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Structural Analysis of *Bacillus subtilis* Spore Peptidoglycan during Sporulation

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A major structural element of bacterial endospores is a peptidoglycan (PG) wall. This wall is produced between the two opposed membranes surrounding the developing forespore and is composed of two layers. The inner layer is the germ cell wall, which appears to have a structure similar to that of the vegetative cell wall and which serves as the initial cell wall following spore germination. The outer layer, the cortex, has a modified structure, is required for maintenance of spore dehydration, and is degraded during spore germination. Theories suggest that the spore PG may also play a mechanical role in the attainment of spore dehydration. Inherent in one of these models is the production of a gradient of cross-linking across the span of the spore PG. We report analyses of the structure of PG found within immature, developing *Bacillus subtilis* forespores. The germ cell wall PG is synthesized first, followed by the cortex PG. The germ cell wall is relatively highly cross-linked. The degree of PG cross-linking drops rapidly during synthesis of the first layers of cortex PG and then increases two- to eightfold across the span of the outer 70% of the cortex. Analyses of forespore PG synthesis in mutant strains reveal that some strains that lack this gradient of cross-linking are able to achieve normal spore core dehydration. We conclude that spore PG with cross-linking within a broad range is able to maintain, and possibly to participate in, spore core dehydration. Our data indicate that the degree of spore PG cross-linking may have a more direct impact on the rate of spore germination and outgrowth.

Sporulation by certain gram-positive bacteria, such as *Bacillus* and *Clostridium* spp., results in the formation of a metabolically dormant cell known as an endospore, which is resistant to severe physical and chemical conditions. Sporulation involves an asymmetric septation to produce the mother cell, which will contribute several components of the mature spore, and the forespore, which will develop into the mature spore. Engulfment of the smaller forespore by the larger mother cell leaves the forespore surrounded by two membranes. Spore peptidoglycan (PG) synthesis occurs within this intermembrane space. Spore PG is clearly required for the maintenance of spore core dehydration, the major factor determining spore heat resistance (4, 19, 26). It has also been suggested that spore PG may play a direct role in the attainment of spore core dehydration (17, 23, 34).

Electron microscopy suggests that spore PG consists of two layers: a thin layer adjacent to the inner forespore membrane, called the germ cell wall, and a thicker outer layer, termed the cortex. The structure of the germ cell wall is believed to be similar to that of vegetative PG (33). Functionally, the germ cell wall is defined as the initial cell wall following spore germination and is possibly a template for the synthesis of vegetative PG. It has been suggested that germ cell wall PG is synthesized from precursors made in the forespore, but this has not been clearly demonstrated (33). Cortex PG is synthesized from precursors that are made in the mother cell and transported across the outer forespore membrane into the intermembrane space (8, 33).

Warth and Strominger first determined the structures of both vegetative cell and spore PG in *Bacillus subtilis* (35–37). In both structures the glycan strands contain alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) residues. Each NAM residue initially has a pentapeptide side chain that can be utilized in the cross-linking of the glycan strands. The cross-linking of these peptides involves removal of a terminal D-alanine by a transpeptidase and the formation of a new peptide bond between the second D-alanine residue of the peptide side chain and the diaminopimelic acid (Dpm) residue of a peptide side chain on another glycan strand. The transglycosylase and transpeptidase activities involved in PG synthesis are contained within the high-molecular-weight (high-MW) penicillin-binding proteins (PBPs) (reviewed in references 10 and 13). The low-MW PBPs are most frequently DD-carboxypeptidases that remove terminal D-alanine residues. This activity can limit the number of peptides available to participate in cross-linking and can thereby decrease the overall percentage of cross-linking (23, 24, 32). More recently, spore PG structure has been analyzed in greater detail and in a number of mutant strains (3, 24). In spore PG the peptide side chains are completely removed from 50% of the NAM residues and these sugars are converted to muramic-δ-lactam. These muramic-δ-lactam residues are produced in a regular pattern at every second NAM position in the glycan strands. The side chains on 24% of the NAM residues have been cleaved to single L-alanine residues. A result of this removal of the peptide side chains is that only 4% of NAM residues are cross-linked whereas 40% are cross-linked in vegetative PG (3, 24, 35–37). Muramic-δ-lactam appears to be found in cortex PG and not in germ cell wall PG (3, 25). Spores produced by *B. subtilis cwlD* mutant strains do not contain muramic-δ-lactam, are unable to degrade cortex PG, and cannot complete germination (3, 25, 31). These findings and in vitro evidence (6, 7) indicate that muramic-δ-lactam functions as a specificity determinant for spore germination lytic enzymes.

A mutant strain lacking the *dacB* gene product, a DD-carboxypeptidase, produces spores that show a fivefold increase in PG cross-linking and that are slightly delayed in spore outgrowth in comparison to wild-type spores (26). These spores exhibit no significant change in spore core dehydration, as

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measured on wet density gradients (23, 26). Spore PG produced by *cwlD* mutant (3, 25, 31) and *cwlD dacB* double-mutant (27) strains have two- and eightfold increases in cross-linking, respectively. However, these spores exhibit normal spore core dehydration (25, 27). A *dacF* mutant which lacks another sporulation-specific DD-carboxypeptidase produces spores with normal PG structure, whereas a *dacB dacF* double-mutant strain produces spores that have approximately a 10-fold increase in PG cross-linking, do not appear to achieve normal spore core dehydration, and are even more delayed in spore outgrowth than the *dacB* single mutant (23).

Theories suggesting that a mechanical activity of spore PG may contribute to the attainment of spore core dehydration are dependent on the low cross-linking of spore PG (17, 23, 34). Loosely cross-linked PG is highly flexible and is capable of expanding and contracting in response to ionic changes (21). Theories suggest that spore PG may contract (17) or expand anisotropically (34) in response to ionic changes in the environment and could thereby exert pressure on the spore core to achieve dehydration. A recent theory proposed a gradient of cross-linking across the layers of *B. subtilis* spore PG in which the innermost layers of spore PG are most loosely cross-linked and the outermost layers are most highly cross-linked (23). This type of structure could potentially carry out the anisotropic expansion proposed by Warth (34). The more loosely cross-linked inner layers would expand more than the highly cross-linked outer layers. The rigidity of the outer layers would direct the expansion inwards and could create pressure on the spore core, thus contributing to its dehydration (23).

