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Analysis of the Peptidoglycan Structure of *Bacillus subtilis* Endospores

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Peptidoglycan was prepared from purified *Bacillus subtilis* spores of wild-type and several mutant strains. Digestion with muramidase resulted in cleavage of the glycosidic bonds adjacent to muramic acid replaced by peptide or alanine side chains but not the bonds adjacent to muramic lactam. Reduction of the resulting muropeptides allowed their separation by reversed-phase high-pressure liquid chromatography. The structures of 20 muropeptides were determined by amino acid and amino sugar analysis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. In wild-type spores, 50% of the muramic acid had been converted to the lactam and 75% of these lactam residues were spaced regularly at every second muramic acid position in the glycan chains. Single L-alanine side chains were found on 25% of the muramic acid residues. The remaining 25% of the muramic acid had tetrapeptide or tripeptide side chains, and 11% of the diaminopimelic acid in these side chains was involved in peptide cross-links. Analysis of spore peptidoglycan produced by a number of mutants lacking proteins involved in cell wall metabolism revealed structural changes. The most significant changes were in the spores of a *dacB* mutant which lacks the sporulation-specific penicillin-binding protein 5*. In these spores, only 46% of the muramic acid was in the lactam form, 12% had L-alanine side chains, and 42% had peptide side chains containing diaminopimelic acid, 29% of which was involved in cross-links.

Bacterial endospores produced by gram-positive species (including *Bacillus* and *Clostridium* species) are metabolically dormant resting-stage cells resistant to a variety of physical and chemical treatments which are rapidly lethal to vegetative cells. These resistance properties derive from a variety of modifications in the cell structure and contents. The major determinant of spore heat resistance has been found to be the relative degree of dehydration of the spore protoplast (1, 14, 17, 18). The relative dehydration of the protoplast is maintained by a surrounding peptidoglycan structure. This structure is composed of a thin inner layer called the germ cell wall and a thicker outer layer termed the cortex. The germ cell wall has a structure similar to that of the vegetative wall peptidoglycan (27) and serves as the precursor of the vegetative wall upon spore germination. The cortex peptidoglycan has a significantly different structure (30, 32) (Fig. 1), with the most dramatic changes being the absence of side chains from approximately 50% of the muramic acid residues which are converted to muramic lactam and the presence of single L-Ala side chains on a large fraction of the muramic acid residues.

Degradation of the cortex peptidoglycan, during spore germination or by artificial means, results in rapid rehydration of the protoplast and a concomitant loss of spore heat resistance and dormancy. Several theories suggest that the cortex peptidoglycan also has a mechanical activity that is involved in the attainment of spore protoplast dehydration. These theories are based on the observations that peptidoglycan expands and contracts in response to changes in pH or ionic strength (16) and that the relatively loosely cross-linked cortex peptidoglycan should have a significant range of motion (19, 32). Such a mechanical activity could perhaps function during sporulation to decrease the volume of the spore core, forcing water out of the protoplast (12, 29). In an attempt to critically examine these theories, we have previously carried out analyses of spore peptidoglycan cross-linking, spore core dehydration, and spore resistance properties in wild-type and several mutant strains (17, 19). The results of these studies indicated that contrary to some theoretical predictions, a significant increase in spore peptidoglycan cross-linking did not affect spore core dehydration. In contrast, the spores of some strains exhibited a defect in protoplast dehydration in the absence of an obvious change in peptidoglycan structure. However, our ability to draw definitive conclusions from these studies was hampered by the fact that we had measured only a few aspects of spore peptidoglycan structure and only the final structure. In order to further define the role of the spore cortex in attaining and maintaining spore dehydration and to determine which aspects of cortex structure are important for these roles, we have developed a rapid and reproducible method for the quantitative analysis of the complete spore peptidoglycan structure. We describe here the use of reversed-phase high-pressure liquid chromatography (HPLC), amino acid and amino sugar analysis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to examine the peptidoglycan structure in wild-type and mutant spores.

MATERIALS AND METHODS

Bacterial strains and sporulation conditions. All of the *B. subtilis* strains described in Table 1 are derivatives of strain 168. Growth for sporulation occurred in 2× SG medium (11) at 37°C. Spores were purified by water washing as previously described (15) and were lyophilized for storage. Microscopic examination indicated that all spore preparations were >98% free of contaminating vegetative cells, sporulating cells, and germinated spores.

