which utilizes electropositive transition metals to specifically bind the histidine residues attached to the protein of interest. First, the resin was prepared through centrifugation at 700 x g for 2 minutes and washed twice in sonication buffer (20 mM Tris HCl pH 8, 100 mM NaCl). The washed resin was then added to 15 ml soluble protein fraction and incubated with gentle agitation at 25°C for 20 minutes. The mixture was then centrifuged at 600

x g for 5 minutes and supernatant was removed. The resin was resuspended in sonication buffer, agitated at 25°C for 10 minutes and centrifuged at 600 x g for 5 minutes. The washing was repeated and the resin was then transferred to a Talon 2 ml disposable column (Clontech). First, the resin bed was washed with sonication buffer. Second, elution buffer (50 mM $NaPO_4$ pH 5, 20 mM MES pH 5, 100 mM NaCl) was slowly added to the resin and the liquid was collected from the bottom of the column. Addition of elution buffer was repeated and a solution of elution buffer containing 1% triton X-100 (Fisher) and 5 mM β-mercaptoethanol was added and the liquid passing through column was collected. The elution buffer, triton, and β-mercaptoethanol solution was again added to the resin bed and collected. Lastly, 100 mM EDTA pH 8.0 was added and collected from the column twice to strip the metal ions from the resin. The collected fractions were then analyzed with SDS-PAGE to determine if the 27 kDa protein believed to be CwlD was obtained.

Purification of CwlD with anion exchange chromatography

E.coli strain DPVE14 was induced for over-expression of CwlD containing the signal peptide. The membrane fraction containing CwlD was obtained and solubilized for use in the purification procedure. Solubilization of the membrane fraction was carried out by adding 50 mM Tris HCl pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol, and 1% triton X-100. The solution was incubated at 4°C for 1 hour and centrifuged at 48,000 x g for 1 hour at 4 °C. The supernatant, which contains the solubilized protein was then diluted 10-fold with buffer A (10 mM Tris HCl pH 8.0, 1 mM β-mercaptoethanol, 0.1 % triton X-100). The diluted, solubilized membrane fraction was loaded on a source 30-Q anionic exchange column (Amersham/Pharmacia) and the protein was eluted with an increasing gradient of buffer B (10 mM Tris HCl pH 8.0, 1 mM β-mercaptoethanol, 1 M NaCl, 0.1% triton X-100). The eluted fractions were collected and analyzed with SDS-PAGE to determine which contained the 27 kDa CwlD protein.

Purification of *Bacillus subtilis* vegetative peptidoglycan

PS832 (*Bacillus subtilis* wild-type strain) was grown in 250 ml 2xYT media to an optical density of 1.0 (Spectronic Genesys 5, A_{600}). The culture was rapidly chilled in an ice water bath and centrifuged at 12,400 x g for 10 minutes at 4°C. The pellets were resuspended in

4 ml cold water, dripped into 100 ml boiling 4% SDS, and boiled for 30 minutes. The cell/SDS solution was left to cool to room temperature and was then centrifuged for 6 minutes (9,800 x g, 25°C). The pellet was resuspended in 40 ml 50°C H_2O and centrifuged as before. After the pellet was washed 7 times with H_2O, the supernatant was tested for SDS (11). If SDS was not completely removed washing was continued as stated above until SDS was no longer detected. The pellet was then resuspended in a total volume of 2 ml with 100 mM Tris HCl pH 7.5 with 200 µg α-amylase (Sigma) and incubated at 37°C for 2 hours. Then 20 µg DNase I (Sigma), 100 µg RNase A (Sigma), and $MgSO_4$ were added (final concentration of 20 mM) and the suspension was incubated again at 37°C for 2 hours. Finally, 200 µg trypsin (Worthington TPCK) and $CaCl_2$ were added (final concentration of 10 mM) and incubated at 37°C for 16 hours. SDS was then added to 1%. The suspension was boiled for 15 minutes and centrifuged at 15,000 x g for 10 minutes at 20 °C. The pellet was washed twice in 16 ml H_2O, once in 8 M LiCl, twice in H_2O, and centrifuged (15,000 x g, 5 minutes). The supernatant was removed and the pellet was lyophilized for 1 hour. The dried pellet was resuspended in 2 ml 49% hydrofluoric acid (HF) to remove teichoic acids and was agitated for 48 hours at 4°C. The HF/wall suspension was then dripped into 26 ml 1 M NH_4HCO while on ice, placed in a polycarbonate centrifuge tube, and centrifuged at 17,000 x g for 15 minutes at 20°C. The pellet was then washed three times with 10 ml water and centrifugation at 15,000 x g for 10 minutes at 20°C. The washed pellet was resuspended in 1 ml 100 mM NH_4HCO_3 with 5 units of alkaline phosphatase and incubated at 37°C for 24 hours. The suspension was then boiled for 5 minutes, centrifuged (15,000 x g, 15 minutes), and the pellet was washed 3 times with 10 ml water as described above. Finally, the pellets were resuspended in a small volume of H_2O and stored at –20°C.

Purification of *E.coli* peptidoglycan

E.coli strain DPVE13 was grown in 2 liters of 2xYT media with 30 µg/ml chloramphenicol to an optical density of 3.0 (Spectronic Genesys 5, $A_{.600}$). The cells were harvested (11,000 x g, 10 minutes, 4°C) and resuspended in a total of 25 ml of cold H_2O. The cell suspension was dripped into 300 ml of boiling 4% SDS, allowed to boil for 30 minutes, and cooled to room temperature. The cell/SDS solution was centrifuged (48,000 x g, 1 hour, 25oC) and washed in a total volume of 60 ml of H_2O until SDS was no longer detected (11). Pellets were then resuspended in a total volume of 5 ml with 100 mM Tris HCl pH 8.0 with α-amylase

(500 µg) and incubated at 37°C for 2 hours. DNase I (50 µg), RNase A (250 µg), and 20 mM $MgSO_4$ was added and incubated again for 2 hours at 37°C. Lastly, trypsin (500 µg) and 10 mM $CaCl_2$ was added and incubated for 16 hours at 37°C. SDS was added to 1%, the solution was boiled for 15 minutes, centrifuged (48,000 x g, 10 minutes, 25°C), and resuspended in a total of 45 ml of H_2O. Washing was continued (48,000 x g, 1 hour, 25°C) until SDS was undetectable. Finally, pellets were resuspended in a total of 3 ml of H_2O and stored at –80°C.

Testing activity of purified CwlD *in vitro*

B.subtilis (PS832) vegetative and *E.coli* (DPVE3) purified peptidoglycan (0.05 O.D. units) was added to CwlD purified from DPVE16 in a total volume of 1 ml with 20 mM Tris HCl pH 7.0 and 2 mM $MgCl_2$. The $O.D._{600}$ was measured every 15 minutes with incubation at 37°C in between readings.

Activity of CwlD purified from DPVE14 through anion exchange chromatography on DPVE13 purified peptidoglycan was detected through HPLC analysis. The protein fraction (50 µl) was mixed with DPVE13 peptidoglycan at an O.D. of 1.0 in a total volume of 58.5 µl and the mixture was incubated at 37°C for 1 hour. Triton X-100 (500 µl) was added to 1 % and the sample was centrifuged at 48,000 x g for 1 hour at 4 °C (Beckman TL-100 ultracentrifuge). The supernatant was removed, the pellet was prepared for HPLC analysis as previously described (20). Separation of muropeptides by reversed-phase HPLC was carried out as described (20).

Detecting CwlD activity *in vivo*

Peptidoglycan was extracted from *E.coli* strain DPVE14, from cells that were induced for over-expression of CwlD and from non-induced cells. The peptidoglycan was prepared for HPLC analysis as described (20) and the purified muropeptides were identified and quantified using amino acid analyses and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (21).

The membrane fraction (50 µl) was isolated from *E.coli* strain DPVE14 and mixed with *E.coli* (DPVE13) purified peptidoglycan (50 µl) in the presence and absence of triton added to 1%. The reactions were incubated at 37°C for 1 hour, and the samples were centrifuged to pellet the peptidoglycan (48,000 x g, 1 hour, 4°C). The peptidoglycan was prepared for HPLC analysis as previously described (20).

Production and Purification of polyclonal anti-CwlD antibodies

The purified CwlD protein which contains a His tag and does not contain its signal peptide region (1 mg) was extracted from an SDS containing polyacrylamide gel and used in injection of rabbits for antibody production (Cocalico Biologicals, Inc.). Antibodies were affinity purified with 15 mg of purified CwlD covalently coupled to a 4 ml of Affi-Gel 10 activated resin (Bio-Rad). The coupling was carried out in 5 ml of 10 mM EDTA pH 8.0, 0.1% triton X-100 buffer with 3 M guanidine HCl for 16 hours at 4^{o}C. The coupling reaction was stopped by adding ethanolamine to 100 mM for 1 hour at 4^{o}C. The unbound protein was removed with washing three times in 10 ml of 20 mM Tris HCl pH 7.2, 150 mM NaCl, 1 mM β-mercaptoethanol, 0.1% tween-20 at 4^{o}C for 10 minutes. An 18% coupling efficiency was obtained based on the amount of protein present in solution before and after coupling (Lowry protein assay). The resin was transferred to a Talon 2 ml disposable column (Clontech), 1ml of serum was added, collected, and passed over resin two more times. Resin was washed with 20 volumes 10 mM Tris HCl pH 7.2, 0.3 M NaCl and 0.05% Tween-20. Antibodies were eluted with 10 volumes of 0.1M glycine pH 2.5 and neutralized with 1/10 volume of 1 M Tris HCl pH 7.2. Immunoreactivity of the purified antibody was tested with western blot analysis.

Results from the Biochemical Analysis of CwlD

Strains analyzed

Two different forms of CwlD, over-expressed by *E.coli* strains DPVE14 and DPVE16, were used in this analysis. Both strains contained the *cwlD* gene under the control of the T7 *lac* promoter and a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. Therefore when IPTG is added, T7 RNA polymerase is expressed, binds to the T7 *lac* promoter and begins transcription of the target gene. DPVE14 over-expresses the full-length CwlD protein containing the signal peptide region and a C-terminal 6x His affinity tag useful in purification. DPVE16 over-expresses the protein without its signal peptide region; however, containing a N-terminal His tag.

Induction of CwlD

Upon addition of IPTG, CwlD was over-expressed which was evident through SDS-PAGE with the cell lysates from an induced and a non-induced culture (Fig. 5). CwlD is believed to be present based on the observation of a protein band in the induced culture which is absent in the non-induced culture. Furthermore, the band expressed only in the induced culture has a molecular weight similar to the predicted weight of CwlD, approximately 27 kDa (25). However, CwlD expressed by DPVE14 upon induction contains the signal peptide region and, therefore, should run slightly higher than CwlD that does not contain the signal peptide region produced by DPVE16. In addition, the CwlD protein over-expressed in both strains should be slightly heavier than the predicted 27 kDa since they are carrying six extra histidine residues due to the addition of the purification tag. Based on these observations we concluded CwlD was successfully over-expressed.

Isolation of protein fractions

After over-expressing the protein we isolated the insoluble, soluble, and membrane protein fractions from the cell lysates with centrifugation. Examination of the protein fractions with SDS-PAGE allowed us to determine approximately where CwlD was localized within the cell (Fig. 6). DPVE14, the *E.coli* strain producing CwlD with its signal peptide, showed the protein to be partially insoluble; however, it appears to be concentrated in the membrane

Fig. 5. SDS-PAGE demonstrating the over-expression of CwlD in *E.coli* strains

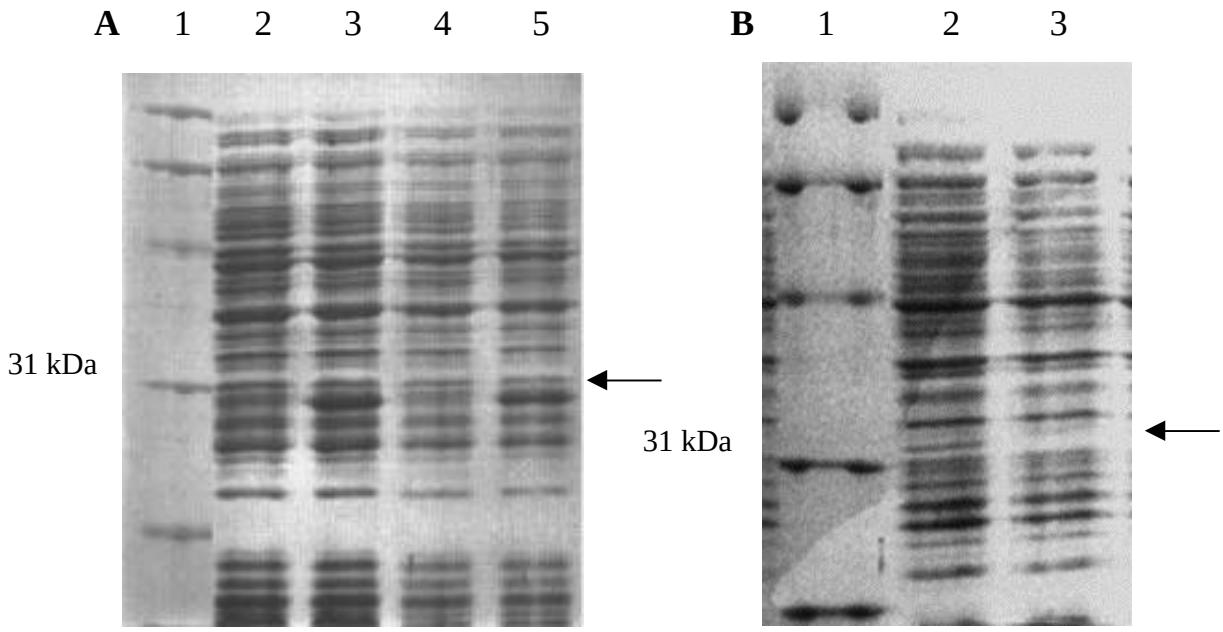


Figure 5. SDS-PAGE demonstrating the over-expression of CwlD from two different *E.coli* strains. Arrows indicate CwlD and 31 kDa molecular weight marker is noted.
Fig. 5A: Over-expression of CwlD without its signal peptide from strain DPVE16. Lane1, molecular weight markers; lane 2 and 4, cell lysate from non-induced culture; lanes 3 and 5, cell lysate from induced culture.
Fig. 5B: Over-expression of CwlD with its signal peptide from strain DPVE14. Lane 1, molecular weight markers; lane 2, cell lysate from induced culture; lane 3, cell lysate from non-induced culture.

Fig. 6. SDS-PAGE of separated protein fractions demonstrating CwlD localization

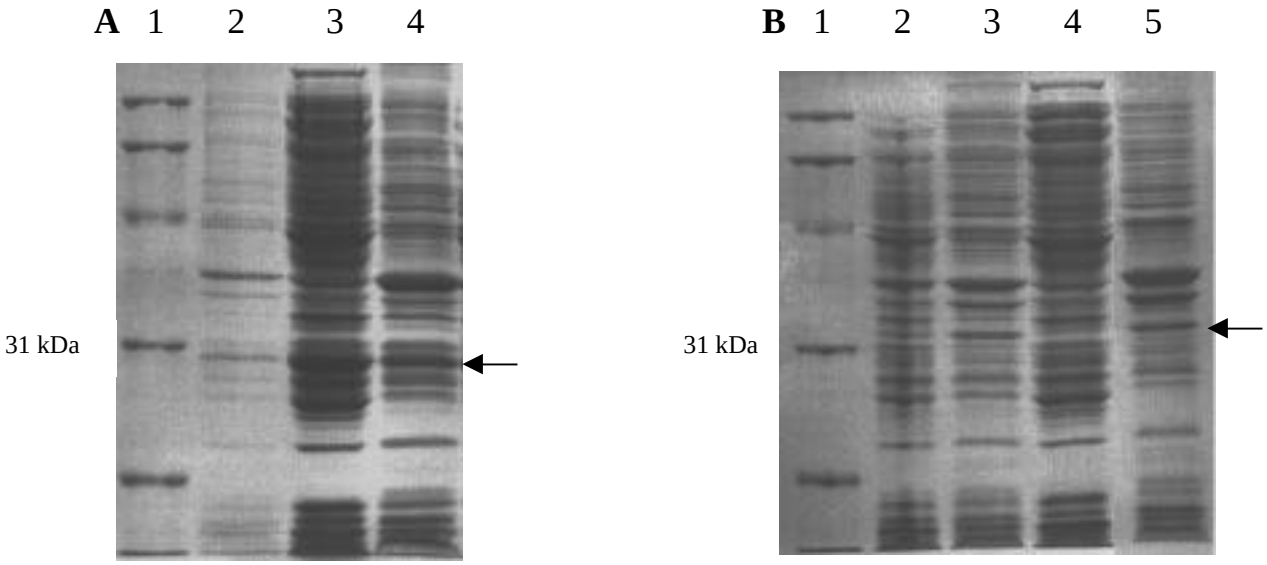


Figure 6. SDS-PAGE of separated protein fractions from *E.coli* strains in which CwlD was over-expressed. Arrows indicate CwlD and 31 kDa molecular weight marker is noted.
Fig. 6A: Protein fractions isolated from *E.coli* strain DPVE16 over-expressing CwlD without its signal peptide. Lane1, molecular weight markers; lane 2, insoluble protein fraction; lane 3, soluble protein fraction; lane 4, membrane protein fraction.
Fig. 6B: Protein fractions isolated from *E.coli* strain DPVE14 over-expressing CwlD with its signal peptide. Lane1, molecular weight markers; lane 2, cell lysate from induced culture; lane 3, insoluble protein fraction; lane 4, soluble protein fraction; lane 5, membrane protein fraction.