The observation that mutant spores with significantly increased PG cross-linking are able to achieve normal spore core dehydration appears to be inconsistent with models that suggest that low cross-linking of the spore cortex allows this structure to actively participate in the process of dehydration. However, previous studies on the structure of spore PG only examined the PG structure in dormant spores and thus could not observe possible temporal changes in PG structure during sporulation or the spatial distribution of PG structural modifications. Small changes in structure that might have a significant effect on spore properties, if concentrated in one area of spore PG, could not be observed. The dramatic changes in spore PG structure produced by *dacB* and *cwlD* mutations might have no effect on spore core dehydration if the theorized gradient of cross-linking was not altered.

We have adapted the spore PG analytical method (3, 24) to examine the PG structure in developing forespores of *B. subtilis* at each stage during its synthesis. We have determined the temporal correlation of changes in spore PG structure with other well-defined sporulation events. We have analyzed forespore PG synthesis in the wild type as well as eight mutant strains. We report that a gradient of cross-linking does span the spore cortex PG. However, the loss of this gradient in some mutant strains indicates that it is not required for spore core dehydration.

MATERIALS AND METHODS

Bacterial strains and growth media. All strains were derived from *B. subtilis* strain PS832, a prototrophic derivative of strain 168. Cultures were induced to sporulate by the nutrient exhaustion method (20). Growth and sporulation were in 2× SG medium (16) in which the concentration of glucose was reduced twofold from the published value in order to achieve the most synchronously sporulating culture.

Phenotypic and biochemical assays. Glucose dehydrogenase (GDH) activity and dipicolinic acid (DPA) accumulation were assayed as previously described (20). Resistance of spores to heating at 80°C for 10 min was measured as described previously (26). The water content of protoplasted cells was determined on density gradients (Nycodenz; Sigma) as described previously (18, 26). To measure total muramic acid and glucosamine content, 0.5-ml culture samples were centrifuged (15,800 × *g* for 45 s), the supernatant was discarded, and the sample was washed in 0.5 ml of cold 1 mM $MgCl_2$. The samples were then resuspended in 0.5 ml of cold 6 N HCl, and 20 μl was transferred to a 1.5-ml tube. The 20-μl samples were hydrolyzed at 95°C for 4 h and subjected to amino acid and amino sugar analysis (11).

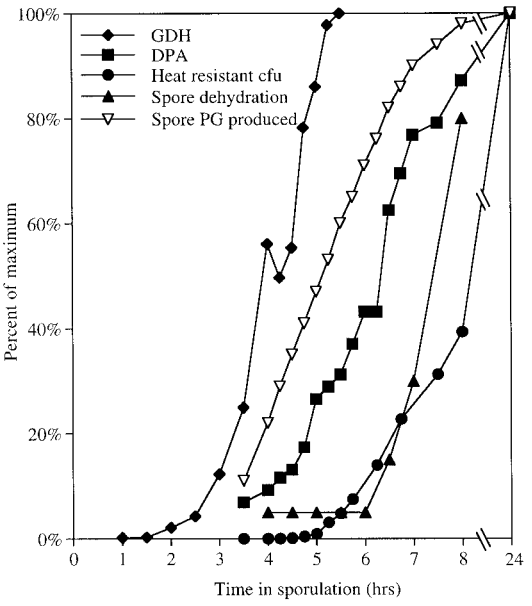

FIG. 1. Appearance of biochemical and phenotypic markers during sporulation. Wild-type cells were grown in 2× SG medium, and samples were collected for assay as described in Materials and Methods. Results for GDH activity, DPA accumulation, spore PG produced, and heat resistant CFU are expressed as percentages of the maximum values reached during the course of the experiment. Spore dehydration is expressed as the percentage of protoplasted cell material that banded on a density gradient at a density greater than that of vegetative cells.

Preparation and analysis of immature forespore PG. Culture samples (30 ml) were centrifuged at 8,000 × *g* for 5 min at 20°C. The supernatant was discarded, and the pelleted cells were resuspended in 5 ml of SMM protoplast solution (5). To degrade the mother cell wall, 25 mg of lysozyme (Sigma) was added and samples were then incubated at 37°C for 15 min. The protoplasted cells were then added to 45 ml of boiling 4% sodium dodecyl sulfate (SDS) (Sigma)–50 mM dithiothreitol (Labscientific, Inc.) solution and boiled for 20 min.

Samples were cooled to room temperature and then transferred to a 50-ml centrifuge tube and centrifuged at 21,000 × *g* for 30 min at 25°C. The supernatant was discarded, and the samples were resuspended in 1 ml of warm (60°C) sterile water. The suspension was boiled for 5 min to solubilize SDS and then centrifuged at 21,000 × *g* for 20 min. The supernatant was discarded, and the washes were repeated until no SDS was detected (12). Each sample was treated with DNase I (10 μg) (Sigma) and RNase A (50 μg) (Sigma) at 37°C for 2 h in a total volume of 1.0 ml of 100 mM Tris–HCl (pH 7.0)–20 mM $MgSO_4$. Trypsin (100 μg) (Worthington TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone]) and $CaCl_2$ (10 mM final concentration) were added, and incubation at 37°C was continued for 16 h. Samples were centrifuged (21,000 × *g* for 20 min), the supernatant was discarded, and the samples were resuspended in 1.0 ml of 1% SDS. The samples were then boiled for 20 min to inactivate the trypsin. Samples were washed with H_2O as described above until no SDS could be detected. The isolated spore PG was digested with 125 U of Mutanolysin (Sigma) in a total volume of 250 μl of 12.5 mM $NaPO_4$ (pH 5.5) for 16 h at 37°C. The solubilized muropeptides were reduced and separated by reverse-phase high-pressure liquid chromatography (HPLC) as previously described (24).