Preparation of spore cortex muropeptides. The muropeptide preparation procedure was adapted from a method we used previously (19). Purified spores (3 to

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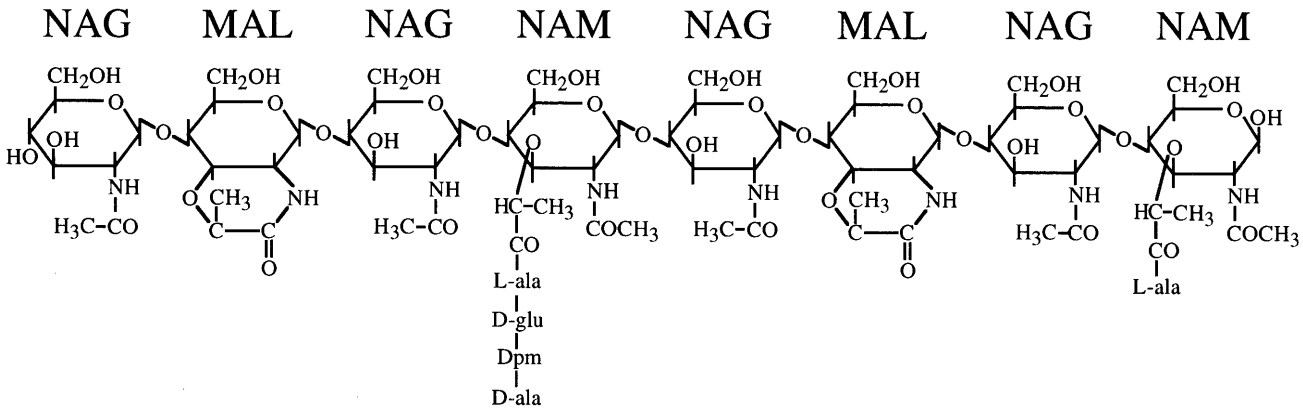

FIG. 1. Structure of the peptidoglycan in *B. subtilis* spores. The basic structure of the spore peptidoglycan was determined by Warth and Strominger (30, 32). Glycan chains are composed of alternating *N*-acetylglucosamine (NAG) and *N*-acetyl-muramic acid (NAM) residues. NAM can carry side chains of either L-Ala or the tetrapeptide L-Ala-γ-D-Glu-Dpm-D-Ala. Approximately 50% of the muramic acid residues have no side chain and have been converted to muramic lactam (MAL). The glycan chains can be cross-linked via the tetrapeptide side chains; a peptide bond is formed between the ε-amino group of Dpm of one peptide and the carboxy-terminal D-Ala of another peptide.

5 mg [dry weight]) were rehydrated in 1 ml of H_2O for 16 h at 4°C. All extraction steps were performed in 1-ml volumes in microcentrifuge tubes, and all centrifugations were at 13,000 × *g* for 30 s (unless otherwise noted). The rehydrated spores were centrifuged and treated with decoating solution (50 mM Tris-HCl [pH 8.0], 8 M urea, 1% [wt/vol] sodium dodecyl sulfate [SDS], 50 mM dithiothreitol) twice for 60 min at 37°C. The decoated spores were washed five times in H_2O. Lytic enzymes were then inactivated by treatment with 5% trichloroacetic acid at 95°C for 6 min (13, 19). The inactivated spores were washed once in 1 M Tris-HCl (pH 8.0) and five times in H_2O. The majority of the protein was removed by digestion with trypsin (Worthington TRTPCK) (0.1 mg/ml in 20 mM Tris-HCl [pH 8.0]–10 mM $CaCl_2$) at 37°C for 16 h. SDS was added to 1%, and the spores were heated at 100°C for 15 min. The extracted spores were then washed in H_2O until no trace of SDS could be detected (7) in the wash supernatant fluid (approximately 15 washes). Peptidoglycan was then digested as previously described (5) with 125 U of mutanolysin (Sigma) in a total volume of 250 μl of 12.5 mM sodium phosphate (pH 5.5) for 16 h at 37°C. Insoluble material was removed by centrifugation at 13,000 × *g* for 15 min, and the supernatant containing the muropeptides was lyophilized. The muropeptides were resuspended in 100 μl of 0.25 M $Na_2B_4O_7$ (pH 9.0), and if necessary, the pH was adjusted to 9 with 1 N NaOH. It has been previously observed that anomerization of the reducing end sugars of muropeptides results in poor resolution during chromatography (6). This problem was eliminated by reduction of the sugar to the corresponding alcohol. However, muramic lactam residues within glycan strands are reduced to two different forms under the conditions normally used for reduction of reducing end sugars (30), and we could not find conditions under which all the muramic lactam was reduced. Partial reduction and differential reduction of muramic lactam resulted in the splitting of muropeptides containing this sugar into multiple peaks during HPLC. We therefore determined the following conditions which allowed full reduction of terminal muramic acid residues with minimal (20 to 30%) reduction of muramic lactam. Muropeptide reduction was initiated by the addition of 25 μl of freshly prepared 25-mg/ml $NaBH_4$. The reaction was allowed to proceed for 5 min at room temperature with frequent vigorous mixing (vortex) and was terminated by the addition of 3.5 μl of H_3PO_4 (approximately 44 N). The final pH was checked and adjusted to ~2 with H_3PO_4. These preparations were stored at −20°C prior to chromatography. No changes in chromatographic patterns were observed after 6-month storage either prior to or after $NaBH_4$ reduction.