fraction. DPVE16, the *E.coli* strain producing CwlD without it signal peptide, showed the majority of the protein to be soluble; however, some of the protein was still present in the membrane fraction. Therefore, this provides evidence that the N-terminal region of the protein is a signal peptide causing CwlD to be membrane associated as previously predicted (25).
Before we went on with the purification of CwlD, we wanted to demonstrate muramoyl-L-alanine activity by the protein *in vivo*. We induced the over-expression of CwlD in DPVE14, the *E. coli* strain expressing CwlD with its signal peptide. Peptidoglycan was then purified from an induced culture and a non-induced culture. We verified the induction with SDS-PAGE and analyzed the muropeptide composition of the extracted peptidoglycan with reversed-phase HPLC (Fig.7). We found that upon induction of CwlD there were increases in a few of the muropeptide peaks, specifically, peaks 2, 7, 11,15, and 18. We then isolated the membrane fraction from the induced cells and mixed the membranes with purified *E. coli* peptidoglycan from strain DPVE13. The peptidoglycan was analyzed with RP-HPLC and we found changes in some of the same muropeptide peaks (# 2, 7, and 11). Next, we solubilized the membrane fraction from the induced cells with detergent (triton X-100) and mixed the soluble protein with the purified peptidoglycan. Upon RP-HPLC analysis, we again saw changes in a few of the same peaks. An amino acid and mass spectrometry analysis was conducted on two of the muropeptide peaks that increased upon induction of CwlD, peaks 2 and 11 (Table 2). The amino acid analysis of peak 2 showed the muropeptide consisted of 2 NAG residues, 1 NAM residue, and 1 NA-muramitol residue, however, there were no alanine, glutamate, or diaminopimelic acid residues, which make up the peptide side chain attached to NAM. We predicted this structure to be a tetrasaccharide with an ion mass $(m+Na)^+$ of 999.4. Mass spectrometry analysis verified our predicted mass with a measured ion mass $(m+Na)^+$ of 1000.7, therefore, we could be certain peak 2 was a tetrasaccharide (Fig. 8). The amino acid analysis of peak 11 showed the muropeptide consisted of 2 NAG residues, 1 NAM residue, and 1 NA-muramitol residue. There were also 2 alanine, 1 glutamate, and 1 diaminopimelic acid residues (Table 2). Therefore, we predicted the structure to be a tetrasaccharide containing a tetrapeptide side chain with an ion mass of 1442.6. Mass spectrometry analysis verified our predicted ion mass with a measured ion mass of 1441.2 indicating muropeptide 11 was a tetrasaccharide-tetrapeptide (Fig. 8). On every NAM residue in *E. coli* peptidoglycan there is a peptide side chain (9), however, the structure of muropeptide 2 shows no side chain present on either NAM residue and there is a peptide side chain present on

Fig. 7. Muropeptide analysis of *E.coli* peptidoglycan following CwlD induction

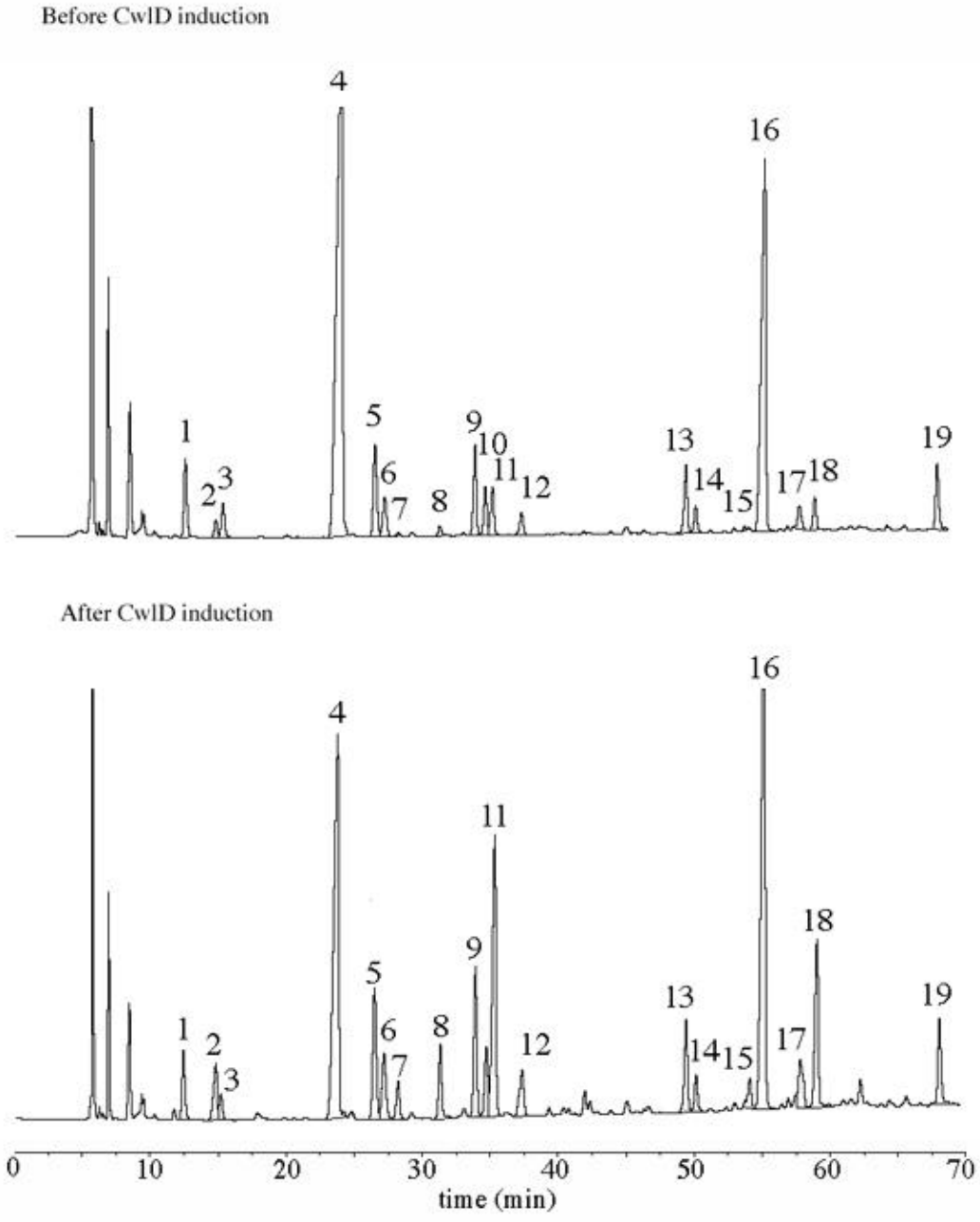


Figure 7. HPLC traces from the muropeptide analysis of *E.coli* peptidoglycan before (top) and after (bottom) CwlD induction in strain DPVE14.

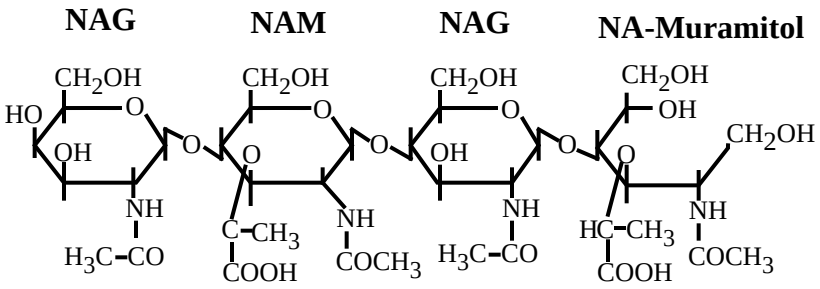
Table 2. Amino acid and mass spectrometry analysis of muropeptides produced upon CwlD induction

	Amino acid analysis nmol detected (predicted molar ratio)								**Mass Spec. analysis**	
Peak	**NAG**	**NAM**	**NA-Muramitol**	**Ala**	**Glu**	**Dpm**	**Predicted structure**	**Predicted mass [m]**	**Predicted ion mass $[m+Na]^+$**	**Measured ion mass $[m+Na]^+$**
2	2	0.6 (1)	1.0 (1)	0	0	0	Tetrasaccharide	976.4	999.4	1000.7
11	2	0.7 (1)	1.0 (1)	1.9 (2)	1.0 (1)	0.9 (1)	Tetrasaccharide-Tetrapeptide	1419.6	1442.6	1441.2

Abbreviations: NAG, N-acetyl glucosamine; NAM, N-acetyl muramic acid; NA-muramitol, N-acetyl muramitol; Ala, alanine; Glu, glutamate; Dpm, diaminopimelic acid.

Fig. 8. Predicted structures of muropeptides 2 and 11

Muropeptide 2



Muropeptide 11

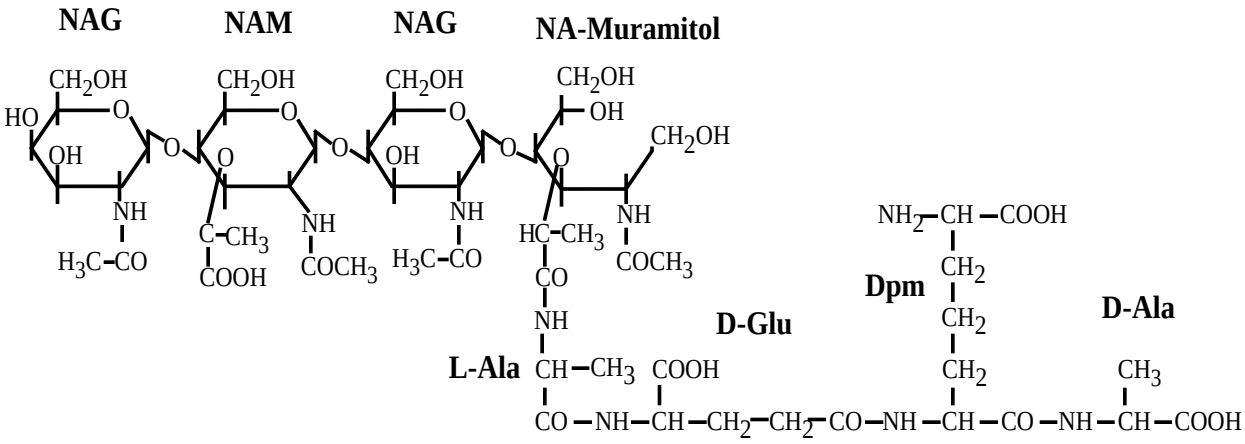


Figure 8. The predicted structures of muropeptide 2 and 11 based on amino acid and mass spectrometry analysis. Abbreviations: NAG, N-acetyl glucosamine; NAM, N-acetyl muramic acid; NA-Muramitol, N-acetyl muramitol; L-Ala, L-alanine; D-Glu, D-glutamate; Dpm, diaminopimelic acid; D-Ala, D-alanine.

only one NAM residue in muropeptide 11. The peptide side chains must have been removed, therefore, demonstrating muramoyl-L-alanine amidase activity is occurring upon induction of CwlD. Since CwlD shows sequence similarities to muramoyl-L-alanine amidases these observations provide strong evidence that the role of CwlD is to carry out the first step of MAL synthesis during sporulation. As a result, we believe it is unnecessary for the peptide side chain to be cleaved to a single L-alanine side chain in order for CwlD to act. We also believe that other enzymes are needed to complete MAL synthesis.

Purification of CwlD

We began the purification of CwlD using *E.coli* strain DPVE16 that produces a soluble CwlD protein without its signal peptide and carrying a histidine tag. Metal affinity chromatography was used with the soluble protein fraction to purify the protein. This method of chromatography was employed based on the interaction of the resin carrying divalent cations such as Co^{2+}, Ni^{2+}, $Cu^{2+,}$ and Zn^{2+} and the six histidine residues attached to the N-terminal region of the protein. It has been found that the imidazole nitrogen of the histidine residues interacts with the electron-deficient orbitals of the electropositive transition metals. The interaction with six histidine residues is very specific and a good method to identify the protein of interest since there are few naturally occurring His-rich proteins (7). We found that CwlD would only elute from the column with EDTA, which strips the metal ions from the column. With this chromatography method we were able to obtain CwlD in a very pure form as seen in the SDS-PAGE of samples taken throughout the purification process (Fig.9). Approximately 3 mg of protein was purified (Lowry Protein Assay).

After purifying CwlD we wanted to determine if the protein was still active. The purified protein was mixed with purified *B. subtilis cwlD* mutant (PS2307) spore peptidoglycan in the presence of Tris HCl, $MgCl_2$ and triton X-100 at a pH of 7.0. The $O.D._{600}$ was read every 15 minutes looking for a decrease. The decrease in O.D. would demonstrate cleavage of peptidoglycan and show the autolytic activity of the protein. We did not see a decrease in O.D. and believed the lack of activity might have been due to the use of the wrong buffer conditions for the protein. It is also possible that the coat proteins surrounding the peptidoglycan could interfere with obtaining correct O.D readings since they have a tendency to aggregate causing O.D. readings to decrease.

Fig. 9. SDS-PAGE demonstrating the purification of CwlD with metal affinity chromatography

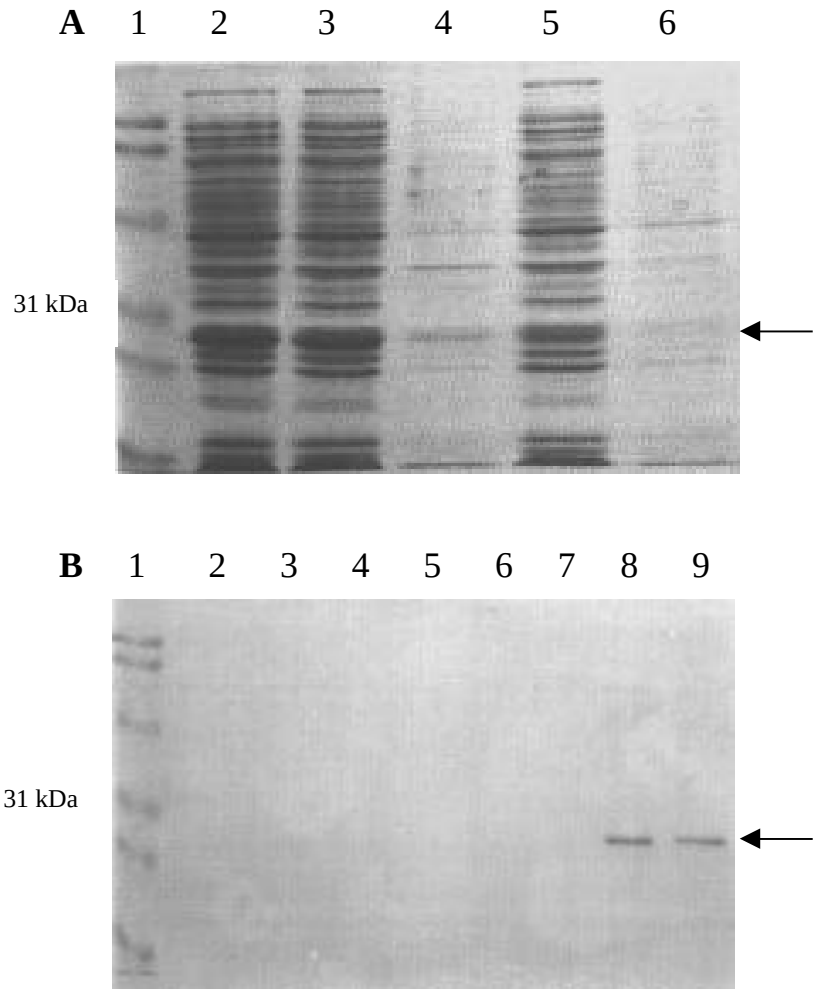


Figure 9. SDS-PAGE of samples collected throughout the purification process of CwlD without its signal peptide from *E.coli* strain DPVE16. Arrows indicate CwlD and 31 kDa molecular weight marker is noted.
Fig. 9A: Lane 1, molecular weight markers; lane 2, cell lysate from induced culture; lane 3, soluble protein fraction; lane 4, insoluble protein fraction; lane 5, proteins that did not bind to the resin; lane 6, sample collected after washing resin with buffer.
Fig. 9B: Lane1, molecular weight markers; lane 2 and 3, sample collected after adding elution buffer to resin; lane 4 and 5, sample collected after adding elution buffer and β-mercaptoethanol; lane 6 and 7, sample collected after adding elution buffer and triton X-100; lane 8 and 9, sample collected after adding EDTA.

Therefore, the purified protein was then mixed with purified *B. subtilis* wild-type vegetative peptidoglycan and purified *E. coli* peptidoglycan to avoid coat protein interaction and to try a different substrate. The reaction was carried out in the presence of Tris HCl, $MgCl_2$ at a pH of 7.0 and without triton X-100. The assay was carried out as before and still there was no decrease in $O.D._{600}$ observed.

Since we were unable to observe activity of the purified protein without its signal peptide *in vitro*, we believed that the protein might need its signal peptide in order to be active. Since wewere able to demonstrate muramoyl-L-alanine amidase activity occurring when CwlD containing its signal peptide is over-expressed we decided to begin purification of the full-length protein from strain DPVE14. We began with metal affinity chromatography using the triton X-100 solubilized membrane fraction. We found the protein was sticking to the resin, but we were unable to recover the protein with any elution treatment short of a SDS-containing buffer. As a result, we turned to anion exchange chromatography to purify CwlD. We solubilized the membrane fraction, performed the chromatography and found we were able to recover approximately 50% of the protein in a relatively pure from (Fig.10). To assay the recovered protein for muramoyl-L-alanine amidase activity we mixed the collected protein with purified *E. coli* peptidoglycan. The peptidoglycan was analyzed with RP-HPLC and we did not see any significant changes occurring in the peptidoglycan structure.