RESULTS

Timing of forespore PG synthesis. Forespore PG synthesis and several sporulation phenotypic and biochemical markers were analyzed in cultures of wild-type *B. subtilis*. All analyses were performed on three separate cultures. The data presented are from one culture; however, the other two analyses produced similar results. Sporulation was induced in cultures of *B. subtilis* by nutrient exhaustion as previously described (16, 20). Figure 1 shows the timing of the different events that occurred during sporulation. GDH activity reached its maximum

during the fifth hour of sporulation (t_5) and then dropped to almost zero (data not shown) as the forespores became resistant to the lysozyme treatment used in the GDH assay. Levels of DPA began to increase around $t_{4.5}$ to t_5 and continued to increase throughout the latter stages of sporulation. The appearance of heat-resistant spores followed both of these events. This order of events has been observed in previous studies (38). The water content of the developing forespore core was determined by measuring the wet density (18) of the protoplasted sporangia. The appearance of higher-density cells roughly paralleled the appearance of heat-resistant CFU (Fig. 1).

The total muramic acid contents of culture samples from each time point were used to estimate the amounts of forespore PG synthesized. It was assumed that there was no significant forespore PG synthesis before t_3 since no forespore PG was recovered from samples taken at t_3 (see results below) and since no engulfed forespores were visible microscopically prior to this time. As seen previously (38), the culture muramic acid content reached a plateau between t_2 and t_3. This was interpreted as the total contribution of mother cell wall PG (and nonsporulating cell wall PG) to the culture, and this value was not expected to change significantly during the remainder of the sporulation process. Previous studies revealed little or no cell wall turnover during starvation of *B. subtilis* under conditions that did not induce lysis (9). Values for muramic acid content for culture samples taken at $t_{2.5}$ and t_3 were averaged to give the mother cell wall PG contribution. This value was subtracted from the muramic acid content for culture samples taken between $t_{3.5}$ and t_8 to give the forespore PG muramic acid content. This value should correspond to the PG produced between the two membranes surrounding the engulfed forespore. This is the forespore PG that was purified, following lysozyme treatment of the culture samples, for structural analysis. The highest forespore PG muramic acid content was set to 100%, and the percentage of forespore PG made at each time point was calculated. These values were graphed against time in sporulation, and a best-fitting line was drawn. The reported percentages of spore PG made were taken from the interpolated line.

Extraction, purification, and analysis of wild-type forespore PG. To extract developing forespore PG, samples from sporulating cultures were centrifuged and the pellets were resuspended in an isotonic solution containing lysozyme to degrade the mother cell wall. Microscopic observation revealed that 99% of the cells had become round after 15 min and that >90% of the developing forespores were still within protective protoplast membranes. The protoplasted cells were added to a boiling solution of SDS and DTT to remove the mother cell material and to prevent autolytic activity on the spore PG. The samples were then washed extensively to remove the SDS and were treated with nucleases and proteases to digest the remaining cell components. The purified spore PG was then treated with a muramidase that specifically cleaves the PG between the NAM and NAG residues but not between muramic-δ-lactam and NAG residues. The resulting muropeptides were reduced and analyzed with reverse-phase HPLC using a methanol gradient (Fig. 2) (24). The muropeptide peaks were tentatively identified based on cochromatography with previously identified peaks (Table 1) (3, 24). Muropeptides were collected from the methanol gradient and were run on a secondary acetonitrile gradient system to verify that each peak represented a single muropeptide (24). The identities of muropeptides collected from the acetonitrile gradient were verified using amino acid analysis (11, 24). Based on elution times and amino acid analyses of muropeptide peaks derived from developing forespore PG, there was no significant contamination with the predominant muropeptides derived from vegetative cell PG: (disaccharide [DS]-tripeptide [TriP] and DS-TriP-DS-tetrapeptide [TP]) with amidations of the Dpm residues (2) (data not shown).

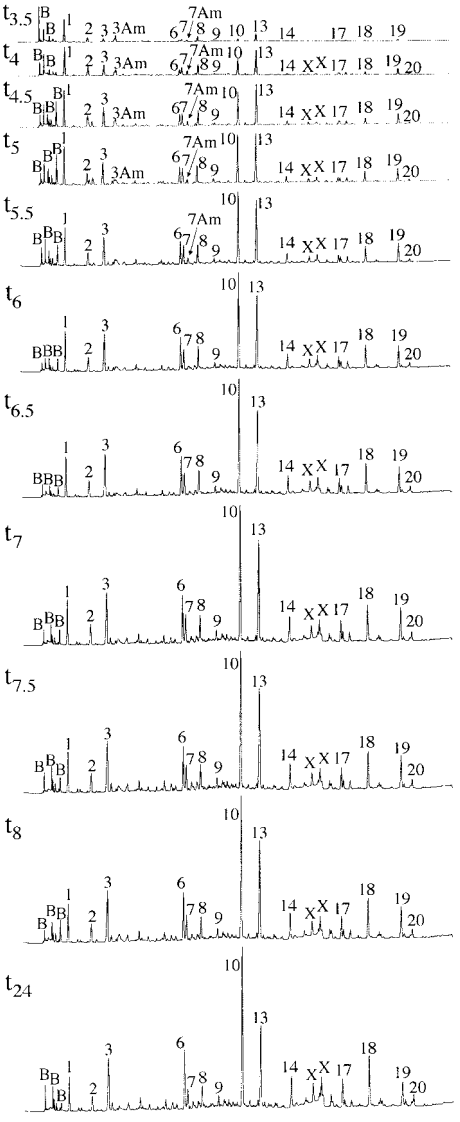

FIG. 2. HPLC analysis of immature forespore PG-derived muropeptides. Forespore PG was purified from culture samples taken every 15 min between t_4 and t_7, but only data for samples taken every 30 min are shown. PG was digested with muramidase, reduced, and separated using a methanol gradient as previously described (24). Peaks are numbered as previously described (24), and muropeptide structures are given in Table 1. Peaks labeled B are buffer components. Peaks labeled X are produced by reduction of muramic-δ-lactam, and corrections for this reduction were made during calculation of PG structural parameters (24).