TABLE 1. *B. subtilis* strains used in this study

Strain	Description or genotype[a]	Gene product(s) missing	Reference or source
PS832	Wild-type strain 168	None	Laboratory stock
PS1804	*pbpE*::Erm^r	PBP4*	20
PS1838	*pbpF*::Cm^r	PBP2c	21
PS1899	*dacB*::Cm^r	PBP5*, SpmA[b,c], SpmB[b,c]	17
PS1900	*dacA*::Cm^r	PBP5	28[d]
PS1901	*dacF*::Cm^r	DacF[c]	33[d]
PS2022	*pbpD*::Erm^r	PBP4	22
PS2029	*spmB*::Cm^r	SpmB[c]	17
PS2030	*spmA*::Cm^r	SpmA[c], SpmB[b,c]	17
PS2031	*ponA*::Cm^r	PBP1	23
PS2066	In-frame Δ*dacB*	PBP5*	17
PS2123	In-frame Δ*prfA*	PrfA[c]	23
PS2185	In-frame Δ*spmA*	SpmA[c]	17

[a] All mutations interupt the indicated gene early in the coding sequence and are therefore null mutations. Abbreviations: Cm^r, resistance to 3 μg of chloramphenicol per ml; Erm^r, resistance to 0.5 μg of erythromycin per ml and 12.5 μg of lincomycin per ml.

[b] This gene product is missing because the indicated insertion mutation in an upstream gene has a polar effect on its expression.

[c] This gene product has not been identified biochemically. The predicted DacF protein has strong sequence similarity to D-alanine carboxypeptidases (33).

[d] The indicated mutation was moved from the strain background described in the reference into strain PS832 by transformation.

Separation of muropeptides by reversed-phase HPLC. Separation of muropeptides by HPLC was carried out in a manner similar to that previously described (5, 6). The HPLC system was composed of a Waters 680 gradient controller, two Waters 501 pumps, and a Waters U6K injector. Separation was carried out on a Hypersil-octyldecyl silane column (250 by 4.6 mm; 3 μm; Keystone Scientific) with a Javelin guard column (Keystone Scientific) containing the same packing. The column was submerged in a water bath and maintained at 51 or 52°C. It was necessary to precondition columns by chromatography of the peptidoglycan derived from approximately 3 mg of spores in order to achieve reproducible separation of compounds with long retention times. Presumably this preconditioning involved the saturation of some reactive sites on the column matrix. No leaching of muropeptides from preconditioned columns was observed during blank gradient runs. Eluted compounds were detected by their A_{206} with a Waters 481 spectrophotometer. Fractions containing the eluted compounds were collected with an Isco 2150 peak separator and an Isco Foxy fraction collector. The areas of absorbance peaks were determined with a Spectra-Physics SP4100 integrator.

Elution was carried out at a flow rate of 0.5 ml/min with a linear gradient initiated 5 min after injection in 100% buffer A and an increase from 0 to 67% for buffer B in 120 min. Two different buffer systems were used. In the methanol system, buffer A was 50 mM sodium phosphate (pH 4.31) and buffer B was 50 mM sodium phosphate (pH 4.95) in 30% methanol; a small amount of sodium azide was added to buffer A to equalize its A_{206} with that of buffer B. In the acetonitrile system, buffer A was 0.1% trifluoroacetic acid (pH 1.5) and buffer B was 0.1% trifluoroacetic acid in 20% acetonitrile (pH 1.5).

Identification and quantitation of peaks. Fractions collected from acetonitrile gradients were dried and resuspended in H_2O, and samples were taken for amino acid and amino sugar analysis and mass spectrometry. Samples for amino acid and amino sugar analysis were dried and hydrolyzed under vacuum in the vapor of 6 N HCl at 105°C for 16 h. This hydrolysis resulted in the conversion of muramic lactam to muramic acid (30). The samples were dried and then loaded on a Beckman System 7300 analyzer by the hydrolysate method. The recovery, quantitation, and elution positions of glucosamine, muramic acid, muramitol