Production and purification of anti-CwlD polyclonal antibodies

A second possible method for demonstration that muramoyl-L-alanine amidase activity is due to CwlD is the use of anti-CwlD polyclonal antibodies. We developed these antibodies as a tool that can be used in future research to demonstrate that anti-CwlD antibodies result in the inactivation of muramoyl-L-alanine amidase activity. If this occurs, then it could be concluded that CwlD is responsible for this activity. We purified CwlD from DPVE16, the *E. coli* strain producing the protein without its signal peptide region, with metal affinity chromatography. We removed the protein from a SDS-polyacrylamide gel and had antibodies raised to the protein in rabbits. We tested the serum we had obtained and found that it was specifically recognizing the purified protein and also bound to the protein within the membrane preparation (Fig. 11). However, the antibody also exhibited non-specific interactions with several proteins in the membrane fraction. As a result, we affinity purified the antibodies to ensure only CwlD-specific

Fig. 10. SDS-PAGE demonstrating the partial purification of CwlD with anion exchange chromatography

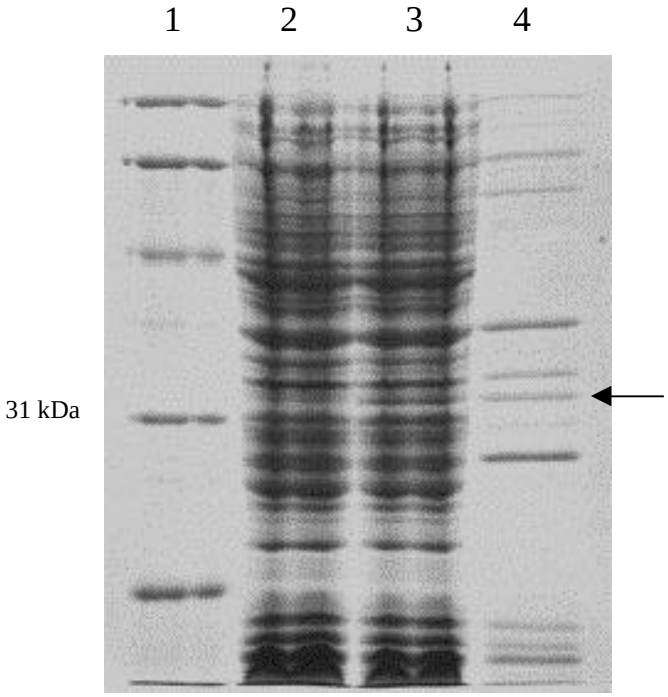


Figure 10. SDS-PAGE demonstrating the partial purification of CwlD containing its signal peptide from *E.coli* strain DPVE14. An arrow indicates CwlD and the 31 kDa molecular weight marker is noted. Lane 1, molecular weight markers; lane 2, cell lysate from non-induced culture; lane 3, cell lysate from induced culture; lane4, sample obtained after anion exchange chromatography.

Fig. 11. Western blot demonstrating the immunoreactivity of antibodies to CwlD

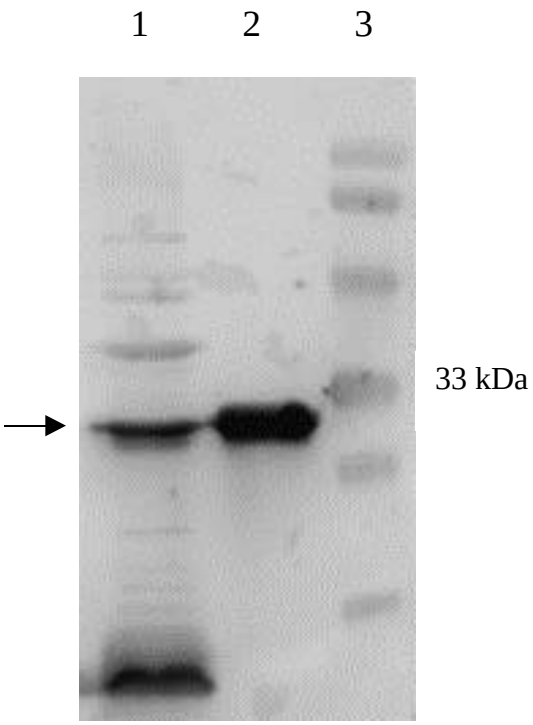


Figure 11. Western blot demonstrating the ability of antibodies to recognize CwlD. The arrow indicates CwlD and the 33 kDa marker is noted. Lane 1, membrane fraction obtained from *E.coli* strain DPVE16 over-expressing CwlD without its signal peptide. Lane 2, sample from the purification of CwlD without its signal peptide with metal affinity chromatography. Lane 3, molecular weight markers.

interactions. A large amount of the non-active CwlD (DPVE16) was purified and bound to a gel matrix. The crude serum was run over the column and the CwlD-specific antibodies interacted with the fixed CwlD protein. The antibodies were then eluted from the column and the immunoreactivity of the purified antibodies was tested with western blotting. DPVE16 membrane preparations were run on a SDS-polyacrylamide gel and two blots were prepared. One was incubated with the crude serum and the other was incubated with the affinity purified antibodies (Fig.12). We found the crude serum showed the same non-specific interactions as previously seen and the affinity purified antibodies bound more specifically to CwlD. We were able to successfully exclude interactions occurring from many of the antibodies that are naturally produced by the rabbit to *E. coli* proteins and obtain antibodies that are more specific to CwlD.

In order to prove the purified antibodies were specific to the CwlD protein we wanted to demonstrate the antibodies ability to detect the protein in *B. subtilis* wild-type (PS832) strain and not in the *B. subtilis* mutant strain lacking *cwlD* (PS2307). The two cultures were grown and the cells were harvested approximately 5 hours after the initiation of sporulation. This time was chosen based on the previous finding that CwlD is transcribed in the mother cell three hours after sporulation initiation and in the forespore 4.5 hours after initiation (25). Therefore, at this point in sporulation the maximum amount of CwlD should be present. The cells were broken with sonication and the lysates were used for western blotting with the purified antibodies. We were unable to detect the protein in wild-type *B. subtilis* and theorized this was a result of the method used to lyse the cells. It is possible that CwlD may be in the intermembrane space and may be tightly associated with the peptidoglycan. As a result, we needed to use a harsher method than sonication to break open the coat layers and be able to extract all the proteins. Therefore, we tried a bead-beating protocol in which lyophilized cells are placed in a thick walled tube with glass powder and a metal ball and shaken vigorously. The broken cells were resuspended in sample buffer and the western blot was repeated. Again, we were unable to detect CwlD in *B. subtilis*.

We also tested the purified anti-CwlD antibodies against cell lysates from the *E.coli* strain (DPVE14) over-expressing CwlD with its signal peptide. A western blot was performed with cell lysates from induced and non-induced cultures (Fig.13). A control was also run with the membrane fraction isolated from the *E.coli* strain (DPVE16) which was used to raise the antibodies and in which CwlD was previously recognized by the purified antibodies. A band

Fig. 12. Western blots demonstrating CwlD-specific antibodies

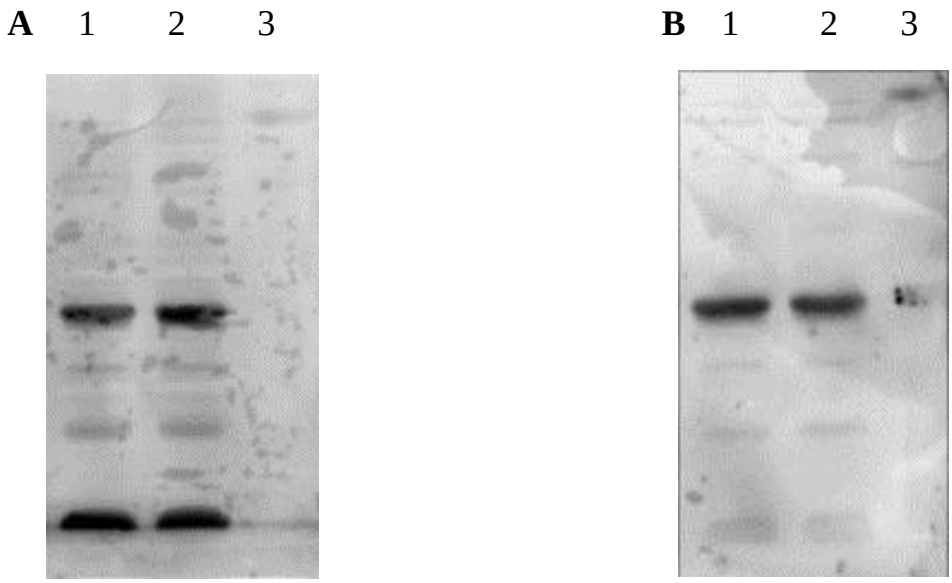


Figure. 12. Western blots demonstrating the specific recognition of affinity purified antibodies.
Fig. 12A: Western blot containing membrane fraction obtained from *E.coli* strain DPVE16 over-expressing CwlD without its signal peptide (lane1 and 2). Blot was incubated with non-purified antibodies. Lane 3 contains molecular weight markers.
Fig. 12B: Western blot containing membrane fraction obtained from *E.coli* strain DPVE16 over-expressing CwlD without its signal peptide (lane 1 and 2). Blot was incubated with affinity purified CwlD-specific antibodies. Lane 3 contains molecular weight markers.

Fig. 13. Western blot demonstrating the identification of CwlD in a *E.coli* cell lysate

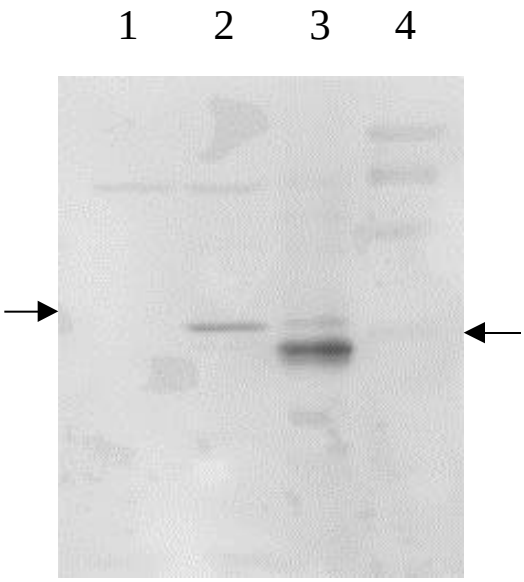


Figure 13. Western blot demonstrating the identification of CwlD in a cell lysate from *E.coli* strain DPVE14 induced for CwlD over-expression. Lane1, cell lysate from non-induced culture; lane2, cell lysate from an induced culture; lane 3, positive control containing DPVE16 membrane fraction; lane 4, molecular weight markers. Blot was incubated with CwlD affinity purified antibodies. Arrows indicate CwlD.

was seen in the induced cell lysate and in the positive control, however, there was no band present in the lane containing the non-induced cell lysate. The band seen in the induced cell lysate lane ran slightly higher than the positive control band. This is expected since the induced culture over-expressed CwlD with its signal peptide and the control was CwlD without its signal peptide. Therefore, this shows that the two different forms of CwlD have the same antigenic determinants and the antibodies are able to recognize CwlD in whole cell lysates.

The CwlD-specific antibodies were also tested against the collected protein fractions from the anion exchange chromatography of CwlD containing its signal peptide from strain DPVE14. This was done to ensure the fractions contained the same protein purified from DPVE16 believed to be CwlD. We also wanted to demonstrate the immunoreactivity of the antibodies with the full-length protein and observe which collected fractions contained the majority of the CwlD protein. The CwlD-specific antibodies were used in a western blot with the anion exchange protein fractions and a DPVE16 membrane preparation as a positive control (Fig.14). The western blot demonstrated the anion exchange fractions do contain the same protein obtained from metal affinity chromatography that was used to produce the CwlD-specific antibodies. From the western blot we were also able to determine which fraction contained the majority of CwlD. This information was correlated with the results of the HPLC analysis of the peptidoglycan that was previously mixed with the anion exchange protein fractions to detect muramoyl-L-alanine activity. The protein fractions that contained a large amount of CwlD did not cause changes in the peptidoglycan structure compared to the negative control, which did not contain any protein.

Fig. 14. Western blot demonstrating CwlD-specific antibodies recognize CwlD purified from anion exchange chromatography

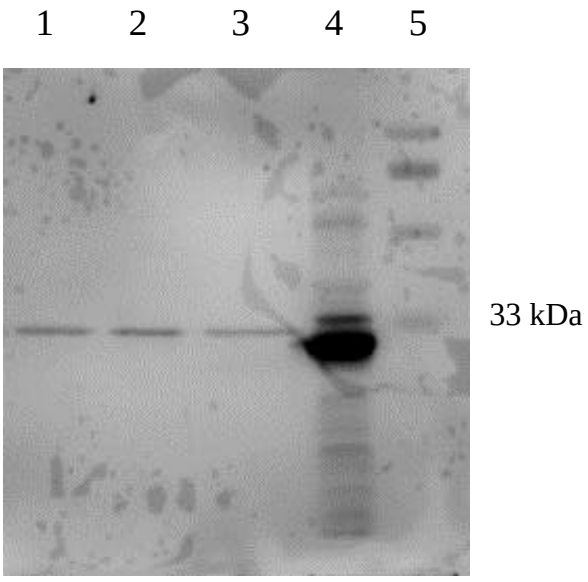


Figure 14. Western blot demonstrating the reactivity of affinity purified CwlD-specific antibodies to CwlD purified with anion exchange chromatography from *E.coli* strain DPVE14 over-expressing CwlD with its signal peptide. Lane1, 2, and 3, samples from the purification of full-length CwlD with anion exchange chromatography. Lane 4, membrane fraction obtained from *E.coli* strain DPVE16 over-expressing CwlD without its signal peptide. Lane 5, molecular weight markers.

Discussion of the Biochemical Analysis of CwlD

Demonstration of muramoyl-L-alanine amidase activity

Through the extraction of peptidoglycan from *E.coli* strain DPVE14 induced for over-expression of CwlD containing its signal peptide and from a non-induced culture, we were able to observe muramoyl-L-alanine activity we believed to be due to CwlD. RP-HPLC analysis of the extracted peptidoglycan showed that upon induction of CwlD there were changes occurring in the muropeptide profile obtained. We then isolated the membrane fraction from the over-expressed culture and assayed for activity using purified *E.coli* peptidoglycan as the substrate. We saw the same changes occurring in the HPLC traces. Lastly, we solubilized the membrane fraction with triton X-100 and assayed for activity. Again, we saw the same changes occurring and concluded that we may be able to purify the protein from a solubilized membrane preparation and still maintain its activity.

To determine if the changes detected in the HPLC analysis upon CwlD induction was due to muramoyl-L-alanine amidase activity, we chose two of the muropeptide peaks, 2 and 11, which showed increases in all three activity assays. These muropeptides were subjected to amino acid and mass spectrometry analysis. The data showed that muropeptide 2 was predicted to be a tetrasaccharide containing two NAG and two NAM residues. However, both NAM residues did not contain peptide side chains that are normally present on every NAM in *E.coli* peptidoglycan (Fig. 8). Muropeptide 11 was predicted to be a tetrasaccharide-tetrapeptide containing two NAG and two NAM residues, however only one NAM residue contained a peptide side chain (Fig. 8). We believe that the predicted structures are correct since we know that mutanolysin, the enzyme used to cut peptidoglycan strands into muropeptides, only cleaves peptidoglycan after NAM residues and before NAG residues. Therefore, each muropeptide should begin with a NAG and end with a NAM. We also know that mutanolysin cuts with greater efficiency next to residues containing tetrapeptide side chains rather than next to residues with single L-alanine or no peptide side chain. Therefore, based on our knowledge of the mutanolysin enzyme we believe our predicted structure of muropeptide 11 is the most probable structure to obtain. We believe there is a peptide side chain present on the end NAM residue while the inner NAM has lost its side chain and is probably a precursor of muramic lactam. However, both of the NAM residues in muropeptide 2 do not contain peptide side chains. There

are two explanations as to how it is possible to obtain the structure of muropeptide 2. First, it is possible that due to the large amount of CwlD acting on the peptidoglycan, the enzyme is able to cleave off more peptide side chains. Second, it is possible that the activity of CwlD is regulated by other enzymes that are not present and normally control which peptide side chain should be cleaved. Both muropeptides showed a loss of peptide side chains demonstrating that muramoyl-L-alanine amidase activity occurred when CwlD was induced. This information leads us to believe CwlD is carrying out the first step of muramic lactam synthesis, the cleaving of the peptide side chain, and other enzymes are needed to complete muramic lactam cyclization. We also believe that it is unnecessary for the peptide side chain to be cleaved down to a single L-alanine side chain in order for CwlD to carry out its activity.

Purification of CwlD

Once we determined the optimal conditions for the over-expression of CwlD with a histidine tag and without its signal peptide from DPVE16 we were able to begin the purification of the protein with metal affinity chromatography. Once we obtained the purified protein we tested it for activity by mixing the protein with purified *B. subtilis cwlD* mutant spore peptidoglycan, *B. subtilis* wild-type vegetative peptidoglycan, and *E. coli* peptidoglycan. An assay was used in which we looked for a decrease in optical density that would indicate autolytic activity by the protein. We were unable to detect activity of the purified protein. Since we had previously observed CwlD activity by the protein containing its signal peptide *in vivo*, we decided that we should attempt to purify the membrane-bound protein. We obtained the protein in a relatively pure form with anion exchange chromatography and assayed for its activity by mixing it with purified *E.coli* peptidoglycan. The peptidoglycan was analyzed with RP-HPLC and we did not see any significant changes occurring in the peptidoglycan.

CwlD-specific antibodies

We purified the inactive CwlD without its signal peptide and had antibodies raised. We affinity purified the antibodies and used them to attempt to detect the protein in wild-type *B. subtilis* and not in a *cwlD* mutant strain. This would prove that our antibodies were specific to CwlD. We were unable to detect the protein in the wild-type *B. subtilis* strain and there are a few possibilities to explain this result. First, we were able to use the antibodies to detect the

protein in the membrane protein fraction from an *E.coli* strain over-expressing the protein. It is possible that the protein in not produced in a large enough concentration in *B. subtilis* and, therefore, must be over-expressed in order to be detected. Second, it is possible that the protein in *B. subtilis* is in a form that is completely insoluble and, therefore, can not be detected by the antibodies. Third, it is possible that when *cwlD* is expressed in *E.coli* it somehow has a different structure than that produced in *B. subtilis* causing the antibodies to not recognize the protein in *Bacillus*.

We believe that the antibodies we obtained are truly CwlD-specific for two reasons. First, we tested the purified antibodies produced from CwlD without signal peptide (DPVE16) against cell lysates from induced and non-induced cultures for over-expression of the protein containing its signal peptide (DPVE14). The western blot demonstrated two important points. First, the antibodies recognized CwlD in the induced cell lysate and not in the non-induced lysate. Second, the antibodies recognize both forms of the CwlD protein, with and without its signal peptide. In addition, the band seen in the lane containing CwlD with the signal peptide ran slightly higher than the band seen in the lane containing CwlD without its signal peptide. Other evidence demonstrating CwlD-specific antibodies was seen when we tested the antibodies against the fractions obtained from anion exchange chromatography of CwlD with its signal peptide. The antibodies recognized the protein we obtained from anion exchange chromatography and which we believed was CwlD. This information suggests that we were able to obtain altered forms of the same protein with two different purification methods.