The muropeptide peaks labeled 3Am and 7Am (Fig. 2) represent unique peaks that are seen only in developing forespore PG and not in dormant-spore PG. Amino acid analyses tentatively identified muropeptide 3Am as DS-TriP-TP and muropeptide 7Am as a tetrasaccharide (TS) with no peptide side chain (Table 2). These predicted identities of muropeptides 3Am and 7Am were confirmed by mass spectrometry (Table 2). These two novel peaks are the result of muramoyl-L-alanine amidase activity, an enzymatic activity normally associated with both vegetative and spore PG metabolism. A

TABLE 1. Identities of wild-type *B. subtilis* muropeptide peaks

Peak[a]	Structure[b]	Reference for structure
1	DS-TriP	24
2	DS-Ala	24
3	DS-TP	24
3Am	DS-TriP-TP	Table 2
6	TS-TP (reduced lactam)	24
7	TS-Ala (reduced lactam)	24
7Am	TS	Table 2
8	DS-TriP-DS-TP	24
9	DS-TP-DS-TP	24
10	TS-TP	24
13	TS-Ala	24
14	DS-TP-TS-TP	24
17	TS-TP-TS-TP	24
18	HS-TP	24
19	HS-Ala	24
20	TS-TP-HS-TP	24

[a] Peaks are numbered as in Fig. 1.
[b] HS, hexasaccharide. Where multiple saccharide moieties are indicated, they are cross-linked via their peptide side chains.

cwlB (*lytC*) mutation which eliminates the most abundant muramoyl-L-alanine amidase in vegetative cells (14, 15) had no effect on the production of muropeptides 3Am and 7Am (data not shown). These two muropeptides were not found in forespore PG purified from a *cwlD* mutant, suggesting that the amidase encoded by this gene (3, 25, 31) is responsible for their production. For two reasons we believe that much of the production of these amidase products is an artifact of unregulated CwlD activity during the forespore PG purification procedure. First, amounts of these muropeptides varied dramatically not only between experiments but also within each experiment. For example, amidase products were sometimes seen in samples taken at t_4 and $t_{4.5}$ but not at $t_{4.25}$. Second, in muropeptide 7Am, peptides were cleaved from two adjacent muramic acid residues. If this muropeptide were a normal component of forespore PG, it would be on the pathway for production of a hexasaccharide which contains two adjacent muramic-δ-lactam residues. However, in several instances, production of muropeptide 7Am was in excess of the eventual amount of hexasaccharide muropeptides produced (data not shown).

A correction for the production of muropeptides 3Am and 7Am was included in the calculation of spore PG structural parameters. Since muropeptide 8 is the only muropeptide ever observed in spore PG that has a TriP-TP cross-link, it was assumed that muropeptide 3Am is derived from muropeptide 8 via amidase activity. In calculating structural parameters, the molar values of muropeptides 3Am and 8 were combined to produce the total amount of TriP-TP cross-links. Muropeptide 7Am can presumably be derived by amidase activity on any of the other muropeptides that contain TS, such as muropeptides 10, 13, 14, 17, and 20. We have no evidence to suggest that muropeptide 7Am is derived preferentially from any subset of these TS-containing muropeptides. To correct for this amidase activity, we calculated the amount of each TS-containing muropeptide as a percentage of total TS. We then divided the molar amount of muropeptide 7Am according to these percentages and added these values to the molar amounts of the other TS-containing muropeptides. The validity of these corrections was determined by comparing two sets of structural parameters. The first set of structural parameters was calculated, with the correction, for a sample that contained significant amounts of muropeptides 3Am and 7Am. The second set of structural parameters was calculated, without the correction, for a similar sample that, by chance, had extremely low levels of these muropeptides. There was no significant difference between the two sets of structural parameters.

HPLC analysis of the purified forespore PG shows increasing amounts of PG from the beginning to the end of spore PG synthesis during sporulation (Fig. 2). We believe that there is little or no degradation and turnover of forespore PG. Turnover of vegetative-cell PG is necessitated by the inside-to-outside growth process of the cell wall, which involves PG synthesis on the inside of the wall with concurrent shedding of PG on the outside (1). Forespore PG is synthesized predominantly from the outside (33), so PG degradation is not required for expansion of this wall. Difficulty in the precise definition of the contribution of mother cell wall PG to the muramic acid content of the culture resulted in poor accuracy in the reported percent spore PG made, especially in samples taken early during forespore PG synthesis. Due to this problem we limit our conclusions about early forespore PG synthesis to observations of relatively large changes in PG structure.

Structural parameters (Table 3) for the forespore PG recovered at each time point revealed that a relatively low level of muramic-δ-lactam observed in the first 5 to 10% of the spore PG made was followed by a rapid increase. A high percentage of TriP side chains was also observed in the first 10% of spore PG made. These two facts suggest that the germ cell wall is synthesized first, adjacent to the inner forespore membrane. The germ cell wall has a high level of cross-linking relative to the spore PG as a whole. A calculation of the total amount of TriP-containing muropeptides (Table 3) and the number of peptide cross-links involving TriPs (data not shown) revealed that these values do not change significantly following synthesis of the first 10 to 15% of spore PG. We interpret this to mean that the TriPs and TriP cross-links made in the germ cell wall are stable during synthesis of the cortex PG.