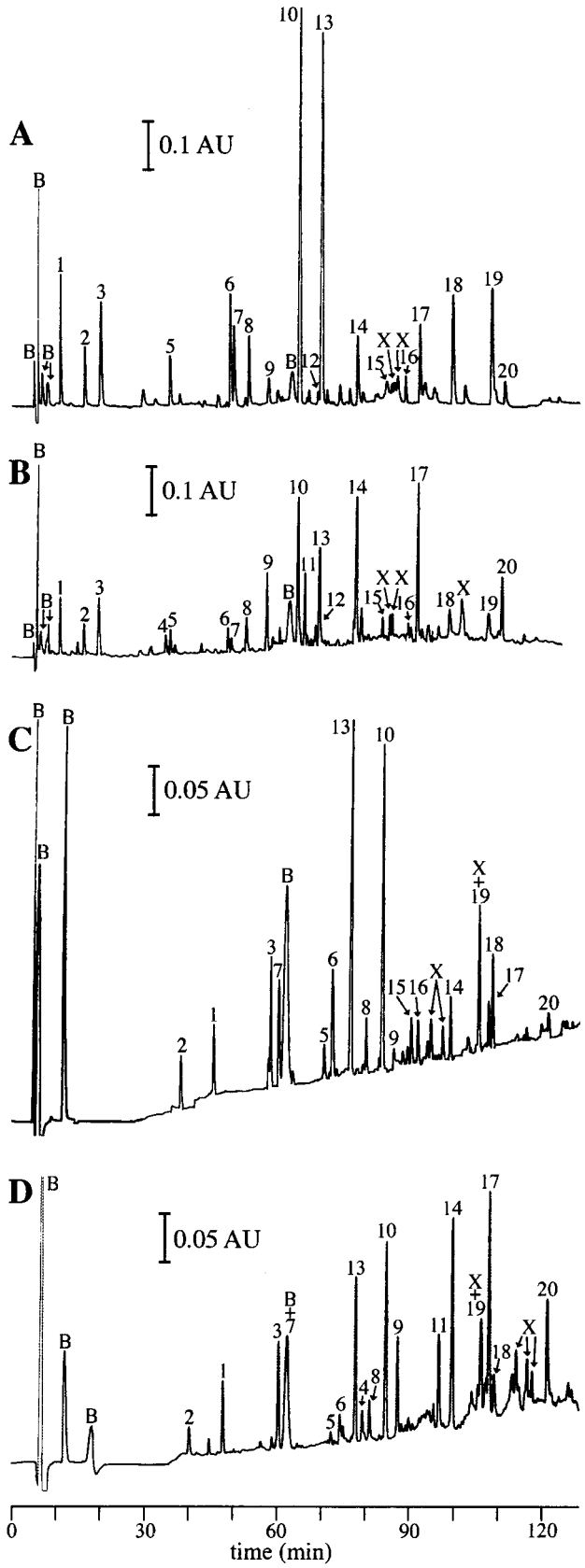

FIG. 2. HPLC separation of spore peptidoglycan muropeptides. (A and B) Separation using the methanol gradient system of the muropeptides produced from wild-type (PS832) and *dacB* mutant (PS2066) spores, respectively. (C and D) Separation of the same muropeptide preparations (PS832 and PS2066, respectively) using the acetonitrile gradient system. Identified peaks are numbered sequentially in panels A and B. The corresponding peaks are indicated by the same numbers in panels C and D. Peaks marked with a B are buffer components observed in blank gradient runs. Peaks marked with an X are unidentified and have been found to arise from the reduction of muramic lactam. The times indicated are from the time of sample injection. AU, absorbance units.

(prepared by reduction of muramic acid), Ala, Glu, and diaminopimelic acid (Dpm) were determined with pure standards.

MALDI-TOF MS spectra were obtained with a Finnigan MAT (now Thermo BioAnalysis, Ltd.) Vision 2000 reflectron TOF MS and a Laser Science, Inc., nitrogen laser with a beam at 337 nm and a 3-ns pulse width. Negative-ion mass spectra exhibits $[(M - H)^-]$ were acquired for most samples because the presence of multiple negatively charged amino acid residues favored stability of the deprotonated species. In a few cases, positive-ion mass spectra exhibits $[(M + Na)^+]$ were acquired. Sample solutions containing approximately 5 pmol of the compound of interest in H_2O were mixed on the probe tip with 1 μl of 10-mg/ml 2,5-dihydroxybenzoic acid in H_2O and air dried. From 3 to 10 spectra were averaged to obtain final values.