CHAPTER THREE

The Genetic Analysis of the *cwlD* Operon

The second part of this research is a genetic analysis of *cwlD* through the construction of a series of mutant *B. subtilis* strains containing altered expression patterns of the *cwlD* operon products. Analyzing the phenotypic characteristics of each mutant strain should demonstrate the function of CwlD produced within the forespore and determine a potential role for Orf1 during spore peptidoglycan synthesis.

Materials and Methods

Construction of strains and plasmids

Plasmid DNA was transformed into *B. subtilis* by making cells competent for DNA uptake (1). Chromosomal DNA was extracted from *B. subtilis* by pelleting 3.0 ml of culture and resuspending the pellet in 1ml lysis solution (0.1 M NaCl, 50 mM EDTA). Samples were then pelleted (15,000 x *g,* 30 seconds) and resuspended in 0.5 ml lysis solution containing 3 mg lysozyme. The solution was incubated at 37^0C for 15 minutes, sarcosyl was added (2% final concentration) with continued incubation at 37^0C for 10 minutes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed violently with shaking and centrifuged at 15,000 x *g* for 3 minutes. The supernatant was removed and the extraction was repeated. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the extraction was done as previously described and repeated. $1/10^{th}$ volume of 3 M sodium acetate pH 5.2 was added and 1 ml ethanol was gently added so phases did not mix. The sample was then shaken violently, the DNA was pelleted, washed with 70% ethanol and vacuum dried. TE was added to the pelleted DNA that was allowed to resuspend for 16 hours at 4^0C.

DPVB47 was made through first constructing plasmid pDPV12. This plasmid contained a PCR fragment from primers cwl I and cwl III (Table 1). These primers produced a fragment containing *orf1* and *cwlD* with a *Bam*HI restriction site approximately 270 bp upstream of *orf1* and a *Sph*I site approximately 18 bp downstream of *cwlD*. The PCR fragment was not cut with restriction enzymes and was cloned into the pGEM-T vector (Promega) at the *Eco*RV site. Next, plasmid pDPV14 was constructed with a 800 bp fragment containing *cwlD* cut from pDPV12 with *Sph*I. This fragment was cloned into pUC19 at the *Sph*I site. Plasmid pDPV12 was cut

with *Bam*HI and *Sca*I to produce a 350 bp fragment containing the first two codons of *orf1*. Plasmid pDPV14 was cut with *Sal*I, treated with Klenow (Promega) to blunt the end, and digested with *Bam*HI to produce a fragment containing the last codon of *orf1* and the entire *cwlD* gene. These fragments were ligated at the *Bam*HI ends while the *Sal*I blunt end was fused to the *Sca*I end to make the in-frame deletion in *orf1*. The resulting plasmid was pDPV19 which contained an in-frame deletion in *orf1* with all but three codons of the gene removed. This sequence was verified with DNA sequencing (Virginia Tech DNA sequencing facility). A 1200 bp spectinomycin resistance cassette was removed from plasmid pDG1727 with digestion with *Eco*RV and *Stu*I. This fragment was ligated into the *Sma*I restriction site of pDPV19 to produce pDPV20.

Plasmid pDPV20 was transformed into PS832 where the plasmid inserts into the chromosome via a single recombination event. In most cases this produces a strain carrying a wild-type copy of the *cwlD* operon along with the Δ*orf1-cwlD* operon obtained from the plasmid. However, one type of recombination event can result in two copies of the Δ*orf1-cwlD* operon inserted into a chromosome. Therefore, PCR with primers cwl I and cwl III was performed to identify transformants carrying two copies of the Δ*orf1-cwlD* operon. A transformant was identified and then grown non-selectively with subculturing approximately every 12 hours to allow excision of the plasmid, therefore, removing one copy of the Δ*orf1-cwlD* operon. After approximately 8 subcultures the cells were plated non-selectively for isolation of single colonies. The resulting colonies were screened for spectinomycin sensitivity, which ensured the plasmid had excised from the chromosome. The chromosomal DNA was then extracted from the drug sensitive isolate and PCR with primers cwl I and cwl III was used to verify a single copy of the Δ*orf1-cwlD* operon was present.

During subculturing it is possible to acquire random mutations within the chromosome. As a result, even though we had obtained the chromosomal DNA with the correct mutation we moved the mutation into a new strain to ensure a clean background. To do this competent cells were made with PS2307, a strain containing a mutation in *cwlD* due to insertion of a chloramphenicol resistance cassette. This strain was used so we could screen for chloramphenicol sensitivity when the mutated *cwlD* is replaced with the wild type *cwlD* that carries in the in-frame *orf1* deletion. First, the PS2307 competent cells were transformed with DPVB36 (*hisA*::Tn 917, MLS^R) to make DPVB48. The resulting strain carries the mutation in

cwlD and chloramphenicol and MLS resistance. DPVB48 was then transformed with the verified chromosomal DNA containing a single copy of the Δ*orf1-cwlD* operon with selection for His^+. This was done to select cells that became competent. The transformants were screened for chloramphenicol sensitivity to identify cotransformants in which the mutated *cwlD* gene was replaced with the wild-type *cwlD* that would often also bring in the in-frame *orf1* deletion mutation. The drug sensitive colonies were screened with PCR to ensure the in-frame *orf1* deletion was present in the chromosome. The resulting strain was DPVB47.

DPVB100, a strain containing *orf1* and *cwlD* under the control of an IPTG inducible pSpac promoter, was made. First, a plasmid was constructed carrying *orf1* and *cwlD* under the control of the pSpac promoter. This was done by digesting plasmid pDPV12 with *Sal*I, treating with Klenow to blunt the ends, and cutting with *Eco*RI to produce a fragment containing *orf1* and *cwlD*. This fragment was inserted into pDG148, a plasmid carrying the pSpac promoter (2), at the *Hin*dIII and *Sal*I restriction sites. The resulting plasmid was pDPV36. PS832 was then transformed with pBL1, a plasmid that over-expresses LacI, the repressor for the *lac* operon. This was done to ensure there was no leaky expression of *cwlD* from the pSpac promoter during vegetative growth that could inhibit the growth of the organism. The resulting strain DPVB75 was then transformed with plasmid pDPV36 carrying *orf1* and *cwlD* with the pSpac promoter.

DPVB77, a strain containing a deletion of both *orf1* and *cwlD* in addition to *orf1* and *cwlD* under the control of an IPTG inducible pSpac promoter was constructed through the transformation of DPVB19 with the plasmid, pBL1. The resulting strain, DPVB76 was then transformed with plasmid pDPV36 containing the pSpac-*orf1cwlD* construct.

A plasmid containing a *cwlD-lacZ* transcriptional fusion, pDPV75, was constructed. First, pDPV28 was constructed with a 720 bp fragment obtained from PCR with CwlD primers 3 and 4 (Table 1). CwlD primer 3 added a *Sal*I site to the N-terminus of *cwlD* and does not include the signal peptide region. CwlD primer 4 adds *Bam*HI and *Hin*dIII restriction sites on to the C-terminus of *cwlD* including the stop codon. This fragment was then inserted into pUC19 at the *Hin*cII restriction site. Plasmid pDPV28 was then digested with *Bam*HI and *Hae*III to obtain a 500 bp fragment containing *cwlD*. This fragment was inserted into the *Bam*HI and *Sma*I sites of pDPC87, a plasmid carrying the *lacZ* gene, to produce plasmid pDPV75. Plasmid pDPV75 was then transformed into strains PS832 (wild-type), DPVB3 (Δ*orf1*::Sp), and DPVB47

(Δ*orf1* in-frame). The resulting strains DPVB70, DPVB71, and DPVB72, respectively, were verified with Southern blotting using a probe containing *orf1* (26).

β-galactosidase assays

The strains were grown in 2xSG media and sporulation was induced through nutrient exhaustion (17)(20). Samples (2 x 1ml) were taken every 30 minutes throughout sporulation (t_0-t_8), resuspended in 25 mM Tris HCl pH 7, and the pellets were stored at –80°C.

β-galactosidase activity in the mother cell was assayed by resuspending the pellets in 1 ml Z buffer (16) containing 0.5 mg/ml lysozyme. The cell extract was obtained by incubating at 37°C for 10 minutes and adding triton X-100 to 0.1% (v/v). Cell extract (400 µl) was added to Z buffer (400 µl), placed at 30°C, and the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) (Jersey Lab Supply) (final concentration of 2 mM) was added. To quench the reaction Na_2CO_3 was added to a final concentration of 0.3 M, all samples were centrifuged at 15,0000 x g for 5 minutes to remove insoluble material, the $O.D._{420}$ was recorded, and Miller units were calculated (16). β-galactosidase activity in lysozyme-resistant forespores was analyzed by, first, resuspending samples in 1 ml decoating solution containing 50 mM Tris HCl pH 8.0, 8 M urea, 1% SDS, 50 mM dithiothreitol (DTT) (Labscientific, Inc.) and incubating at 37°C for 90 minutes. Samples were centrifuged (15,800 x g, 15 seconds) and washed 5 times in 150 mM NaCl. Pellets were then resuspended in Z buffer and lysozyme and assay was carried out as described above.

Preparation and analysis of developing spores

Peptidoglycan analysis

Strains were grown at 37°C in 2xSG media and sporulation was induced through nutrient exhaustion (17). A 30 ml culture sample was taken at 15 or 30 minute intervals throughout sporulation. The sample was centrifuged (8,000 x g, 20°C, 5 minutes), the supernatant was removed and the pellet was resuspended in 5 ml SMM protoplast solution (4). Lysozyme (25 mg) was added, the solution was incubated at 37°C for 15 minutes, and added to 45 ml of a boiling 4% SDS, 50 mM DTT solution, and boiling was continued for 20 minutes. The solution was centrifuged (20,000 x g, 25°C, 30 minutes), the supernatant was removed, and the pellet was resuspended in 1 ml sterile H_2O. The suspension was then boiled for 5 minutes and centrifuged

at 21,000 x g for 20 minutes. The pellet was washed in 1 ml 50°C sterile H_2O until SDS was no longer detected (11). The pellet was then resuspended in a total of 1 ml of 100 mM Tris HCl pH 7, 20 mM $MgSO_4$ containing 15 µg DNase I and 75 µg RNase A, and incubated at 37°C for 2 hours. $CaCl_2$ was added to a final concentration of 10 mM, 150 µg trypsin was added, and the suspension was incubated at 37°C for 16 hours. The samples were then pelleted as above, resuspended in 1 ml 1% SDS and boiled for 20 minutes. The samples were washed with centrifugation at 21,000 x g, 20 minutes, 20°C and resuspended in sterile H_2O until SDS was no longer detected (11). The pellet was then digested in a total volume of 250 µl containing 12.5 mM $NaPO_4$ pH 5.5 with 125 units of mutanolysin (Sigma) for 16 hours at 37°C. The solubilized muropeptides were prepared for HPLC analysis as described (20).

Biochemical analysis

Glucose dehydrogenase (GDH) activity and dipicolinic acid (DPA) accumulation were assayed as previously described (17) and spore heat resistance was measured as described (22). To measure hexosamine content, 0.5 ml culture samples were taken, centrifuged (15,800 x g, 45 seconds), and washed in 0.5 ml cold 1mM $MgCl_2$. The samples were then resuspended in 0.5 ml cold 6 N HCl and a 20 µl sample was removed. The 20 µl samples were hydrolyzed at 95°C for 4 hours and subjected to amino acid analysis as described (10).

Analysis of dormant spores

Mutant strains were grown in 2xSG media and sporulation was induced through nutrient exhaustion (17). Spores were purified through frequent washing in H_2O over 4 days and storage at 4°C with gentle agitation (21). Spore viability was determined by plating dilutions of untreated spores on Luria Bertani (LB) agar plates (19). Spore chloroform resistance was carried out by vortex mixing spores in a 10% (vol/vol) suspension of chloroform for 1 minute prior to plating on LB (19). The spore heat resistance was measured by heating samples at 80°C for 10 minutes, preparing dilutions, and plating cells on LB media. The surviving spores produced colonies that were enumerated (14). Germination rate was determined by heat activating spores in H_2O at 65°C for 20 minutes and inoculating into 2xYT media containing 4 mM L-alanine. $O.D._{600}$ values were taken at specific time points throughout germination (19).

Results from the Genetic Analysis of the *cwlD* Operon

Strains analyzed

The genetic analysis of the *cwlD* operon involved the examination of four mutant *B. subtilis* strains with altered expression patterns of *cwlD* and *orf1* (Table 3). The first strain analyzed, DPVB71, contains a deletion in *orf1* accompanied by the insertion of a spectinomycin resistance cassette. This mutation will block expression of *orf1* and *cwlD* from the σ^G-dependent promoter that is specific to the forespore. Therefore, this strain allowed the observation of the phenotypic characteristics of spores produced by cells expressing only CwlD and only within the mother cell. The second strain analyzed was DPVB72 containing an in-frame deletion in *orf1*. This mutation allowed expression of CwlD in the mother cell and in the forespore, however, there is no expression of *orf1*. The third strain analyzed was DPVB100 containing a wild-type copy of the chromosome and a plasmid carrying *orf1* and *cwlD* under the control of an IPTG-inducible pSpac promoter. The plasmid is present within the mother cell and the forespore, therefore, upon induction with IPTG both *orf1* and *cwlD* are expressed in both cell compartments. However, this strain also expresses Orf1 and CwlD from the chromosomal copies of the genes. To observe the phenotypic effects on spores produced by a strain that only expresses Orf1 and CwlD from the plasmid within both the mother cell and the forespore and not from the chromosomal copies of the genes, strain DPVB77 was constructed. This strain contains a deletion in *orf1* and *cwlD* within the chromosome and carries the plasmid containing *orf1* and *cwlD* under the control of an IPTG-inducible pSpac promoter.

Verification of strains analyzed

Before the analysis of the four mutant strains began, their *cwlD* expression patterns were verified. Two of the mutant strains, the *orf1* insertion mutant and the *orf1* in-frame deletion mutant, carry a *lacZ* transcriptional fusion to *cwlD* along with the mutation. Therefore, an assay for β-galactosidase activity was used to determine in which cell compartment, the mother cell or the forespore, *cwlD* was being transcribed. A third strain was also constructed, DPVB70, containing the wild-type chromosome with *lacZ* fused to *cwlD.* This strain was used for a wild-type comparison during the β-galactosidase assays. All three *lacZ* fusion strains were grown and

Table 3. *Bacillus subtilis* strains used in the genetic analysis of the *cwlD* operon

Strain[a]	Genotype[b]	Mother cell[c]	Forespore[d]
DPVB70	Wild type + *cwlD-lacZ*	CwlD	CwlD Orf1
DPVB71	*orf1*::Sp + *cwlD-lacZ*	CwlD	
DPVB72	In-frame Δ*orf1*	CwlD	CwlD
DPVB77	Δ(*orf1cwlD*) + pSpac-*orf1cwlD*	CwlD[e] Orf1[e]	CwlD[e] Orf1[e]
DPVB100	Wild type + pSpac-*orf1cwlD*	CwlD Orf1	CwlD Orf1

[a] Laboratory stock strain numbers. [b] Abbreviations: Sp, spectinomycin
[c]Expression patterns of CwlD and Orf1 in the mother cell compartment.
[d]Expression patterns of CwlD and Orf1 in the forespore compartment.
[e]CwlD and Orf1 expressed from a plasmid only

two samples were taken every 30 minutes throughout sporulation. The first set of samples was used in assaying for β-galactosidase activity within the mother cell. It is possible to measure the enzyme's activity only within the mother cell while excluding any activity exhibited in the forespore in the later stages of sporulation due to the lysozyme resistance of the forespore. Therefore, lysozyme treatment will cause the mother cell and the forespore to lyse in the initial stages of sporulation, approximately t_0 to $t_{3.5}$. However, the forespore will not lyse in the later stages of sporulation, approximately $t_{3.5}$ to t_8. Measuring β-galactosidase activity only within the forespore is possible through treating the cells with a decoating solution containing SDS and DTT, which makes the forespores sensitive to lysozyme and also inactivates any β-galactosidase present in the mother cell.