There was a short decreasing gradient of cross-linking during synthesis of the first 30% of spore PG, followed by a slightly increasing gradient across the outer 70% of the spore PG. The cross-linking values in Table 3 are for the total spore PG

TABLE 2. Structural data for muropeptides 3Am and 7Am[a]

Peak	Amt (nmol) of:						Predicted structure	$(M-H)^-$ m/z[b]	
	Glucosamine	Muramitol	Muramic-δ-lactam	Ala	Glu	Dpm		Calc.	Obs.
3Am	1 (1)	0.95 (1)	0	2.94 (3)	1.84 (2)	2.25 (2)	DS-TriP-TP	1311.56	1312.47
7Am	1 (2)	0.48 (1)	0.35 (1)	0	0	0	TS	915.36	917.10

[a] Peaks are numbered as in Fig. 1. All nanomole values were determined using amino acid and amino sugar analyses (24) and were normalized to a value of 1 for NAG. NAM residues at the reducing ends of muropeptides and muramic-δ-lactam were detected as muramitol and muramic acid, respectively, in the amino acid analysis. Values in parentheses are predicted numbers of moles of the residues within the molecule.
[b] $(M-H)^-$ is the deprotonated molecular ion observed in the negative-ion mode. Calc., predicted from the amino acid and amino sugar analyses, with the average mass values given; obs., measured by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

TABLE 3. Structural parameters for wild-type forespore PG

Time in sporulation (h)	% Spore PG made[a]	% Muramic acid with:					% Total spore muramic acid with TriP[c]
		Side chains of:				Cross-link	
		Lactam[b]	Ala	TriP	TP		
3.5	11	29.2	29.8	29.1	11.9	6.0	3.2
4	22	39.1	33.6	13.7	13.6	3.5	3.0
4.25	29	42.2	31.8	9.3	16.6	2.9	2.7
4.5	35	43.8	30.7	7.2	18.3	2.5	2.5
4.75	41	44.1	30.2	6.8	18.9	2.7	2.8
5	47	45.2	27.9	5.3	21.6	2.7	2.5
5.25	53	45.5	27.0	4.7	22.7	2.9	2.5
5.5	60	45.5	27.0	4.2	23.3	2.8	2.5
5.75	65	46.4	25.5	3.7	24.4	3.1	2.4
6	71	46.6	25.3	3.6	24.5	3.2	2.6
6.25	76	46.9	24.6	3.4	25.2	3.2	2.6
6.5	82	46.9	24.5	3.2	25.4	3.2	2.6
6.75	86	47.4	23.8	2.7	26.1	3.5	2.3
7	90	47.5	23.5	2.6	26.4	3.4	2.3
7.5	97	47.7	23.0	2.5	26.8	3.5	2.4
8	100	47.7	23.2	2.4	26.6	3.4	2.4
24	100	46.1	20.0	2.6	31.2	3.6	2.6

[a] These values are derived from the interpolation of cell muramic acid contents as determined by amino acid analyses.
[b] Lactam, muramic-δ-lactam.
[c] The amount of muramic acid with a TriP side chain detected in each sample is expressed as a percentage of the total muramic acid found in spore PG at t_{24}.

present in each sample and do not indicate the cross-linking within the spore PG made between samples. For example, a cross-linking value of 6.0% was observed for the first 11% of the spore PG made, and a cross-linking value of 3.5% was observed for the entire first 22% of the spore PG made (Table 3). The cross-linking in the PG made between 11 and 22% must be lower than 3.5% in order to bring the average value over the total 22% of spore PG down to 3.5%. These raw values for cross-linking were graphed against percent spore PG made, and a line was drawn through the points. Cross-linking values were determined from the interpolated line for each 10% of spore PG made. These new cross-linking values were used to calculate the amount of cross-linking in each 10% "slice" of spore PG made (Fig. 3). These calculations indicate that cross-linking is relatively high in the first 10% of spore PG synthesized (in the region of the germ cell wall) and rapidly decreases during synthesis of the next 20% of the spore PG, correlating with the production of larger amounts of muramic-δ-lactam. An assumption inherent in this conclusion is that cross-links formed in the first PG synthesized are not removed during subsequent synthesis. We believe this is true due to the apparent stability of the TriP-containing cross-links found in the first 10 to 15% of the spore PG made. Cross-linking increases progressively during synthesis of the final 70% of the spore PG. This increase in cross-linking must be due to higher cross-linking in the new PG synthesized because we never detected significant amounts of the pentapeptide side chains required for cross-link formation. Repetition of this analysis with forespore PG derived from three different wild-type cultures indicated that the decrease in cross-linking between the 0- to 10% and 20- to 30% slices of spore PG ranged between three- and sevenfold. The increases in cross-linking between the 20- to 30% and 90- to 100% slices ranged between two- and sixfold. This relatively large range in our reporting of the degree of the cross-linking gradient is a result of the difficulties in determining precise values for the amount of forespore PG synthesized at early time points.

Forespore PG synthesis in mutant strains. The method for the analysis of developing forespore PG structure was applied to eight *B. subtilis* mutant strains lacking various enzymes involved in PG synthesis. Analyses were performed on two independent cultures of each strain. Results from one analysis of each strain are shown, and results from the duplicate analyses were similar. The timing of appearance of GDH activity, DPA accumulation, heat resistance, an increase in spore core wet density, and spore PG synthesis in all eight mutant strains was

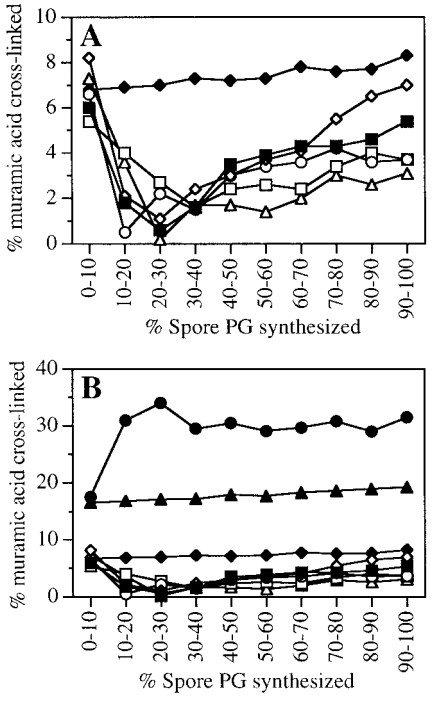

FIG. 3. Cross-linking across the span of the spore PG in wild-type and mutant *B. subtilis* strains. The percentage of muramic acid residues with cross-linked peptides was calculated for each 10% slice of spore PG isolated from cultures of eight different strains. (A) Data for the wild-type (■), *dacA* (◇), *dacF* (△), *dacA dacC dacF* (○), *ponA* (□), and *cwlD* (◆) strains. (B) Same data as in panel A plus those for the *dacB* (▲) and *cwlD dacB* (●) strains on an expanded vertical axis.

similar to that observed for wild-type spores (data not shown). Wet density analysis was not done for *dacA* and *dacF* strains, but normal spore core dehydration is expected since normal heat resistance was observed. Heat resistance for strains with mutations in *cwlD* was not measured because the spores of these strains cannot complete germination and form colonies. However, a normal increase in spore wet density was observed (data not shown), and normal spore heat resistance was previously demonstrated for these strains (25, 27). A complete set of forespore PG structural parameters was derived for each of the strains (Table 4), except for the *dacB dacF* strain, which will be discussed below. For each strain, samples were collected every 15 min between t_4 and t_7, as was done for the wild type. For brevity, only data for samples taken every 30 min are presented in Table 4.