RESULTS

Preparation and identification of spore muropeptides. Purified spores were subjected to chemical extraction to inactivate lytic enzymes and to remove small molecules (13, 19). The extracted spores were treated with trypsin and then SDS in order to remove as much protein as possible. Quantitative analyses of Dpm and hexosamine indicated that very little peptidoglycan was solubilized by these extraction steps; the major losses of material were simply due to the large number of manipulations (centrifugations) required (19) (data not shown). Electron-microscopic examination suggested that the remaining insoluble material consisted of the spore peptidoglycan, some spore coat proteins, and some insoluble material in the spore core (19). Digestion with mutanolysin resulted in the solubilization of >95% of the spore's Dpm and hexosamine but very little protein (19) (data not shown). The soluble muropeptides were concentrated, reduced, and subjected to reversed-phase HPLC using a methanol gradient producing the pattern of peaks seen in Fig. 2A. This pattern of peaks was not affected by the substitution of isopropanol reflux (8) for trichloroacetic acid extraction or by substitution of pronase for trypsin during peptidoglycan preparation (data not shown). Each of the peaks resulting from chromatography using a methanol gradient at pH 4.3 to 4.95 (see Materials and Methods) was collected and rechromatographed (data not shown) by using an acetonitrile gradient at pH 1.5 (Fig. 2C). The changes in pH and solvent resulted in significant changes in the retention times of most peaks. This procedure had three results. First, it allowed the determination of the retention times of each compound in both buffer systems. Second, it verified that each peak collected from the methanol gradient was in fact a single compound. Given that the buffer change resulted in both increases and decreases in retention times of various peaks, we believe it to be unlikely that two compounds would coelute in both buffer systems. This assumption was borne out by MS results (see below). Finally, rechromatography in the trifluoroacetic acid-acetonitrile system allowed the collection of each compound in a volatile solvent mix which facilitated subsequent analyses.

Purified peaks were subjected to amino acid and amino sugar analysis and MALDI-TOF MS. The results of these analyses (Table 2) allowed the identification of peaks corresponding to the three disaccharides (DS), two tetrasaccharides (TS), and two hexasaccharides (HS) identified by Warth and Strominger (32). The side chains on the muramic acid residues in the three DS were L-Ala (DS-Ala [peak 2 in Table 2]), and

TABLE 2. Muropeptide analysis

Peak[a]	Amt (nmol)[b]						Predicted structure	$(M - H)^-$[c]	
	NAG	NAM	Muramic lactam	Ala	Glu	Dpm		Calculated *m/z*	Observed *m/z*
1	1	0.93 (1)	0	0.69 (1)	0.76 (1)	1.11 (1)	DS-TriP	869.5	870.2
2	1	1.05 (1)	0	0.85 (1)	0	0	DS-Ala	592.3[d]	592.0[d]
3	1	0.85 (1)	0	1.68 (2)	0.81 (1)	0.81 (1)	DS-TP	940.5	940.7
4	1	1.27 (1)	0	3.38 (4)	1.86 (2)	1.89 (2)	DS-TP-TP	1,383.6	1,382.7
5	1 (2)	0.62 (1)	0.49 (1)	0.92 (2)	0.46 (1)	0.51 (1)	(TS-TP) or (TS-TP + H_2O)	1,358.6	1,358.4
								1,376.8	1,376.2
6	1 (2)	0.64 (1)	0[e] (1)	1.52 (2)	0.75 (1)	0.82 (1)	TSred-TP[f]	1,344.8	1,344.6
7	1 (2)	0.66 (1)	0[e] (1)	0.72 (1)	0	0	TSred-Ala[f]	972.7	972.5
8	1 (2)	1.01 (2)	0	1.40 (3)	0.96 (2)	1.01 (2)	DS-TriP-DS-TP	1,792.8	1,790.7
9	1 (2)	1.06 (2)	0	1.80 (4)	0.95 (2)	0.96 (2)	DS-TP-DS-TP	1,863.8	1,861.0
10	1 (2)	0.51 (1)	0.51 (1)	1.19 (2)	0.59 (1)	0.60 (1)	TS-TP	1,358.6	1,358.4
11	1 (2)	0.59 (1)	0.67 (1)	1.84 (4)	1.03 (2)	1.02 (2)	TS-TP-TP	1,801.8	1,800.6
12	1 (3)	0.82 (2)	0[e] (1)	1.49 (4)	0.79 (2)	0.80 (2)	DS-TP-TSred-TP[f]	2,269.3	2,267.0
13	1 (2)	0.49 (1)	0.49 (1)	0.61 (1)	0	0	TS-Ala	986.6	986.0
14	1 (3)	0.75 (2)	0.31 (1)	1.10 (4)	0.56 (2)	0.58 (2)	DS-TP-TS-TP	2,283.3	2,281.2
15	1 (3)	0.49 (1)	0.36[e] (2)	0.70 (2)	0.38 (1)	0.39 (1)	HSred-TP[f]	1,762.8	1,761.8
16	1 (3)	0.46 (1)	0.32[e] (2)	0.71 (2)	0.37 (1)	0.39 (1)	HSred-TP[f]	1,762.8	1,761.1
17	1 (2)	0.64 (1)	0.63 (1)	1.05 (2)	0.59 (1)	0.53 (1)	TS-TP-TS-TP	2,701.7	2,700.4
18	1 (3)	0.41 (1)	0.71 (2)	0.60 (2)	0.33 (1)	0.32 (1)	HS-TP	1,776.8	1,774.4
19	1 (3)	0.39 (1)	0.67 (2)	0.31 (1)	0	0	HS-Ala	1,404.9	1,403.9
20	1 (5)	0.50 (2)	0.52 (3)	0.81 (4)	0.57 (2)	0.51 (2)	TS-TP-HS-TP	3,118.9	3,119.2