The β-galactosidase assays allowed us to determine in which cell compartment *cwlD* was being expressed. The analysis of the enzyme's activity produced in the wild-type/*cwlD-lacZ* fusion strain showed high levels in the mother cell in samples taken in the early stages of sporulation and a low level of activity in samples from the later stages. Similar results were obtained with the strain containing an insertion mutation in *orf1*containing the *cwlD-lacZ* fusion and the in-frame *orf1*deletion mutant with the *cwlD-lacZ* fusion (Fig. 15). Therefore, CwlD was produced in the mother cell in all three strains as predicted. After assaying for β-galactosidase activity only within the forespore we found high levels of activity in the later stages of sporulation for the wild-type strain carrying the *cwlD-lacZ* fusion strain and the in-frame *orf1*deletion mutant carrying the *cwlD-lacZ* fusion. However, there was no β-galactosidase activity detected in the *orf1* insertion mutant carrying the *cwlD-lacZ* fusion (Fig. 16). Therefore, there is CwlD production in the forespore in the wild type and the in-frame *orf1* deletion mutant and no expression of the protein in the forespore in the *orf1* insertion mutant. These results are consistent with the expression patterns expected in the mutant strains. When the results from the β-galactosidase assays from the mother cell and the forespore compartments are combined, the wild-type strain and the in-frame *orf1* deletion mutant showed increasing levels of activity throughout sporulation (Fig. 17). Therefore, these strains are producing CwlD in the mother cell and within the forespore as predicted. However, the *orf1* insertion mutant shows a high level of activity in samples taken early in sporulation with a dramatic decrease in activity in the later samples. These data show CwlD is being expressed in the mother cell and not in the forespore as

Fig. 15. β-galactosidase activity in the mother cell of *cwlD-lacZ* fusion strains during sporulation

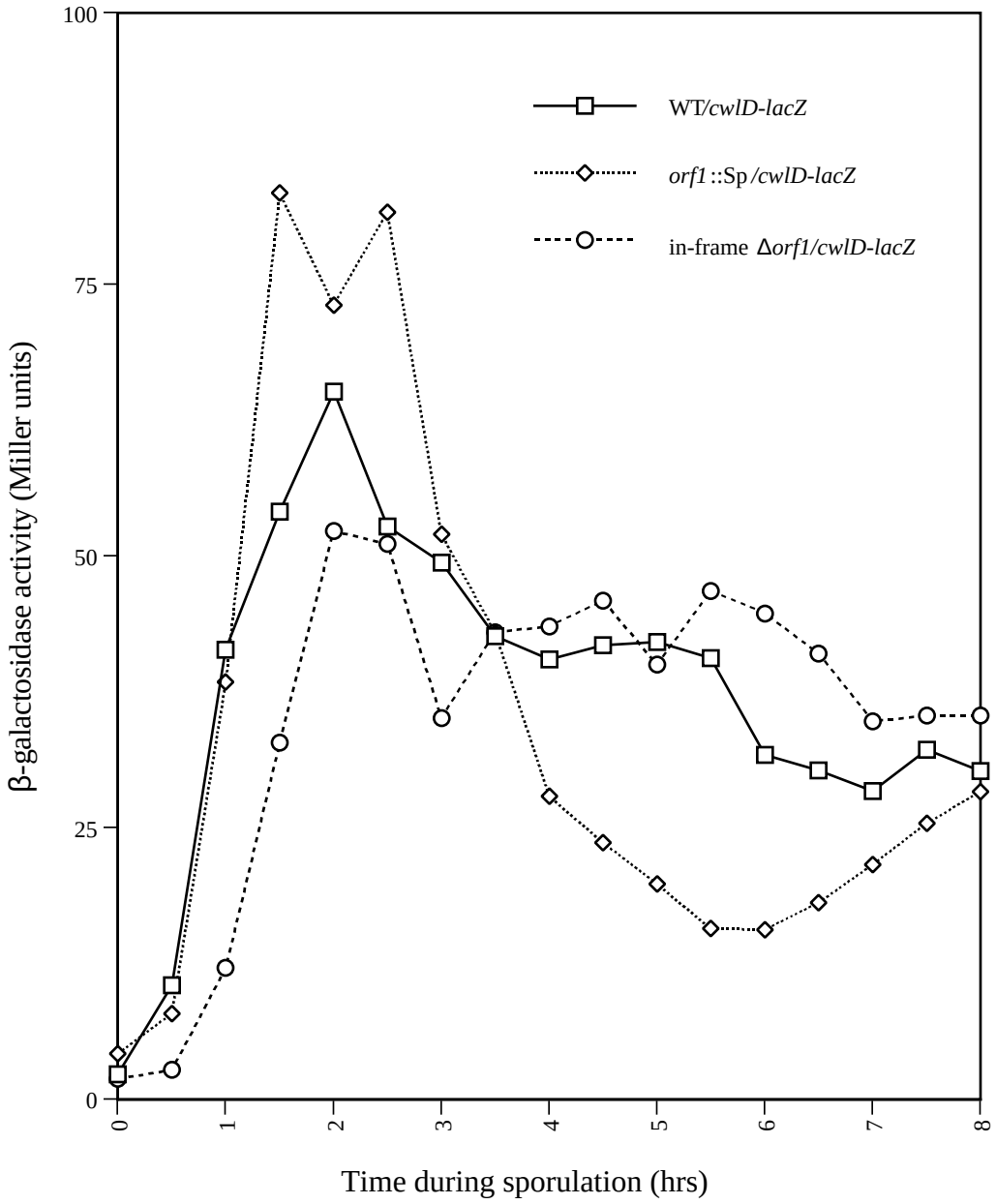


Figure 15. β-galactosidase activity was measured within the mother cell of wild-type and mutant strains containing *cwlD-lacZ* transcriptional fusions.

Fig. 16. β-galactosidase activity within the forespore of *cwlD-lacZ* fusion strains during sporulation

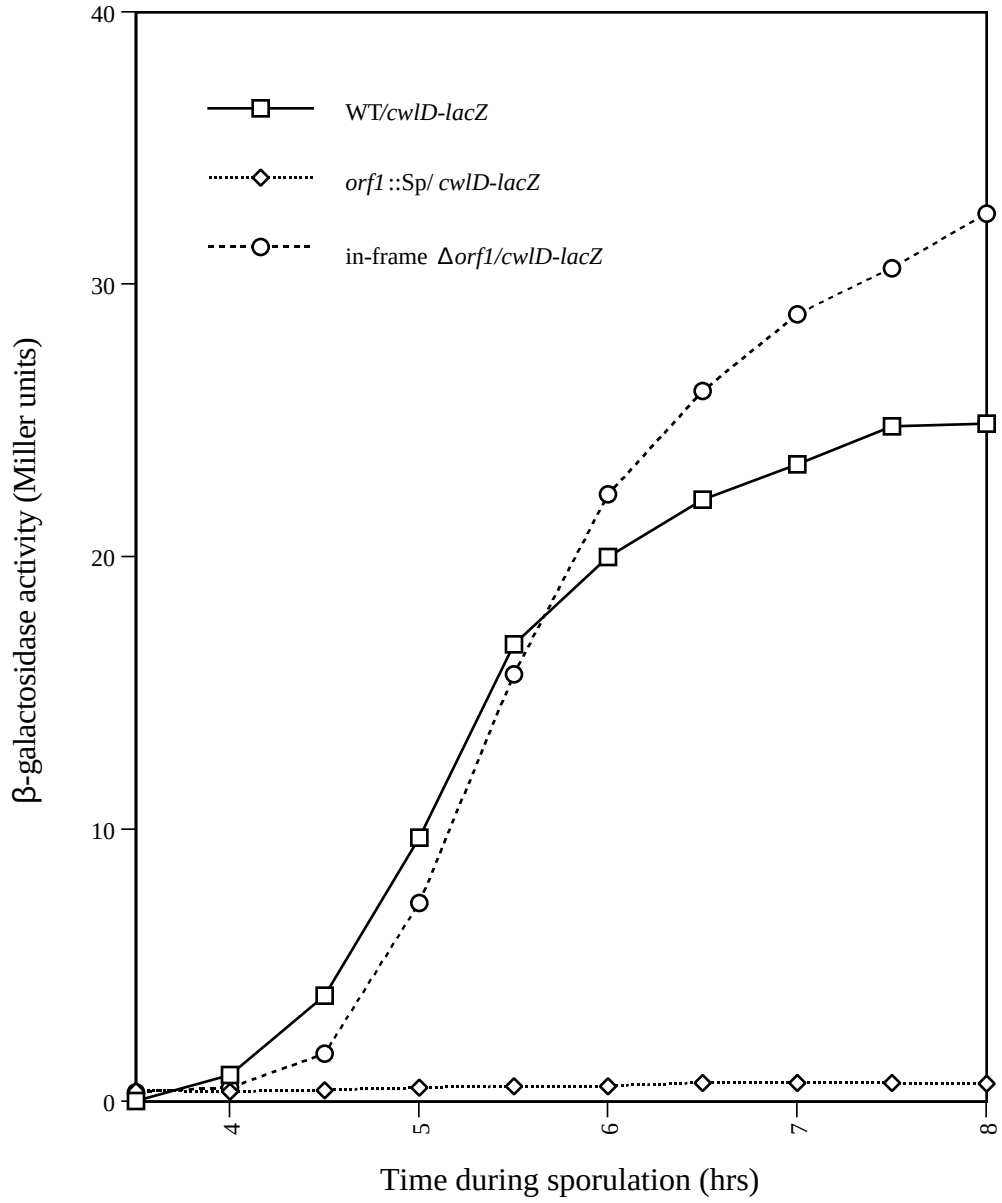


Figure 16. β-galactosidase activity was measured in the forespore in wild-type and mutant strains containing *cwlD-lacZ* transcriptional fusions.

Fig. 17. β-galactosidase activity in the mother cell and forespore of *cwlD-lacZ* fusion strains throughout sporulation

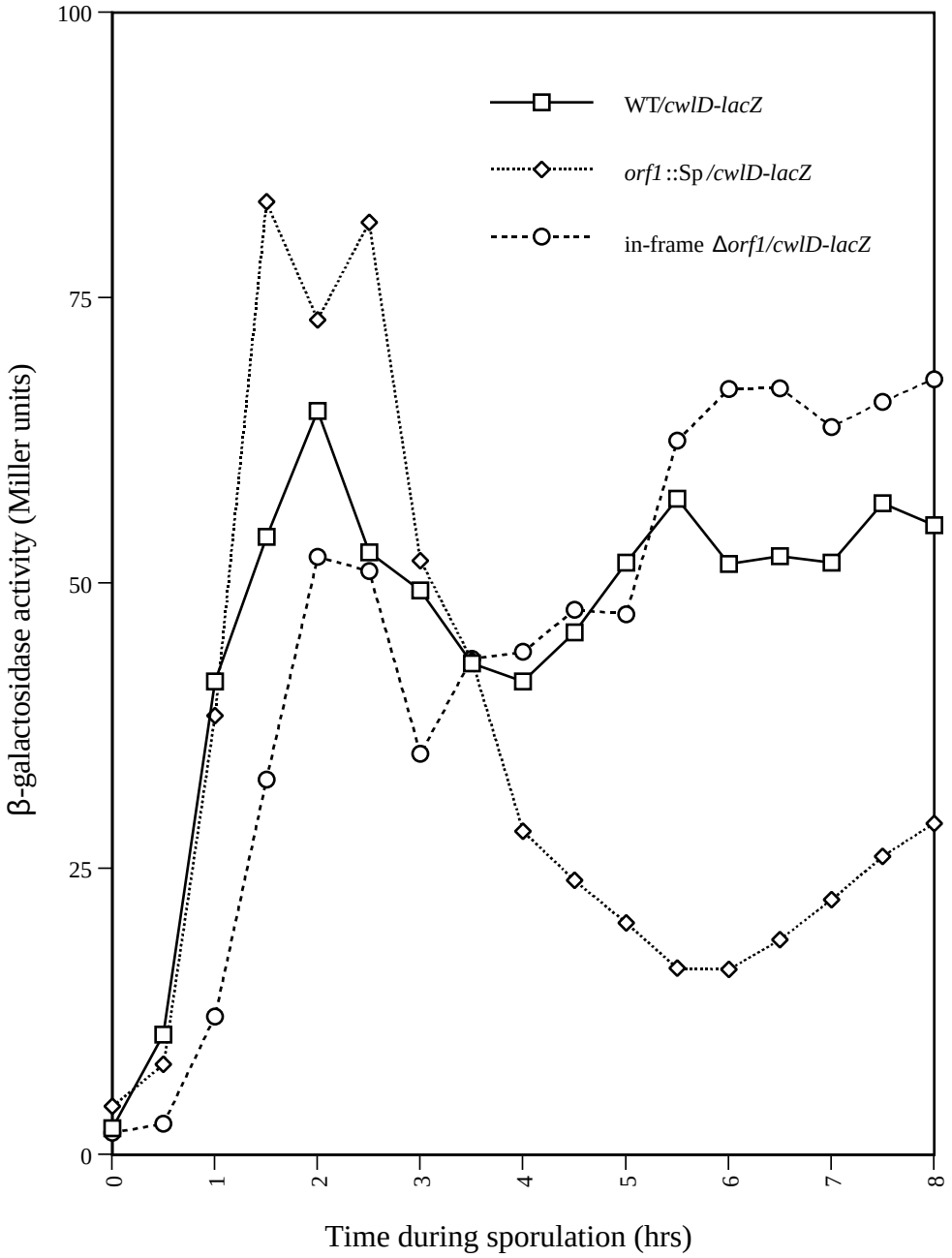


Figure 17. The combined results from the measurement of β-galactosidase activity within the forespore and mother cell of wild-type and mutant strains containing *cwlD-lacZ* transcriptional fusions.

predicted. Samples were also taken for the measurement of DPA, a late sporulation marker. This was done to rule out the possibility that the lack of activity seen in the *orf1* insertion mutant late in sporulation was due to the strain not sporulating. All strains including the *orf1* insertion mutant showed wild-type levels of DPA produced throughout sporulation (Fig. 18). Therefore, these assays were able to successfully verify the mutant strains were expressing *cwlD* within the expected cell compartments.

Analysis of developing forespores in *cwlD* operon mutant strains

The *cwlD* operon mutant strains were analyzed for the phenotypic effects of a mutation altering the expression patterns of *cwlD* and *orf1* during spore peptidoglycan synthesis. The strains analyzed included a wild-type strain carrying a *cwlD-lacZ* fusion (DPVB70) for comparison. Four mutant strains were analyzed, an *orf1* insertion mutant carrying the *cwlD-lacZ* fusion (DPVB71), an in-frame *orf1* deletion mutant carrying the *cwlD-lacZ* fusion (DPVB72), a strain containing a wild-type chromosome carrying a plasmid expressing *orf1* and *cwlD* (DPVB100), and a strain with deletions in *cwlD* and *orf1* also carrying a plasmid expressing *orf1* and *cwlD* (DPVB77). The strains containing the *cwlD-lacZ* transcriptional fusion were used since we could be certain they expressed *orf1* and *cwlD* in the desired cell compartments. Even though the *cwlD-lacZ* fusion is present in three of the strains analyzed, it did not play a role in this analysis. The cultures were grown and sporulation was induced through nutrient exhaustion. IPTG was added to the strains carrying the IPTG-inducible plasmid at approximately $t_{2.5}$, 30 minutes before the first sample is taken for peptidoglycan analysis. Samples were taken throughout sporulation and used for the analysis of certain biochemical and phenotypic markers. Samples were also taken and used for the extraction and analysis of the structure of developing forespore peptidoglycan. Lastly, dormant spores were purified and used in the examination of resistance properties and the germination ability. The analyses of each strain were performed in duplicate. The results presented below are from one analysis of each mutant strain, however, the other examinations produced similar results.

Fig. 18. The percentage of DPA produced in the *cwlD* operon mutant *cwlD-lacZ* fusion strains throughout sporulation

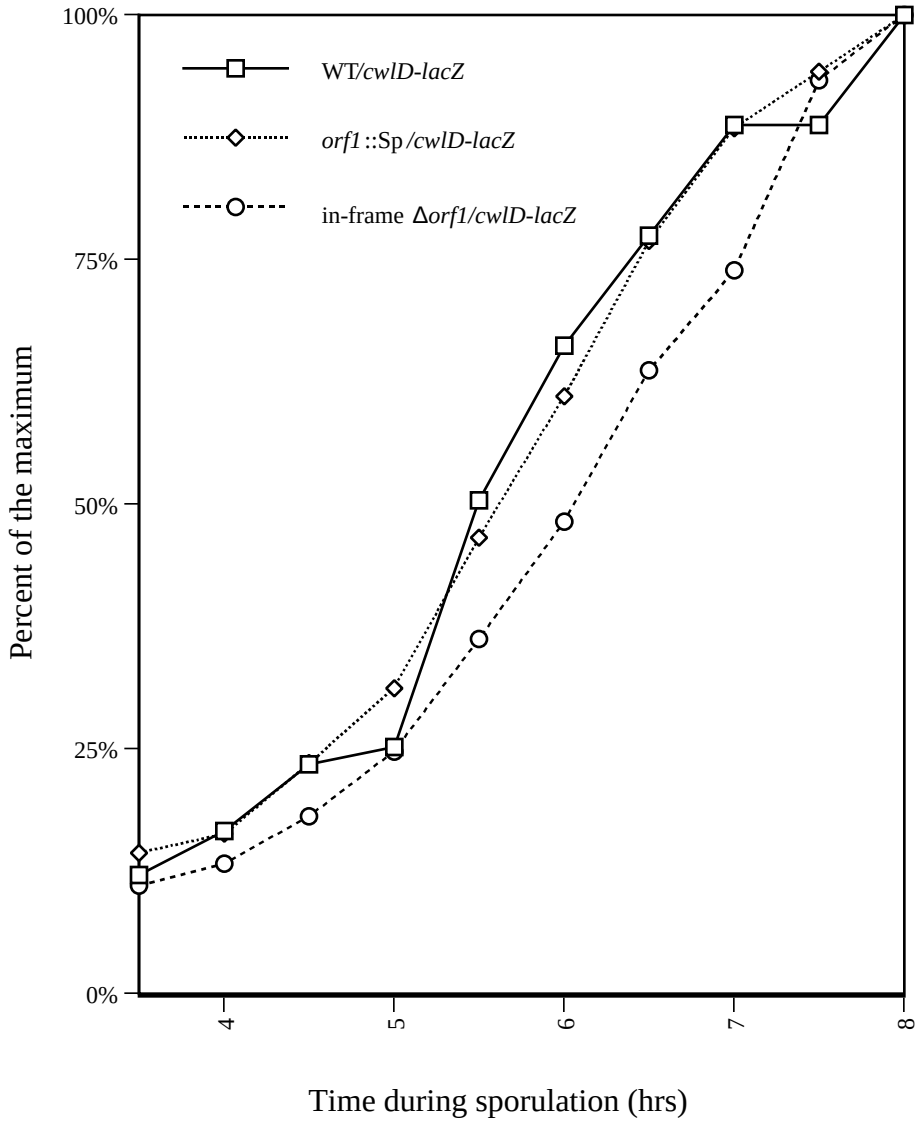


Figure 18. Measurement of the amount of DPA produced in developing forespores of wild-type and mutant strains containing *cwlD-lacZ* transcriptional fusions. Maximum values: WT */cwlD-lacZ,* 75 ug/ml; *orf1*::Sp / *cwlD-lacZ,* 68 ug/ml; in-frame Δ *orf1* / *cwlD-lacZ,* 75 ug/ml.

Comparison of mutant dormant spore resistance properties and germination ability

Dormant spores were obtained from the wild type and all four mutant strains after 48 hours of incubation (>40 hours after initiation of sporulation). The spores were used to test the heat resistance and the chloroform resistance of the mutant strains. For spore heat resistance, the culture samples were heated at 80^{o}C for 10 minutes, diluted, plated and the colonies were counted. For spore chloroform resistance, the culture samples were mixed with chloroform, put at room temperature for 10 minutes, diluted, plated, and the resulting colonies were counted. A viable count was also obtained for comparison by diluting and plating an unheated culture sample. The *orf1* insertion mutant and the in-frame *orf1* deletion mutant showed colony forming units per milliliter of culture to be equivalent to that seen in the wild-type strain for the heat resistance and the chloroform resistance assays (Table 4). The viable count of the two strains also showed similar numbers to that of wild type. The strain carrying a deletion in *orf1* and *cwlD* along with the plasmid expressing *orf1* and *cwlD* and the wild-type strain carrying the same plasmid showed a four- to six-fold reduction in viable count, heat resistance, and chloroform resistance colony forming units. Therefore, the strains carrying the IPTG-inducible plasmid expressing *orf1* and *cwlD* are not producing as many fully matured spores as compared to wild-type, however, the spores that are able to fully mature do exhibit the same heat resistance and chloroform resistance as demonstrated by wild-type.