Structural parameters of developing forespore PG from a *dacF* mutant (strain PS1901, lacking DacF, a forespore-specific putative DD-carboxypeptidase [24, 39]) and a *ponA* mutant (strain PS2062, lacking PBP1, the major class A high-MW PBP [28, 30]) were similar to those determined for the wild-type strain (Table 4). This finding is consistent with previous structural analyses on dormant-spore PG from these two mutant strains (24, 27). Previous studies of strains with mutations in *dacA* (strain PS1900 *dacA* and strain DPVB17 *dacA dacC dacF* [24, 27]) demonstrated that dormant-spore PG produced by these mutants had a twofold reduction in the amount of TriP side chains (24, 27). The *dacC* mutation alone has no effect on spore PG structure, and the *dacF* and *dacC* mutations had no effect in the *dacA* mutant background (22, 23) (data not shown). Structural analyses of forespore PG isolated from developing spores of *dacA* strains showed that a gradual decrease in the amount of TriP side chains occurred during spore PG synthesis relative to amounts observed during the synthesis of wild-type spore PG (Table 4). This analysis also revealed that spore PG from these strains contained a significant number of pentapeptide side chains. Pentapeptide-containing muropeptides were identified based on amino acid analysis data (11) and cochromatography with pentapeptide-containing muropeptides observed in *cwlD* mutant spore PG (27). Pentapeptide side chains have also been found in vegetative cell wall PG isolated from a *dacA* mutant strain (2). As observed previously (23, 24), these changes in spore PG structure had no effect on spore dehydration or heat resistance.

Synthesis of forespore PG in strains with mutations in *dacB* (strain PS2066 [26]), *dacB* and *dacF* (strain PS2421 [27]), *cwlD* (strain PS2307 [25, 31]), and *cwlD* and *dacB* (strain PS2422 [27]) differed substantially from that in the wild type. Relative to that from the wild type, developing forespore PG from the *dacB* strain had an approximately twofold reduction in NAM residues with single L-alanine side chains and a corresponding increase in TP side chains throughout spore PG synthesis (Table 4). A slight decrease in the amount of muramic-δ-lactam and the large increase in peptide side chains may be the direct cause of a fivefold increase in total spore PG cross-linking seen in this strain (Table 4). In addition, there was no decreasing or increasing gradient of cross-linking across the span of the spore PG (Fig. 3).

Complete analysis of spore PG synthesis in the *dacB dacF* double mutant was prevented by the appearance of many novel peaks that were eluted later in the gradient (23), especially in samples taken during the later stages of sporulation (data not shown). The structures of these new peaks were not determined. However, we were able to identify peaks corresponding to approximately 90% of the muropeptide material in the earliest samples, and we were able to calculate structural parameters for the first 20% of the spore PG made in two independent cultures. Structural changes, relative to the wild type, seen in the first PG synthesized include (i) a threefold reduction in the amount of single L-alanine side chains, (ii) a greater-than-twofold increase in the amount of TP side chains, and (iii) an approximately threefold increase in cross-linking. These structural parameters are similar to those for early forespore PG from the *dacB* single mutant. We used an alternate method for determining the degree of PG cross-linking to analyze later samples from *dacB dacF* double-mutant cultures (data not shown). These and previous results (23) indicated that although the levels of cross-linking in the first spore PG produced by *dacB* and *dacB dacF* mutant strains were similar, the later PG produced by the double mutant was more highly cross-linked than that of the single mutant.

Previous studies on the structure of PG from *cwlD* mutant spores demonstrated that it contained no muramic-δ-lactam and contained small amounts of pentapeptide side chains and peptide side chains that contained glycine (3, 25, 27). Analysis of a *cwlD* mutant revealed the presence of small amounts of pentapeptide side chains throughout forespore PG synthesis (Table 4). Levels of cross-linking in *cwlD* mutant and wild-type forespore PG are similar during synthesis of the first 10% of forespore PG (Table 4). This similarity is due to the low level of muramic-δ-lactam in the first layers of forespore PG produced in the wild type. Differences between the two strains become more apparent during synthesis of the cortex PG. The absence of muramic-δ-lactam in the last 90% of the spore PG synthesized in the *cwlD* mutant is tied to the following structural differences relative to the wild type: (i) a slow progression to 60% more single-L-alanine side chains, (ii) an increase to approximately threefold more TriP side chains; (iii) a twofold increase in the amount of TP side chains, (iv) a twofold increase in PG cross-linking (Table 4), and (v) the absence of any increasing or decreasing gradient of cross-linking across the span of the spore PG (Fig. 3).

Dormant-spore PG from a *cwlD dacB* double-mutant strain demonstrated a combination of the structures observed in the two single mutants (25, 27). Analyses of developing forespore PG in the *cwlD dacB* double mutant revealed a similar pattern (Table 4). A twofold decrease in the amount of single-L-alanine side chains, relative to the wild type, throughout the period of *cwlD dacB* mutant forespore PG synthesis was also observed in the *dacB* single mutant. The first 20% of forespore PG synthesized in the double mutant had amounts of TriP side chains similar to those observed during early wild-type and *cwlD* mutant forespore PG synthesis. However, the amount of TriP side chains increased twofold over wild-type values during synthesis of the last 80% of the spore PG in the *cwlD dacB* mutant, just as observed in the *cwlD* strain. Cross-linking in the first layers of spore PG produced was higher than that found in the wild type, similar to what was found for the *dacB* mutant. Cross-linking increased a further twofold in the remainder of the spore PG, and no gradient of cross-linking was observed (Fig. 3).