[a] As numbered in Fig. 2A.

[b] Values determined in amino sugar and amino acid analyses were normalized with the result for *N*-acetylglucosamine (NAG) set at 1. Values in parentheses are predicted molar ratios. NAM, *N*-acetyl-muramic acid.

[c] $(M - H)^-$ is the deprotonated molecular ion observed in the negative-ion mode. Calculated *m/z* is the mass/charge predicted from amino acid and amino sugar analyses. Below *m/z* 2,000, monoisotopic mass values are given; above *m/z* 2,000, average mass values are given. Observed *m/z* is the MALDI-TOF MS-measured value.

[d] These calculated and observed *m/z* values are for $(M + Na)^+$ observed in the positive-ion mode.

[e] In these analyses, a peak corresponding to reduced muramic lactam was observed. The size of this peak, relative to the Glu and Dpm peaks, was the same in each case, suggesting that it was present in a 1:1 molar ratio with these amino acids. For peaks 15 and 16, there was 1 mol of muramic lactam and 1 mol of reduced muramic lactam.

[f] These are molecules containing reduced (red) muramic lactam.

the tripeptide L-Ala-γ-D-Glu-Dpm (DS-TriP [peak 1 in Table 2]), and the tetrapeptide L-Ala-γ-D-Glu-Dpm-D-Ala (DS-TP [peak 3 in Table 2]). The two TS had the side chains L-Ala (TS-Ala [peak 13 in Table 2]) and the tetrapeptide (TS-TP [peak 10 in Table 2]). The same two side chains were found on the two HS (HS-Ala and HS-TP [peaks 19 and 18 in Table 2, respectively]). Two very minor peaks eluting at the end of the methanol gradient (peaks 21 and 22 [not apparent in Fig. 2]) were subjected to MS and produced results consistent with the structures octasaccharide-tetrapeptide [peak 21; $(M + Na)^+$ *m/z* observed, 2,220.6; *m/z* calculated, 2,220.2] and octasaccharide-alanine [peak 22; $(M + Na)^+$ *m/z* observed, 1,846.9; *m/z* calculated, 1,847.0]. In addition, we identified a number of cross-linked compounds. On the basis of previous work, we presume all of these cross-links are between the D-Ala of one side chain and the Dpm of the second side chain (32). The identified cross-linked compounds were DS-TriP-DS-TP (peak 8 in Table 2), DS-TP-DS-TP (peak 9), DS-TP-TS-TP (peak 14), TS-TP-TS-TP (peak 17), and TS-TP-HS-TP (peak 20). The DS-TP-TS-TP molecule can exist in two types, one in which the DS-TP moiety is the cross-link acceptor and another in which it is the cross-link donor. We found no evidence of two peaks corresponding to these structures so presumably they cochromatograph. We assume that there were additional cross-linked compounds (DS-TP-HS-TP, HS-TP-HS-TP, and TS-TP-TS-TP-TS-TP) present in very small amounts.

A number of additional peaks were found to result from reduction of muramic lactam residues with $NaBH_4$ (30) during sample preparation. TS-TP and TS-Ala in which the muramic lactam had been reduced to the cyclic secondary amine (TSred-TP and TSred-Ala) (30) were identified as peaks 6 and 7 (Table 2), respectively. Peaks corresponding to secondary, minor (16%) muramic acid reduction products (acrylic primary amino alcohols [30]) were not identified. The identifications of TSred-TP and TSred-Ala were based on the molecular masses and the conversion of purified TS-TP and TS-Ala to TSred-TP and TSred-Ala, respectively, upon further reduction with $NaBH_4$ (data not shown). In the amino acid and amino sugar analysis, the reduced lactam appeared as a compound with mobility similar to that of histidine (data not shown). The presence of this compound and MALDI-TOF MS results indicated that peak 12 was DS-TP-TS-TP in which the muramic lactam had been reduced and that peaks 15 and 16 were HS-TP in which a single muramic lactam had been reduced. The two peaks of HS-TP containing reduced muramic lactam presumably differ in the position of the reduced sugar (position 2 versus position 4 in the six-sugar chain). The observation of changes in chromatograms in response to variations in reduction conditions and the presence of reduced lactam in amino acid and amino sugar analysis results allowed the identification of a number of peaks (marked with X's in Fig. 2) as the results of muramic lactam reduction (data not shown); although the exact identities of these molecules were not determined, presumably some of them represent reduced HS-Ala, TS-TP-TS-TP, and TS-TP-HS-TP. For the preparation of subsequent samples for quantitative analysis, the reduction conditions were adjusted to achieve complete reduction of terminal muramic acid residues with minimal reduction of muramic lactam. Complete reduction of terminal muramic acid residues was judged to be the point at which the areas of DS peaks, which