The mutant dormant spores were then purified and used to test the germination ability of each strain. Purified spores were heat activated at 65^{o}C for 20 minutes and added to fresh media containing L-alanine to induce germination. The $O.D._{600}$ was followed for 2 hours and the percentage of the initial $O.D._{600}$ at each time point was calculated. Wild-type spores show a 40 % loss of optical density in the first 20 minutes and then the values begin to increase (Fig. 19). The initial loss of optical density is due to the rehydration of the spore core causing a loss of refractility, degradation of spore cortex peptidoglycan, and the release of spore solutes. All the mutant strains showed the ability to germinate approximately equal to that of wild type except for the strain carrying deletions in *cwlD* and *orf1* with the plasmid expressing *orf1* and *cwlD*. This mutant strain showed approximately a two-fold decrease in percentage of the optical density lost during germination and a 2-fold decrease in the subsequent cell growth.

Table 4. Analysis of mutant dormant spore resistance properties

Strain analyzed	Heat^R (cfu/ml)	Chloroform^R (cfu/ml)	Viable count (cfu/ml)
Wild type	1.4×10^9	1.9×10^9	1.3×10^9
orf1::Sp	2.1×10^9	1.9×10^9	1.7×10^9
In-frame Δ*orf1*	2.7×10^9	2.0×10^9	9.0×10^8
Δ(*orf1cwlD*) + pSpac-*orf1cwlD*	3.3×10^8	3.4×10^8	2.4×10^8
Wild type + pSpac-*orf1cwlD*	2.2×10^8	3.0×10^8	3.0×10^8

Fig. 19. Germination ability of the *cwlD* operon mutant spores

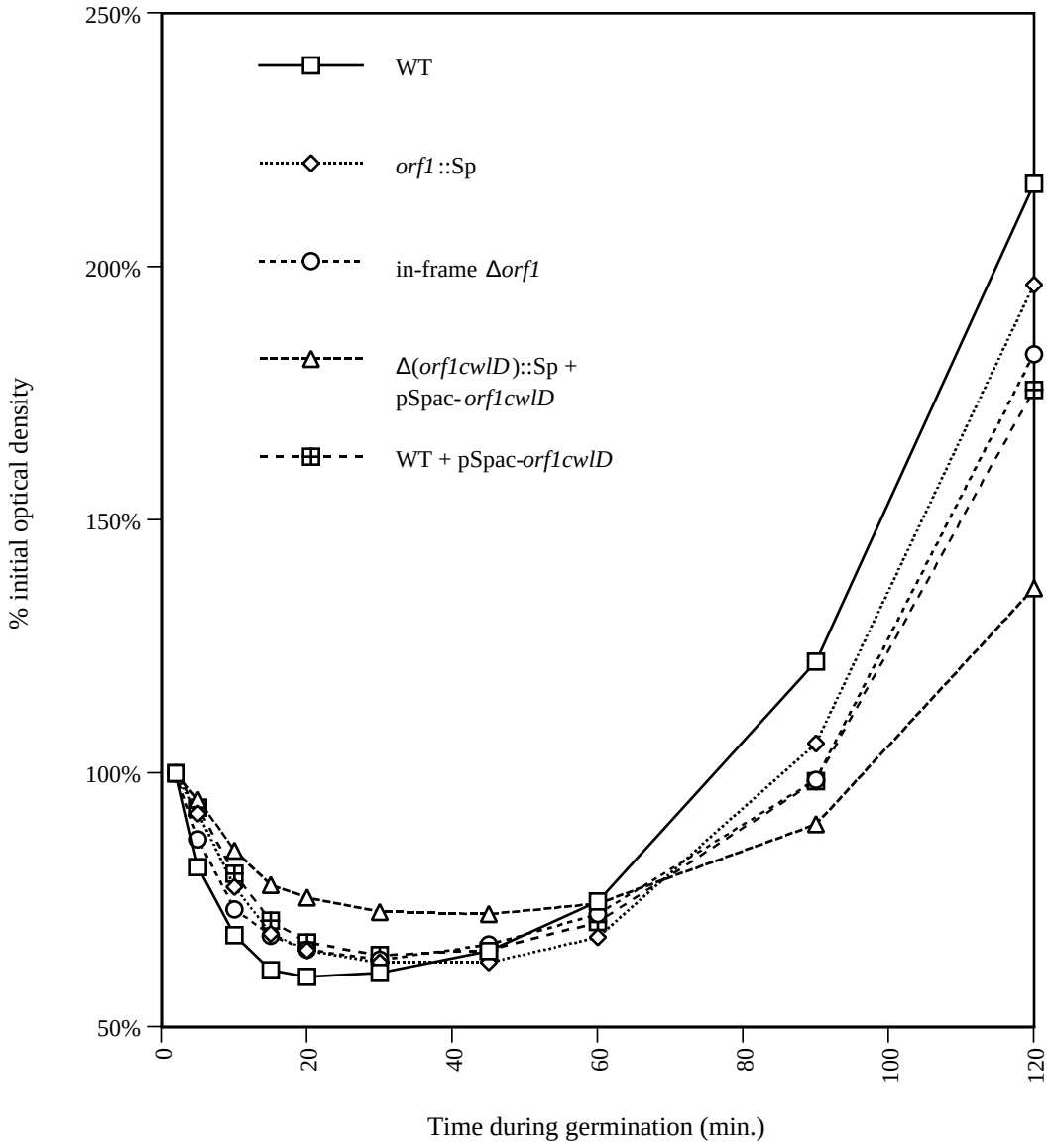


Figure 19. Dormant spores were purified and the germination ability of the wild-type and mutant strains was measured.

Timing of biochemical and phenotypic sporulation markers

Sporulation was induced through nutrient exhaustion in the wild type and mutant strain cultures. At specific time points throughout sporulation two samples were taken to measure the amount of DPA and GDH produced in the developing forespore. GDH is an enzyme produced in the forespore early in its development and can act as a marker for sporulation initiation. DPA is a spore solute that acts as a late sporulation marker as it accumulates in the developing forespore. Meador-Parton *et al.* (15) demonstrated that under these sporulation conditions GDH activity of wild-type developing forespores reaches its maximum during the fifth hour of sporulation (t_5) and drops to zero with the assay used since the forespores become lysozyme resistant. DPA begins to accumulate at $t_{4.5}$ to t_5 and continues to increase throughout sporulation. Both the *orf1* insertion mutant and the in-frame *orf1* deletion mutant showed GDH activity and DPA accumulation to be equivalent to that of the wild-type strain (Fig. 20,21,22). It was also previously shown that the appearance of heat resistant spores occurs after GDH and DPA accumulation in the wild-type strain (15). Wild-type heat resistance was also observed for the two *orf1* mutant stains.

The wild-type strain carrying the plasmid expressing CwlD and Orf1 and the strain containing deletions in *cwlD* and *orf1* and carrying the same plasmid showed results for the timing of biochemical and phenotypic sporulation markers similar to each other but different from wild type. Both strains produced spores which demonstrated the same order of events occurring as found in the wild-type strain (Fig. 23, 24). First, there is the appearance of GDH, then the accumulation of DPA, and finally the appearance of heat resistance. However both of these strains showed a delay in spore development. GDH did not reach its maximum until approximately t_7-t_8 while the maximum is reached at t_5 in wild type. DPA accumulation did not begin until approximately t_6-t_7 while wild type showed accumulation from $t_{4.5}$-t_5. Lastly, the appearance of heat resistance is also delayed. At t_8 in the wild-type strain 50% of the heat resistance has been acquired. However, at t_8 the mutant strains carrying the plasmid expressing CwlD and Orf1 have only acquired approximately 5% of the heat resistance. As a result, the strains carrying the IPTG-inducible plasmid demonstrate a delay in sporulation.

Fig. 20. Appearance of phenotypic and biochemical markers during sporulation of a wild type strain of *Bacillus subtilis*

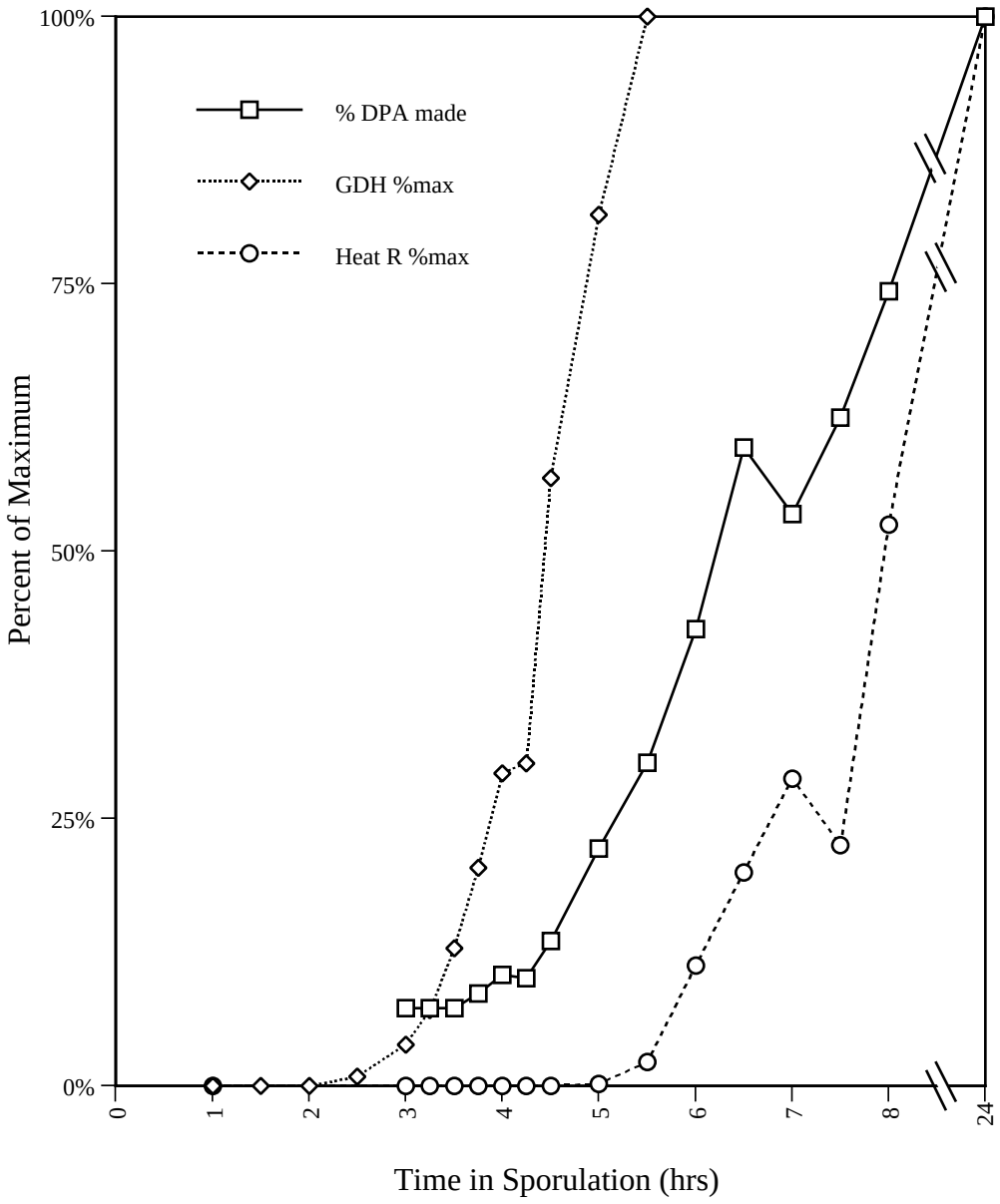


Figure 20. Cells were grown in 2xSG media and allowed to sporulate. Samples were taken to measure DPA, GDH, and the heat resistance of wild-type developing forespores.

Fig. 21. Appearance of phenotypic and biochemical markers during sporulation of an *orf1* insertion mutant strain of *Bacillus subtilis*

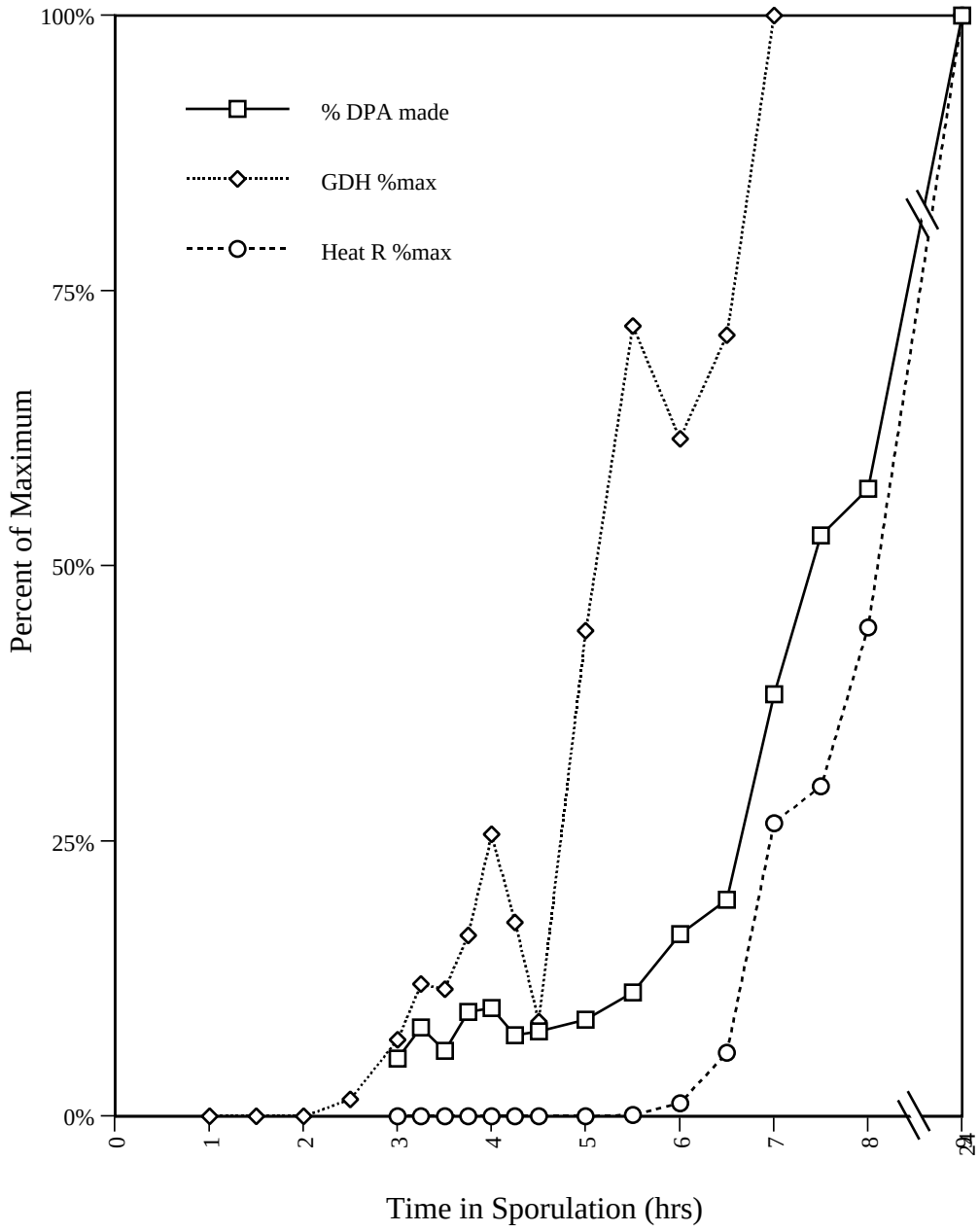


Figure 21. Cells were grown in 2xSG media and allowed to sporulate. Samples were taken to measure DPA, GDH, and the heat resistance of an *orf1* insertion mutant strain.