DISCUSSION

A method for analyzing the structure of dormant-spore PG using reverse-phase HPLC (24) was adapted for examination of the structure of PG at each stage of its synthesis in developing forespores of *B. subtilis*. Structural parameters derived for wild-type forespore PG suggest that the structure of the first 10% of spore PG made has a high percentage of TriP side chains and a small amount of muramic-δ-lactam relative to later samples. These two facts lead to the conclusion that the structure found in the first 10% of spore PG made represents the germ cell wall, which is predicted to have a structure

TABLE 4. Structural parameters for mutant strains' forespore PG[a]

Genotype	Time in sporulation (h)	% Spore PG made[b]	% Muramic acid with:					
			Side chains of[c]:					Cross-link
			Lactam	Ala	TriP	TP	PP	
ponA	3.5	6	31.0	27.9	28.7	12.4	0	5.7
	4	13	39.6	31.3	14.0	15.1	0	5.1
	4.5	21	39.4	31.8	12.4	16.4	0	4.6
	5	31	43.3	28.4	7.2	21.1	0	3.6
	5.5	42	45.1	26.3	5.0	23.6	0	3.3
	6	58	45.8	23.2	3.8	27.2	0	3.7
	6.5	76	46.3	22.9	3.5	27.3	0	3.0
	7	88	47.1	20.7	2.8	29.5	0	3.1
	7.5	94	47.3	19.4	2.7	30.7	0	3.1
	8	100	47.5	19.1	2.3	31.0	0	3.2
	24	100	47.9	17.8	2.1	32.1	0	3.1
dacA	3.5	6	0.0	3.7	61.6	21.5	13.3	9.6
	4	11	20.6	22.1	33.0	15.7	8.6	7.9
	4.5	19	34.0	31.0	14.8	15.1	5.1	5.3
	5	30	40.2	31.4	7.7	17.4	3.3	3.8
	5.5	45	44.3	28.5	4.1	20.9	2.1	3.4
	6	58	45.5	26.2	3.0	23.8	1.5	4.1
	6.5	71	46.2	25.0	2.7	24.6	1.5	3.8
	7	83	47.7	24.8	1.8	24.7	1.0	3.5
	7.5	93	47.1	22.6	2.0	27.0	1.2	4.1
	8	100	47.3	21.8	1.8	27.7	1.4	4.3
	24	100	48.3	19.7	1.4	29.6	1.0	4.4
dacF	3.5	10	28.0	29.3	30.9	11.7	0	7.3
	4	24	37.9	35.0	15.5	11.6	0	4.7
	4.5	38	42.4	33.2	9.3	15.0	0	3.3
	5	54	45.2	32.6	5.5	16.7	0	2.8
	5.5	67	47.4	30.5	3.4	18.7	0	2.5
	6	76.5	47.8	29.4	3.1	19.7	0	2.7
	6.5	84.5	48.0	29.0	2.9	20.1	0	2.8
	7	92.5	48.4	28.3	2.9	20.4	0	2.6
	7.5	96	48.5	28.2	2.7	20.5	0	2.7
	8	100	49.2	29.6	2.3	18.9	0	2.3
	24	100	49.5	23.9	2.0	24.6	0	3.4
dacA dacC dacF	3.5	8	20.1	21.4	24.2	22.7	11.7	7.2
	4	18.5	36.8	34.1	9.9	14.8	4.4	3.6
	4.5	29	39.9	34.0	7.2	15.5	3.4	3.3
	5	41.5	44.6	31.8	3.6	18.1	2.0	2.7
	5.5	54	45.7	32.2	3.0	17.6	1.6	2.4
	6	69	46.3	28.9	2.3	21.1	1.4	2.9
	6.5	78	46.9	27.5	2.1	22.2	1.3	3.2
	7	87.5	47.6	25.0	1.5	24.8	1.1	3.2
	7.5	94.5	47.7	25.0	1.5	24.8	1.0	3.2
	8	100	48.0	24.9	1.3	24.8	0.9	3.1
	24	100	48.9	23.0	1.1	26.3	0.7	3.5
dacB	3.5	4	12.6	11.8	48.5	27.0	0	16.5
	4	9	32.1	15.8	17.8	34.3	0	16.3
	4.5	16	37.3	13.7	10.0	39.0	0	16.8
	5	33.5	39.4	13.4	7.5	39.7	0	16.0
	5.5	65	40.5	11.4	5.9	42.2	0	17.3
	6	78	42.1	9.8	4.3	43.8	0	18.1
	6.5	86	42.3	10.3	5.5	41.9	0	16.5
	7	91	42.4	9.6	4.9	43.1	0	17.3
	7.5	96	42.8	9.0	4.2	44.0	0	17.7
	8	100	43.1	8.6	3.8	44.5	0	18.0
	24	100	44.0	7.4	2.6	46.0	0	19.1
cwlD	3.5	4.5	0	33.6	45.1	21.3	0.0	6.3
	4	10	0	38.4	15.7	44.8	1.1	6.8
	4.5	24	0	36.7	10.0	52.0	1.3	6.8
	5	41	0	37.2	7.8	54.0	1.1	6.8
	5.5	58	0	37.1	7.1	54.8	1.0	7.1