were not subject to muramic lactam reduction, failed to increase with additional reduction.

The identity of the relatively minor peak 5 is not clear. Its area did not vary with increased reduction, indicating that it is not a muramic lactam reduction product. Amino acid and amino sugar analyses produced a molar composition identical to that of TS-TP (Table 2). MALDI-TOF MS of this compound produced two mass values, one of which was identical to that of TS-TP and the other was 18 mass units greater (Table 2), suggesting the introduction of a water molecule. One possibility for this is the hydrolysis of muramic lactam to muramic acid. We hypothesize that the compound in peak 5 contains a modification of muramic lactam which is hydrolyzed to muramic acid during amino sugar analysis and which can revert to muramic lactam or hydrolyze to muramic acid during MALDI-TOF MS. The odd nature of this compound was also indicated by the altered ratio of its extinction coefficients in the two HPLC gradient systems (see below). For quantitative analyses, peak 5 was considered to be equivalent to TS-TP.

We also examined the effects of variations in the extent of muramidase digestion on chromatogram profiles (data not shown). Minimal amounts of muramidase were required to solubilize all peptidoglycan, but larger amounts were required to achieve cleavage of all susceptible bonds. With increasing amounts of muramidase, we first observed complete cleavage of all the glycosidic bonds adjacent to muramic acid-TP and muramic acid-TriP. The glycosidic bond adjacent to muramic acid-Ala was more resistant to cleavage; larger amounts of muramidase were required to complete cleavage of these bonds. In no case did we observe any evidence for cleavage of the glycosidic bonds adjacent to muramic lactam. Digestion with 16-fold-more muramidase than that required to cleave all the glycosidic bonds adjacent to muramic acid-alanine resulted in no further change in the chromatogram. This indicates that there were no contaminating lytic enzymes in the muramidase preparation. For the preparation of subsequent samples for quantitative analysis, the muramidase concentration was set at fourfold above that found to be necessary to achieve cleavage of all susceptible bonds.

Quantitative analysis of spore peptidoglycan structure. Amino acid and amino sugar analyses allowed the determination of extinction coefficients correlating the nanomoles of compound with peak area in the acetonitrile gradient system (Table 3). Chromatography of identical samples in the methanol and acetonitrile gradient systems allowed the determination of conversion factors for peak areas in the two systems and thus the determination of extinction coefficients relating peak areas to nanomoles in the methanol gradient system (Table 3). Extinction coefficients varied with pH and solvent conditions; the ratio of area in the methanol system to area in the acetonitrile system was 1.58 for all compounds except peak 5, for which it was 2.75. These conversion factors were determined by comparing methanol and acetonitrile gradient system chromatograms for 20 different samples. The extinction coefficients we determined exhibited the incremental increases produced by the addition of each amide bond that had been described previously (6). Interestingly, the addition of a DS unit containing a muramic lactam residue, a mass increase from DS-TP or DS-Ala to TS-TP or TS-Ala, produced a decrease in extinction coefficient, whereas the similar mass increase from TS-TP or TS-Ala to HS-TP or HS-Ala resulted in a large increase (Table 3). For quantitative analysis of the cortex peptidoglycan structure, identical samples of muropeptides were chromatographed in both gradient systems. The use of two gradient systems allowed the determination of areas for all peaks under conditions of optimum resolution. Peak areas in each chromatogram were then converted to nanomoles for each well-resolved peak (Table 3). The values for two compounds (TS-TP and TS-Ala) which were well resolved in both gradient systems were used to correct for slight variations in the sizes of the two samples loaded in the different gradient systems.