Fig. 22. Appearance of phenotypic and biochemical markers during sporulation of an in-frame *orf1* deletion mutantstrain of *Bacillus subtilis*

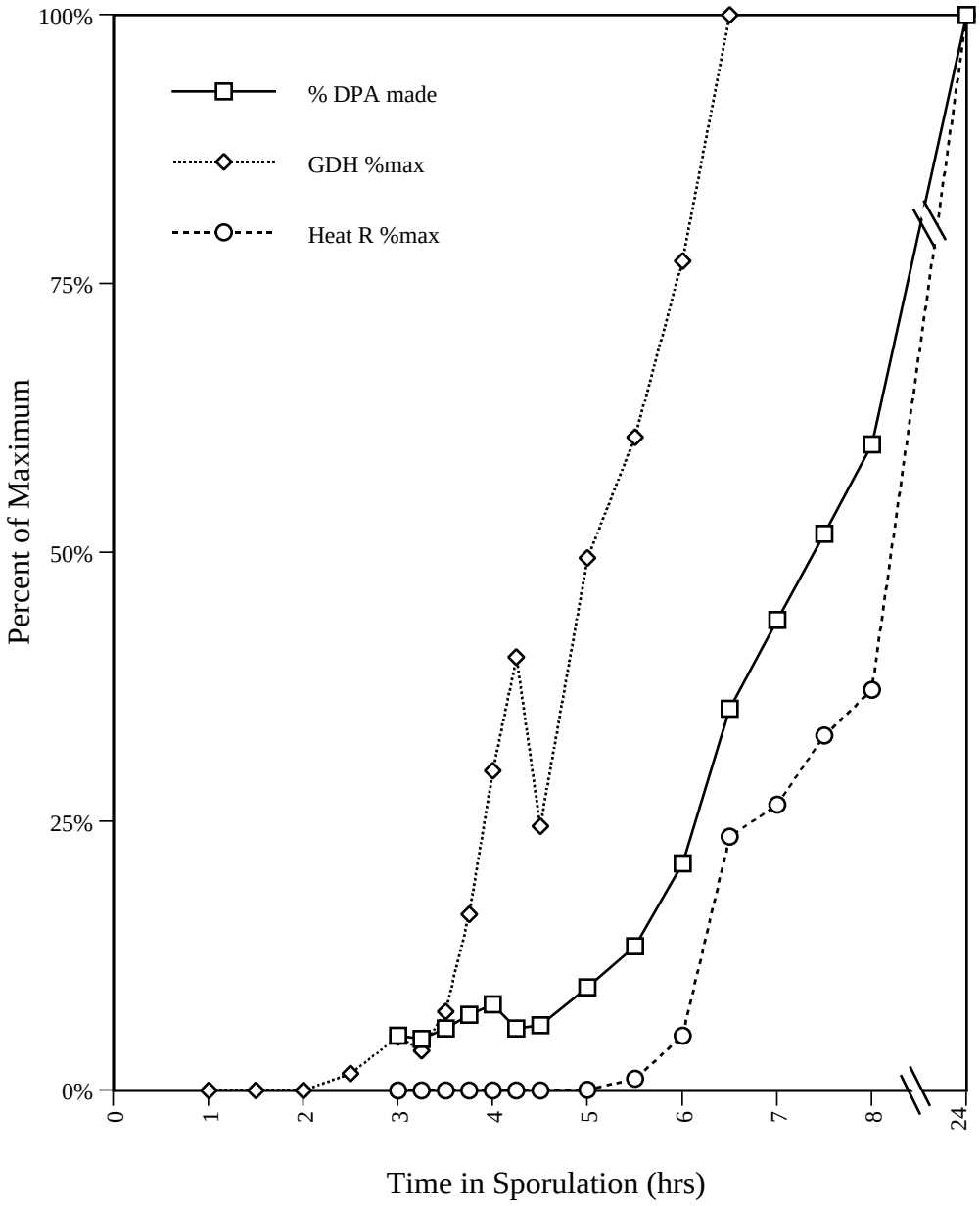


Figure 22. Cells were grown in 2xSGmedia and allowed to sporulate. Samples were taken to measure DPA, GDH, and the heat resistance of an in-frame *orf1* deletion mutant strain.

Fig. 23. Appearance of phenotypic and biochemical markers during sporulation of a *Bacillus subtilis* wild type strain carrying a plasmid expressing *orf1* and *cwlD.*

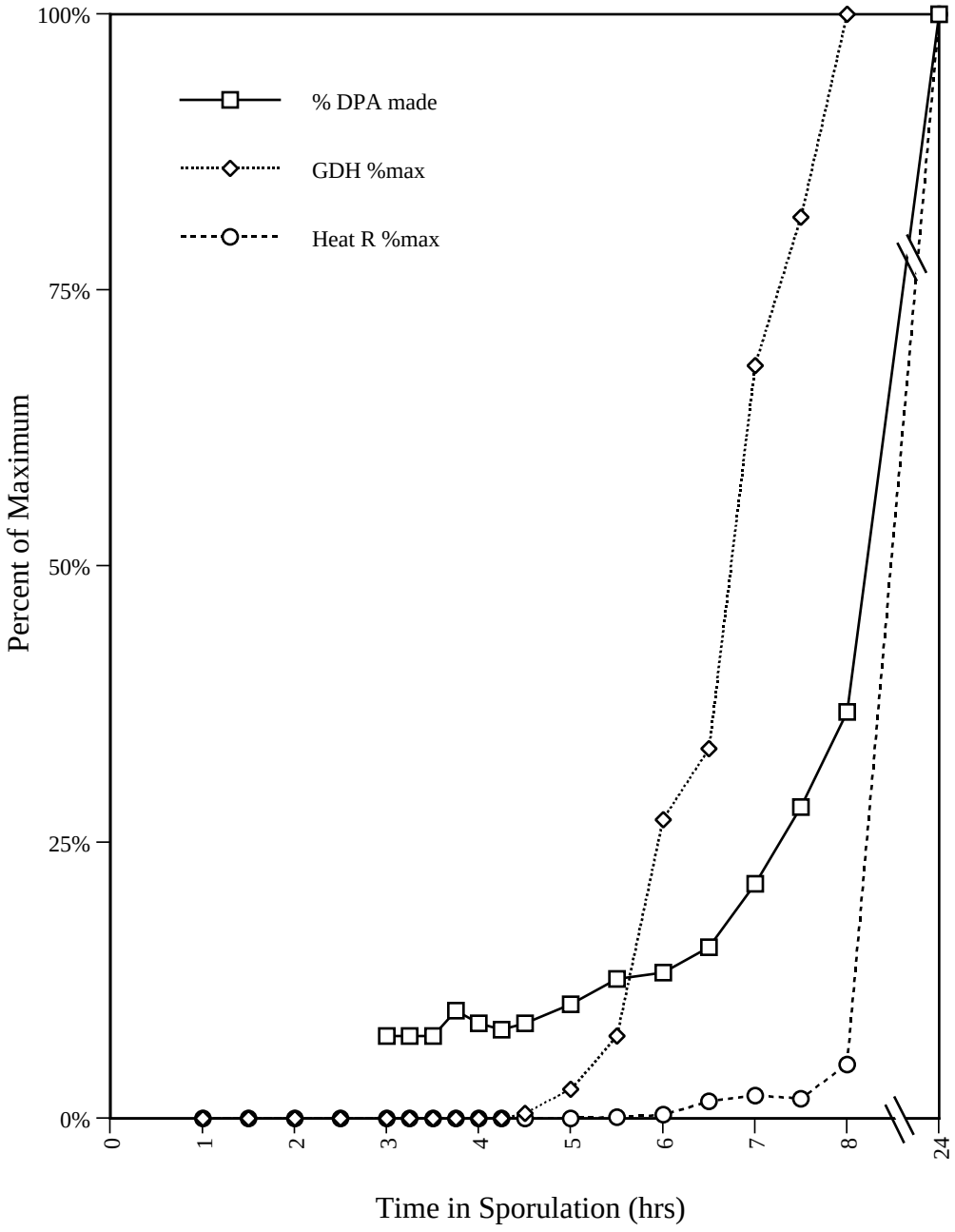


Figure 23. Cells were grown in 2xSGmedia and allowed to sporulate. Samples were taken to measure DPA, GDH, and the heat resistance of an a wild-type strain carrying a plasmid expressing CwlD and Orf1

Fig. 24. Appearance of phenotypic and biochemical markers during sporulation of a *Bacillus subtilis* strain containing deletions in *orf1* and *cwlD* and carrying a plasmid expressing *orf1* and *cwlD*

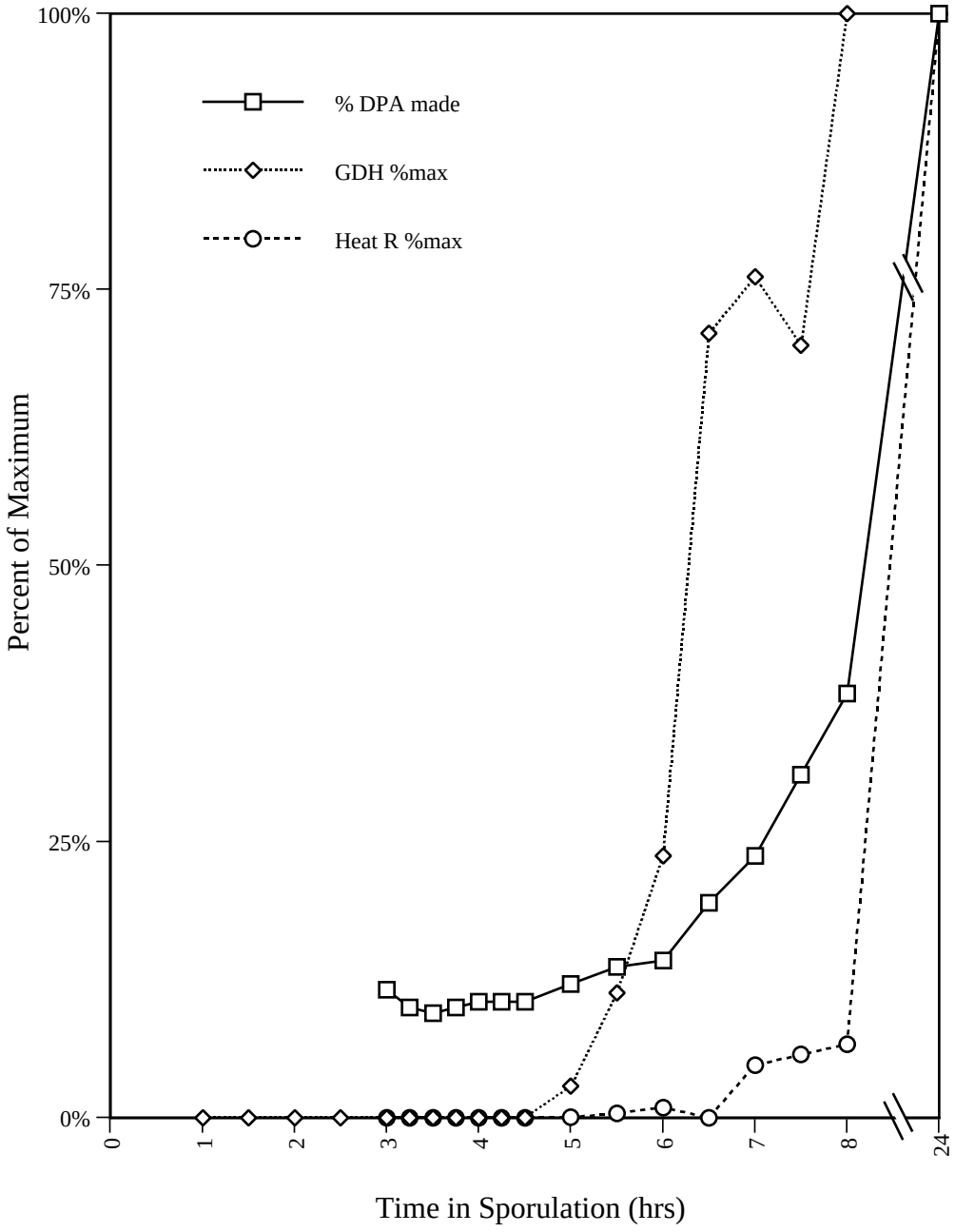


Figure 24. Cells were grown in 2xSGmedia and allowed to sporulate. Samples were taken to measure DPA, GDH, and the heat resistance of an strain containing deletions in *orf1* and *cwlD* and carrying a plasmid expressing Orf1 and CwlD.

Structural comparison for mutant forespore peptidoglycan throughout sporulation

Wild-type

When performing the mutant analysis experiments a sample was taken to measure the total muramic acid content of the developing peptidoglycan. This value is obtained after an amino acid and amino sugar analysis is performed for each sample. The percent of spore peptidoglycan made at each time point throughout sporulation can then be calculated as previously described (15). This value is used when comparing structural parameter values between wild type and mutant strains.

A large culture sample was also taken from which the forespore peptidoglycan was purified and analyzed with RP-HPLC as previously described (15). Analysis of the HPLC traces allowed the identification of peaks which correspond to specific muropeptides which were previously identified through amino acid and mass spectrometry analysis (15, 20). Through the quantification of each peak different structural parameters of the isolated peptidoglycan could be compared between the analyzed strains at specific time points throughout sporulation. Meador-Parton *et al.* (15) developed this method of developing forespore peptidoglycan analysis throughout sporulation and determined the structural parameters of wild-type spore peptidoglycan. They found that there is a low level of muramic lactam in the first 5-10 % of spore peptidoglycan synthesized followed by a rapid increase. They also saw that there was a high percentage of tripeptide side chains in the first 10%. These data led them to believe that the first 10% of spore peptidoglycan synthesized represents the germ cell wall which is made adjacent to the inner forespore membrane. They also found that there is a gradient of cross-linking across the spore peptidoglycan. It was observed that the first 5-10% of peptidoglycan synthesized is highly cross-linked, then there is a rapid decrease in cross-linking over the next 30% followed by a slight increasing gradient over the last 60% of spore peptidoglycan made (15).

***orf1cwlD* operon mutants**

The analysis of the structural parameters of the *orf1* insertion mutant and in-frame *orf1* deletion mutant developing forespore peptidoglycan showed values similar to that of the wild-type strain (Tables 5,6,7).

Table 5. Structural parameters for forespore PG from (#9) mutant *Bacillus subtilis*

		% Muramic acid with side chains of[b]:					
Time in sporulation (hrs)	% spore PG made[a]	% lactam	% Ala	% TriP	% TP	% peptide cross-linked	% muramic acid with cross-link
6	21	48.1	45.6	3.7	2.6	15.1	0.9
6.5	39	47.4	45.6	4.7	2.3	22.6	1.6
7	62	49.1	35.6	3.2	12.0	8.8	1.3
7.5	85	49.0	29.9	3.2	17.9	11.4	2.4
8	100	48.8	25.4	2.9	22.8	11.6	3
24	100	49.9	19.4	1.9	28.8	11.2	3.4

[a] This data is derived from the interpolation of muramic acid peak values as determined by amino acid analyses.

[b] Abbreviations: lactam, muramic-δ-lactam; Ala, alanine; TriP, tripeptide; TP, tetrapeptide.

Table 6. Structural parameters for forespore peptidoglycan from an *orf1* insertion mutant *Bacillus subtilis* strain

		% Muramic acid with side chains of[b]:					
Time in sporulation (hrs)	% spore PG made[a]	% lactam	% Ala	% TriP	% TP	% peptide cross-linked	% muramic acid with cross-link
3.75	2	24.3	34.2	35.1	6.2	15.1	6.3
4	4	32.5	42.4	20.6	4.5	17.9	4.5
4.25	6	24.9	34.0	34.7	6.3	15.4	6.3
4.5	9	31.8	35.4	26.7	6.1	17.6	5.8
5	15	39.3	42.3	14.2	4.2	20.9	3.8
5.5	25	44.9	45.2	6.3	3.5	20.6	2.0
6	42	45.2	43.0	5.9	5.8	20.6	2.4
6.5	76	45.5	29.8	5.0	19.7	13.5	3.3
7	91	46.7	28.6	4.0	20.8	12.8	3.2
7.5	98	47.1	28.2	3.6	21.0	13.1	3.2
8	100	47.3	27.5	3.4	21.8	12.6	3.2
24	100	48.4	24.7	2.4	24.5	12.7	3.4

[a] This data is derived from the interpolation of muramic acid peak values as determined by amino acid analyses.

[b] Abbreviations: lactam, muramic-δ-lactam; Ala, alanine; TriP, tripeptide; TP, tetrapeptide.

Table 7. Structural parameters for forespore peptidoglycan from an in-frame *orf1* deletion mutant *Bacillus subtilis* strain

		% Muramic acid with side chains of[b]:					
Time in sporulation (hrs)	% spore PG made[a]	% lactam	% Ala	% TriP	% TP	% peptide cross-linked	% muramic acid with cross-link
3.5	1	29.0	40.1	23.8	7.1	18.2	5.6
3.75	2	30.6	40.1	22.1	7.22	18.4	5.4
4	3.5	37.7	44.7	13.2	4.5	19.5	3.4
4.25	5	38.9	43.8	12.9	4.3	17.9	3.1
4.5	7	44.2	44.1	8.6	3.0	19.2	2.2
5	14	44.9	42.4	8.0	4.7	20.6	2.6
5.5	27	44.3	35.4	7.9	12.3	13.2	2.7
6	58	44.8	30.3	5.5	19.4	12.1	3.0
6.5	81	47.0	29.6	3.3	20.0	11.9	2.8
7	91	47.4	28.9	3.1	20.7	11.7	2.8
7.5	96	47.7	28.4	2.8	21.2	12.0	2.9
8	100	47.8	27.9	2.6	21.6	11.5	2.8
24	100	48.6	24.9	2.0	24.6	11.1	3.0

[a] This data is derived from the interpolation of muramic acid peak values as determined by amino acid analyses.

[b] Abbreviations: lactam, muramic-δ-lactam; Ala, alanine; TriP, tripeptide; TP, tetrapeptide.

The analysis of the structural parameters of the strain containing a wild-type chromosome and the plasmid expressing *orf1* and *cwlD* showed wild-type results for the percent alanine, tripeptide and tetrapeptide side chains (Table 8). However, the percent of muramic lactam was increased in the first layers of spore peptidoglycan produced and there was a decrease in the percent of muramic acid residues involved in cross-linking. The inverse relationship between the amount of muramic lactam and the percent of muramic acid residues cross-linked is expected due to the cleavage of the peptide side chains from the muramic acid residues during the synthesis of muramic lactam. There appears to be a delay in spore peptidoglycan synthesis following IPTG addition.

The analysis of the structural parameters of the strain containing a deletion of *orf1* and *cwlD* and carrying the plasmid expressing *orf1* and *cwlD* showed wild-type results for the percent of alanine, tripeptide, and tetrapeptide side chains (Table 9). However, this strain exhibited a reduced amount of muramic lactam throughout all layers of peptidoglycan produced. There was also an increase in the percent of muramic acid residues involved in cross-linking as expected with lower amounts of muramic lactam present. This strain also exhibited a delay in sporulation that we believe is due to the addition of IPTG.

The amount of cross-linking in each 10% "slice" of peptidoglycan synthesized throughout sporulation was calculated as previously described (15). The values in tables 5-9 indicate the percentage of muramic acid involved in cross-linking in the total peptidoglycan synthesized up to each specific time point. However, this calculation allows us to look at the percent of muramic acid involved in cross-linking in each 10% segment or "slice" of peptidoglycan made. Therefore, values were determined for the percent of cross-linking in the new spore peptidoglycan synthesized within each 10% segment of the developing forespore peptidoglycan. Meador-Parton *et al.* (15) used this calculation to determine that the wild-type strain does show an increasing gradient of cross-linking across the outer layers of the spore peptidoglycan as previously theorized (15, 19).