Continued on following page

TABLE 4—*Continued.*

Genotype	Time in sporulation (h)	% Spore PG made[b]	% Muramic acid with:					
			Side chains of[c]:					Cross-link
			Lactam	Ala	TriP	TP	PP	
	6	74	0	36.4	6.7	55.6	1.3	7.3
	6.25	80	0	36.6	6.5	55.4	1.5	7.3
	6.75	87	0	36.6	6.5	55.3	1.5	7.1
	7	90	0	37.6	6.7	54.3	1.4	7.2
	7.5	96	0	37.3	6.7	54.5	1.4	7.3
	8	100	0	36.9	6.3	55.4	1.3	7.1
	24	100	0	34.1	5.7	59.0	1.2	7.7
cwlD dacB	3.5	5	0	16.6	54.7	28.7	0.0	9.3
	4	10	0	17.8	25.0	56.4	0.8	20.6
	4.5	18	0	15.0	13.7	69.9	1.4	27.3
	5	26	0	15.0	9.2	74.5	1.3	28.6
	5.5	38	0	14.2	14.6	70.0	1.2	27.1
	6	50	0	13.9	10.2	74.2	1.7	27.3
	6.5	65	0	12.9	7.4	78.0	1.7	28.9
	7	78	0	13.3	6.7	78.4	1.6	29.3
	7.5	90	0	13.7	6.3	78.4	1.6	28.7
	8	100	0	13.4	6.1	78.5	1.9	29.7
	24	100	0	12.0	4.7	81.6	1.6	31.6

[a] Forespore PG was purified from culture samples taken every 15 min between t_4 and t_7, but only data for samples taken every 30 min are shown.
[b] These values are derived from the interpolation of cell muramic acid contents as determined by amino acid analyses.
[c] Lactam, muramic-δ-lactam; PP, pentapeptide.

similar to that of vegetative PG (2) and also suggests that the germ cell wall is synthesized first, adjacent to the inner forespore membrane. Our data do not address whether the substrates for synthesis of these first layers of spore PG are derived from the forespore or from the mother cell. Previous studies have demonstrated that muramic-δ-lactam is a specificity determinant for cortex-specific lytic enzymes (3, 6, 7, 25), which is consistent with the conclusion that the germ cell wall has little, if any, muramic-δ-lactam. Structural analyses of germinating *dacA* mutant spores demonstrated that the amount of DS muropeptides containing TriP side chains remained constant throughout the process of spore PG degradation during germination (2), providing further support for the conclusion that the germ cell wall has a high percentage of TriP side chains. There appears to be very few TriP side chains produced in the remaining spore PG, the cortex; and the TriP and TriP-containing cross-links within the germ cell wall appear to be stable during formation of the remainder of the spore.

A recently proposed model suggested that a gradient of cross-linking, in which the innermost layers of spore PG are most loosely cross-linked and the outermost layers are most highly cross-linked, was formed during spore PG synthesis and that this gradient could be involved in the attainment of spore core dehydration (27). Cross-linking values calculated for each section of spore PG suggest that the region of the germ cell wall, or the first 10% of spore PG made, is highly cross-linked in comparison to the remainder of the spore PG (cross-linking of the germ cell wall is still very low relative to that found in vegetative-cell PG [2, 29, 36]). The level of cross-linking within the germ cell wall appears to be partly determined by the activity of the *dacB* product, PBP5*, a DD-carboxypeptidase. Loss of PBP5* resulted in a greater-than-twofold increase in germ cell wall cross-linking. The further loss of *dacF* or *cwlD* in the *dacB* background did not raise germ cell wall cross-linking above 16 to 17%, even though cross-linking in later spore PG synthesis was increased. Germ cell wall cross-linking may be limited by a transpeptidase activity specifically involved in synthesis of this PG layer. It was surprising that the level of germ cell wall cross-linking was not affected by the product of *dacF*, a putative DD-carboxypeptidase that is expressed in the forespore and that was presumed to cross the inner forespore membrane and remain associated with the outer surface of that membrane (39).

A rapid decrease in cross-linking was observed within the 20% of the spore PG synthesized after the germ cell wall. A major factor in this decrease appears to be the *cwlD* product, a muramoyl-L-alanine amidase involved in formation of muramic-δ-lactam (3, 25, 31). Loss of CwlD specifically prevents the decrease in cross-linking following germ cell wall synthesis but only slightly increases the overall degree of cross-linking. PBP5* also appears to play a role in generating this decrease in cross-linking; the decrease was not present in the *dacB* mutant. The large increase in cross-linking in the *dacB* mutant has only a slight effect on muramic-δ-lactam formation by CwlD, suggesting that either CwlD activity is not substrate limited in the *dacB* mutant or CwlD is able to remove cross-linked peptides. The facts that both CwlD and PBP5* can affect the degree of cross-linking and that their effects are additive suggest that they function independently.

A two- to eightfold increase in cross-linking was observed during synthesis of the final 70% of the wild-type spore PG (Fig. 3). The process driving this increase is unclear. It could be affected by a decrease in the activity of a presumed endopeptidase involved in the production of L-Ala side chains, with a corresponding increase in peptide side chains available for cross-linking or a decrease in removal of cross-linked peptides. Alternatively, a decrease in the specific activity of DD-carboxypeptidases would allow more of the remaining peptides to be cross-linked. Interestingly, the loss of *dacF* in the *dacB* background led to an increase in cross-linking outside of the germ cell wall. One explanation might be that DacF is not anchored to the membrane as previously suggested (39). This could also explain the failure of repeated efforts to detect this putative PBP in membrane preparations (data not shown).

Unresolved is the issue of whether spore PG is actively involved in spore core dehydration. Our data can rule out some

possible models. While a gradient of cross-linking is produced across the span of the spore PG, the absence of this gradient in forespore PG from *dacB*, *cwlD*, and *cwlD dacB* strains, which produce spores with normal dehydration (25, 27), indicates that the gradient is not required. Formation of a spore that can achieve and maintain core dehydration may simply require PG cross-linking within a broad range. The upper limit of the range required for achieving dehydration may have been surpassed in the *dacB dacF* double mutant. While the *dacB* mutant spores achieve normal core dehydration, they are unable to maintain it upon heating (26). These spores may define a spore PG cross-linking limit required for maintenance of dehydration. Another factor affecting the evolution of the specific level of spore PG cross-linking may be the rate of spore outgrowth. The higher degrees of spore PG cross-linking in the *dacB* and *dacB dacF* mutants correlate with slowed outgrowth of their spores. Difficulty in degrading the highly cross-linked spore PG may present a physical barrier to growth of these germinated cells. PG cross-linking during sporulation may thus be a balancing act between achieving the wall stability required for maintaining spore core dehydration and the wall flexibility required for rapid outgrowth and attainment of spore core dehydration.

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