The *orf1* insertion mutant showed the same pattern of muramic acid involved in cross-linking per each 10% "slice" of peptidoglycan as seen for wild-type (Fig. 25). There is a high degree of cross-linking in the first 10% of peptidoglycan made, a rapid decrease in the next 10-20% made, and a steady increase in the last 70% synthesized (15). In one experiment the in-frame *orf1* deletion mutant showed a decrease in the percentage of cross-linking seen in the first

Table 8. Structural parameters for forespore peptidoglycan from a mutant *Bacillus subtilis* strain containing a wild-type chromosome and carrying a plasmid expressing *orf1* and *cwlD*

		% Muramic acid with side chains of[b]:					
Time in sporulation (hrs)	% spore PG made[a]	% lactam	% Ala	% TriP	% TP	% peptide cross-linked	% muramic acid with cross-link
6	5	44.5	31.1	6.9	17.5	6.6	1.6
6.5	50	45.6	30.8	6.5	17.1	9.5	2.2
7	71	48.3	37.1	4.1	10.6	8.0	1.2
7.5	85	48.6	29.9	3.4	18.0	11.8	2.5
8	100	49.1	27.6	2.9	20.4	11.1	2.6
24	100	50.7	17.3	1.4	30.6	10.4	3.3

[a] This data is derived from the interpolation of muramic acid peak values as determined by amino acid analyses.

[b] Abbreviations: lactam, muramic-δ-lactam; Ala, alanine; TriP, tripeptide; TP, tetrapeptide.

Table 9. Structural parameters for forespore peptidoglycan from a mutant *Bacillus subtilis* strain containing deletions in *orf1* and *cwlD* and carrying a plasmid expressing *orf1* and *cwlD*

		% Muramic acid with side chains of[b]:					
Time in sporulation (hrs)	% spore PG made[a]	% lactam	% Ala	% TriP	% TP	% peptide cross-linked	% muramic acid with cross-link
6	10	30.9	42.7	7.4	19.1	9.1	2.4
6.5	48	36.4	39.3	5.3	19.0	8.3	2.0
7	82	37.3	33.6	4.4	24.7	11.4	3.3
7.5	93	38.7	30.0	3.5	27.8	12.1	3.8
8	100	37.9	28.4	3.3	30.4	12.0	4.0
24	100	39.6	20.7	2.1	37.6	11.9	4.7

[a] This data is derived from the interpolation of muramic acid peak values as determined by amino acid analyses.

[b] Abbreviations: lactam, muramic-δ-lactam; Ala, alanine; TriP, tripeptide; TP, tetrapeptide.

Fig. 25. Comparison of cross-linking within each section of spore peptidoglycan

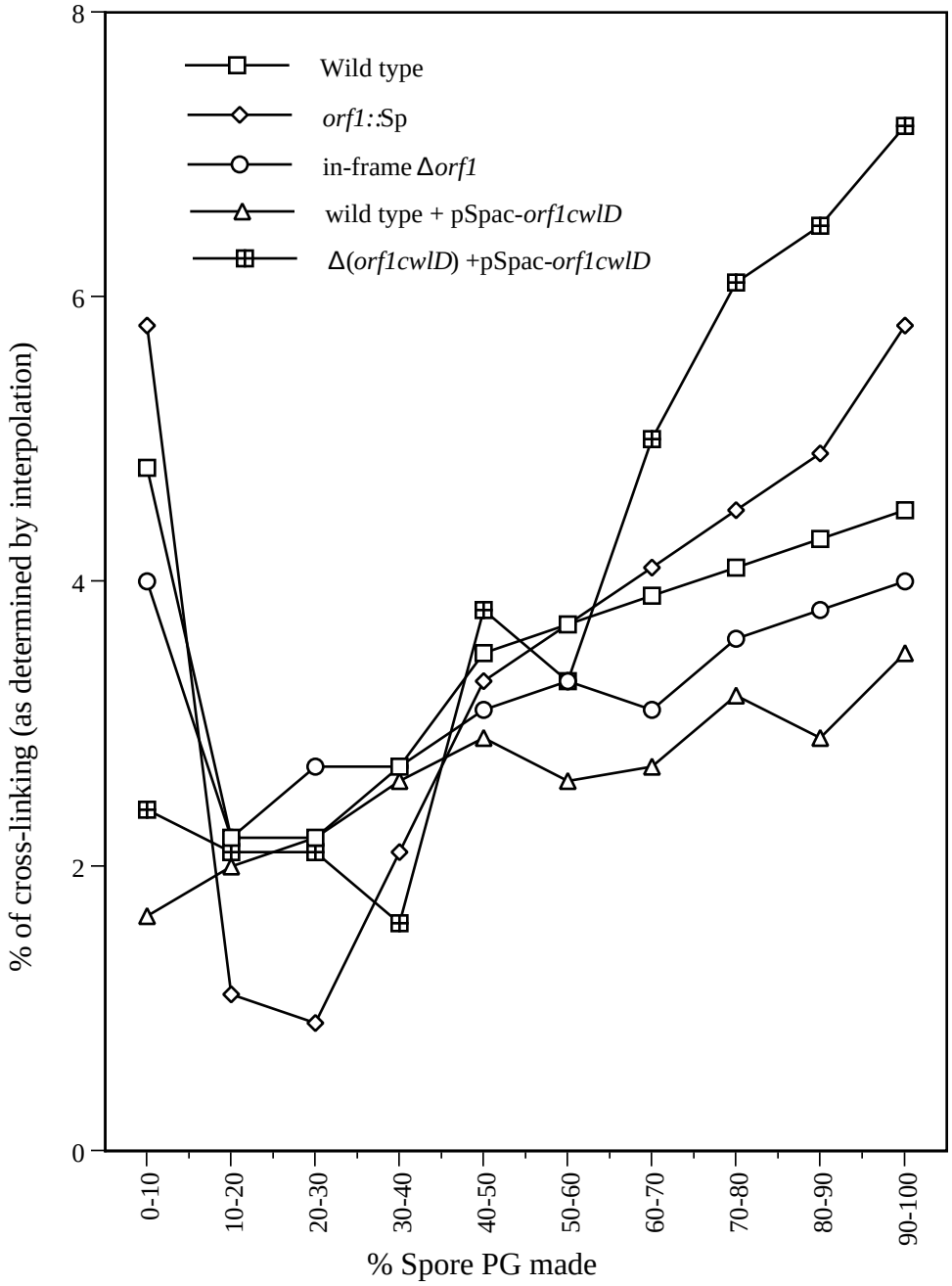


Figure 25. Comparison of cross-linking for wild-type and all *cwlD* operon mutant strains

10% of spore peptidoglycan synthesized. However, this result was not reproducible and, therefore, we believe the strain has percentages similar to that of wild type. The mutant strain carrying a wild-type chromosome and the plasmid expressing *orf1* and *cwlD* showed approximately a two-fold decrease in cross-linking in the first 10% spore peptidoglycan made. Therefore, there is apparently not a rapid decrease in cross-linking as seen in wild type, instead, there is simply a steady increase in cross-linking throughout all of the peptidoglycan synthesized. The strain containing deletions in *orf1*and *cwlD* and carrying the plasmid expressing *orf1*and *cwlD* also showed approximately a two-fold decrease in cross-linking in the first10% of peptidoglycan made. The mutant strain did show an increase in cross-linking over the last 60% of peptidoglycan made, however, the cross-linking increased to almost two-fold more than wild type in the final 10%.

Discussion of the Genetic Analysis of the *cwlD* Operon

The genetic analysis of the *cwlD* operon was carried out through the construction and analysis of a series of mutant *B. subtilis* strains. The expression patterns of the *cwlD* operon products were altered in order to determine a reason for expressing CwlD in the forespore and a possible role for Orf1.

Comparison of mutant dormant spore resistance properties and germination ability

Dormant spores were obtained after 48 hours of growth from the four *cwlD* operon mutant strains. The mutant spores were tested for their heat resistance and chloroform resistance. The spores were then purified and used to test the germination ability of the mutants. It was found that the *orf1* insertion mutant and the in-frame *orf1* deletion mutant strain made as many spores as wild type and the spores had equal heat resistance and chloroform resistance. The *orf1* insertion mutant expresses CwlD only within the mother cell while neither Orf1 nor CwlD are expressed in the forespore. Therefore, the absence of Orf1 and CwlD within the forespore does not affect the heat resistance, chloroform resistance, or germination ability of this mutant. Even if CwlD is expressed in the mother cell and within the forespore, while Orf1 is not expressed at all which is the case in the in-frame *orf1* deletion mutant, there are still no affects on resistance properties or germination ability. It was previously found that a mutant strain that does not produce CwlD in the mother cell or within the forespore is unable to germinate but exhibits wild-type heat resistance (21). From these three mutant strains we can conclude that CwlD must be present in the mother cell in order to produce spores that are able to germinate while CwlD in the forespore does not play a role in the spores' ability to germinate.

The strain carrying a deletion of both *orf1* and *cwlD* along with the plasmid expressing Orf1 and CwlD and the wild-type strain carrying the same plasmid showed a four-fold and six-fold reduction, respectively, in the number of heat resistant spores as produced by the wild-type strain. These strains also showed a six-fold reduction in the number of chloroform resistant spores as compared to wild type. Therefore, the mutant strains carrying the IPTG-inducible plasmid expressing Orf1 and CwlD are not producing as many fully matured spores as compared to wild type. However, the spores that are fully mature do exhibit the same heat resistance and chloroform resistance as demonstrated by wild type. The inability of the spores to reach full

maturation could be due to the addition of IPTG that somehow interferes with spore development. The purified spores from the wild-type strain carrying the plasmid expressing Orf1 and CwlD showed wild-type germination ability. However, spores from the strain carrying deletions in *orf1* and *cwlD* and carrying the same plasmid exhibited a two-fold decrease in the percentage of the optical density lost during germination along with a two-fold decrease in the rate of optical density increased in the outgrowing culture. This data could be interpreted in two ways. It is possible that the reduction in optical density loss is due to a decrease either in the rate of the spores germinating or in the number of spores germinating. We are unable to conclude which explanation is correct without further experimentation. However, we can conclude that the germination ability of this mutant is not equivalent to wild type. These results are not surprising since Orf1 and CwlD are not being expressed from the wild-type promoters and, instead, are produced from the plasmid. The plasmid-produced Orf1 and CwlD may not be as abundant as in the wild type. If this strain is producing a lower amount of CwlD, then there would be less muramic lactam produced. If there is less muramic lactam in the cortex then it would be harder for the germination lytic enzymes to degrade the cortex peptidoglycan during germination. If the cortex peptidoglycan is degraded slower then we would see slower germination and subsequently slower outgrowth of the mutant spores.

Timing of biochemical and phenotypic sporulation markers

Samples were taken to measure the amount of GDH, the accumulation of DPA, and the appearance of heat resistance in the developing forespores of the four *cwlD* operon mutants. All of the mutant strains showed results similar to wild type except the strains carrying the IPTG-inducible plasmid. The wild-type strain carrying the plasmid expressing CwlD and Orf1 and the strain containing deletions in *orf1* and *cwlD* while carrying the same plasmid showed the same order of events as wild-type, however, they exhibited a delay in the appearance of these sporulation markers. This delay could be due to the addition of IPTG somehow interfering with spore development.

Structural comparison for mutant forespore peptidoglycan throughout sporulation

The structural parameters for the spore peptidoglycan of each mutant strain were determined throughout sporulation. The *orf1* insertion mutant showed results similar to wild

type. The in-frame *orf1* deletion mutant showed a decrease in the percentage of muramic acid involved in cross-linking in the first 10% of spore peptidoglycan synthesized. If our hypothesis stating that the role of *orf1* is to inhibit CwlD activity within the forespore is correct, then we would expect to see this decrease in cross-linking. However, this result was not reproducible and, as a result, suggests that our hypothesis is not correct.

The wild-type strain carrying the plasmid expressing CwlD and Orf1 showed wild-type percentages of alanine, tripeptide and tetrapeptide side chains. However, this strain showed an increase in the percentage of muramic lactam found in the first layers of peptidoglycan synthesized. A decrease in the percentage of muramic acid involved in cross-linking was also observed. The increase in the percentage of muramic lactam found in the early layers produced could be due to the fact that there is more CwlD produced in the mother cell and within the forespore due to the expression of the plasmid carrying the gene. If there is more muramic lactam in the early layers of peptidoglycan synthesized, which represents the germ cell wall we would expect that the germ cell wall would be degraded along with the cortex by the germination lytic enzymes during germination. If this was to occur, it could be possible that the cells might lyse before outgrowth begins. However, we did not observe obvious cell lysis during germination and saw wild type outgrowth. Therefore, we believe that the cells did not lyse even if the germ cell wall is degraded since the spore coat layers are still present and could retain the pressure exerted by the cell until a vegetative peptidoglycan wall is produced.

The strain containing deletions in *cwlD* and *orf1* while carrying the plasmid expressing CwlD and Orf1 also showed wild-type percentages of alanine, tripeptide, and tetrapeptide side chains. However, this mutant strain showed a decrease in the percentage of muramic lactam produced throughout all layers of peptidoglycan produced. An increase in the percentage of muramic acid involved in cross-linking was also seen. The decrease in the amount of muramic lactam produced could be a result of not expressing CwlD from the wild-type promoter. The CwlD expressed in this strain is from the plasmid only and, therefore, may be produced in lower concentration. As a result, the plasmid-produced CwlD would not be able to cleave as many peptide side chains from muramic acid residues to form muramic lactam as the wild-type CwlD. If less muramic lactam is produced then the germination lytic enzymes would degrade the cortex peptidoglycan slower. This would explain why we observed the delay in germination and outgrowth of this mutant strain. There is a second explanation concerning why we saw a

decrease in the percentage of muramic lactam. It is possible that our hypothesis is correct concerning Orf1, it inhibits the activity of the CwlD produced in the forespore and that is why we do not see muramic lactam in the germ cell wall layers. Therefore, the plasmid expressed Orf1 could be somehow inhibiting some of the activity of CwlD produced in the mother cell and, as a result, less muramic lactam is produced in the cortex. In order to determine which explanation is correct a wild-type strain carrying an IPTG-inducible plasmid only expressing Orf1 both in the mother cell and forespore compartments should be constructed and analyzed.

Final Conclusions

Through the biochemical and the genetic analysis of the *cwlD* operon products, a few final conclusions can be drawn. The biochemical analysis allowed us to detect muramoyl-L-alanine amidase activity when CwlD is induced *in vivo*. Further analysis of the peptidoglycan structure told us that CwlD is carrying out the first step of muramic lactam synthesis, cleaving of the peptide side chain. From this information we believe that it is unnecessary for the peptide to be cleaved down to a single L-alanine side chain in order for CwlD to act. We also believe that other enzymes are needed to complete lactam cyclization. We were also able to obtain antibodies specific for CwlD. Use of the antibodies revealed that we had purified two different forms of the same protein since they demonstrated the same antigenicity. When both of the proteins, CwlD with and without the signal peptide, were purified we were unable to detect muramoyl-L-alanine activity *in vitro*. Therefore, the activity of both forms of the purified proteins was unable to survive the purification process.

Future research on the activity of CwlD could be directed towards identifying proteins that interact with CwlD. The interacting proteins may be a necessary component for either maintaining the protein's activity or completing MAL cyclization. In vitro assays will also be performed to examine the specific substrate of CwlD. Is it a single L-alanine, a tripeptide, or tetrapeptide side chain?

The genetic analysis of the *cwlD* operon products showed us that the absence of Orf1 and CwlD or just Orf1 within the forespore has no effect on the spore resistance properties or germination ability. In addition, CwlD must be present in the mother cell in order for germination to occur while CwlD in the forespore is not needed. We hypothesized that the role of Orf1 is to inhibit CwlD activity within the forespore explaining why there is no muramic lactam found in the germ cell wall. We found evidence that did not support our hypothesis when we analyzed the amount of muramic acid involved in cross-linking in the *orf1*in-frame deletion mutant. However, the strain containing deletions in *orf1* and *cwlD* while carrying the plasmid expressing Orf1 and CwlD showed decreased amounts of muramic lactam in the spore cortex peptidoglycan. It is a possibility that the reason for the decreased amounts of muramic lactam is due to the inhibition of CwlD activity by Orf1. It is also possible that the decrease in muramic lactam was due to expressing CwlD from the plasmid and not from the wild-type promoter.

Therefore, there could be less CwlD available to produce muramic lactam. These explanations could be tested by constructing and analyzing a strain carrying an IPTG-inducible plasmid only expressing Orf1 both in the mother cell and the forespore compartments. This mutant strain would allow us to see the action of Orf1 on CwlD expressed from the wild-type chromosome. We would then be able to determine if the decrease in muramic lactam is due to simply producing less CwlD or if Orf1 is really inhibiting CwlD produced in the mother cell.

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Meghan E. Gilmore

e-mail: mgilmore@vt.edu

EDUCATION

Master of Science, Microbiology August 2000
Virginia Polytechnic Institute and State University, Blacksburg, VA
- **Thesis:** Analysis of the roles of the *cwlD* operon products during sporulation in *Bacillus subtilis*
- *Project includes:* PCR, Gene manipulation, Protein purification, SDS-PAGE, HPLC analysis, FPLC analysis, Southern blotting, Western blotting
- **Major Advisor: Dr. David Popham, Assistant Professor of Microbiology**

Bachelor of Science, Biology, May 1998
Virginia Polytechnic Institute and State University, Blacksburg, VA; GPA: 3.6

PROFESSIONAL EXPERIENCE

Research

Virginia Polytechnic Institute and State University, Blacksburg, VA Undergraduate research, January 1998 – May 1998
Laboratory technician, May 1998 – August 1998
- Studies of Low-Molecular-Weight Penicillin-Binding proteins in *Bacillus subtilis*

Worcester Foundation for Biomedical Research, Shrewsbury, MA
Research fellow, June 1996 – August 1996
- Research project: Antisense oligonucleotide reversal of quinine resistance in *Plasmodium falciparum* malaria.

Teaching

Virginia Polytechnic Institute and State University, Blacksburg, VA
Graduate Teaching Assistant, Laboratory instructor,
August 1999 – December 1999
- Taught laboratory sections in General Microbiology

PUBLICATION

D.L. Popham, M. E. Gilmore, 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.

PROFESSIONAL MEMBERSHIPS/HONORS

American Society for Microbiology (ASM)
Research Assistantship, 1998, 1999, 2000
Golden Key National Honor Society

Phi Eta Sigma National Honor Society
Phi Sigma Biological Honor Society
Graduated cum laude, 1998