TABLE 3. Muropeptide quantitations and wild-type spore peptidoglycan compositions

Peak[a]	Structure	Area/ nmol[b]	PS832 (wild type)		Corrected amt in PS2066 (Δ*dacB*) (nmol)[c,d]
			Amt (nmol)[c]	Corrected amt (nmol)[c,d]	
1	DS-TriP	66.7	0.60	0.60	0.54
2	DS-Ala	47.6	0.57	0.57	0.44
3	DS-TP	71.4	1.05	1.05	0.86
4	DS-TP-TP	101	0[e]	0[e]	0.14
5	(TS-TP)	80.6	0.29	0.37	0.23
6	TSred-TP	46.5	1.41[f,g]	0	0
7	TSred-Ala	29.4	1.82[f,g]	0	0
8	DS-TriP-DS-TP	143	0.17	0.17	0.18
9	DS-TP-DS-TP	152	0.13	0.13	0.36
10	TS-TP	56.2	4.46[g]	5.74	2.65
11	TS-TP-TP	87.0	0[e]	0[e]	0.77
13	TS-Ala	33.3	7.05[g]	9.07	3.22
14	DS-TP-TS-TP	135	0.24	0.31	1.11
17	TS-TP-TS-TP	120	0.24	0.40	1.35
18	HS-TP	111	0.62	1.03	0.27
19	HS-Ala	90.9	1.10	1.82	0.91
20	TS-TP-HS-TP	175	0.09	0.20	0.52

[a] As numbered in Fig. 2. Peaks 12, 15, and 16, which contained reduced muramic lactam, are not included because the amounts of the respective non-reduced compounds were corrected for this reduction.

[b] Relative peak area per nanomole of compound in the methanol gradient system. The units are arbitrary units.

[c] The data are for a single sample of muramidase-digested spore peptidoglycan. For each sample, the data arose from approximately 0.75 mg (dry weight) of spores. However, because of losses of whole spores during sample preparation, this is probably not a valid estimate of total spore peptidoglycan content.

[d] The data were corrected for the degree of reduction of muramic lactam and the number of lactam residues in each molecule as described in the text.

[e] These compounds were seen only in *dacB* mutant strains.

[f] These values were corrected for the fact that 16% of the reduced muramic lactam was in a form that was not detected (30).

[g] These values were used to calculate the average degree of reduction of muramic lactam. For the wild-type sample, the average value was 22%; for the *dacB* mutant, the average value was 15%.

The percentages of TS-TP and TS-Ala which had been reduced to TSred-TP and TSred-Ala were averaged, and the resulting value was used to correct the values (in nanomoles) of all compounds which contained muramic lactam (Table 3). The amounts of TSred-TP and TSred-Ala used in this calculation were adjusted to account for the fact that 16% of the reduced muramic lactam was in a different form that was not detected. It was assumed that the rate of lactam reduction was the same for all muramic lactam residues regardless of their context. A comparison of the degrees of reduction of TS-TP and HS-TP suggested that this assumption was valid (data not shown). With the reduction conditions used, the degree of muramic lactam reduction varied from 15 to 36% but was between 20 and 30% for >70% of the samples. The degree of this correction varied for compounds containing one, two, and three muramic lactam residues. For instance, when it was determined that 10% of the lactam had been reduced, then the values (in nanomoles) of compounds containing a single muramic lactam residue were increased by 10%; the values for compounds containing two muramic lactam residues were increased by 19%; and the values for compounds containing three muramic lactam residues were increased by 27% (Table 3).

TABLE 4. Spore peptidoglycan structure in wild-type and mutant strains[a]

Strain	Description or genotype	No. of samples[b]	% of muramic acid with side chain of:				% of peptide as TriP[c]	% of Dpm in cross-links	% of lactam in TS[d]
			Lactam	L-Ala	TP	TriP			
PS832	Wild type	8	50 ± 1	25 ± 1	24 ± 1	1.8 ± 0.1	6.9 ± 0.4	11 ± 1	75 ± 1
PS1899	*dacB*::Cm^r	3	47 ± 1	15 ± 1	36 ± 2	2.3 ± 0.3	5.5 ± 0.5	31 ± 1 (21 ± 1)[e]	79 ± 3
PS2066	In-frame Δ*dacB*	4	46 ± 1	12 ± 2	40 ± 2	2.5 ± 0.1	5.3 ± 0.2	36 ± 2 (29 ± 3)[e]	79 ± 2
PS2185	In-frame Δ*spmA*	2	50 ± 1	27 ± 1	21 ± 1	1.9 ± 0.4	7.9 ± 1.0	8 ± 1	77 ± 1
PS2029	*spmB*::Cm^r	2	50 ± 1	27[f]	22 ± 1	1.7 ± 0.2	7.3 ± 1.1	8[f]	76 ± 1
PS2030	*spmA*::Cm^r	3	49[f]	27 ± 1	22 ± 1	1.8 ± 0.3	7.6 ± 1.2	7 ± 1	78 ± 3
PS1900	*dacA*::Cm^r	3	50[f]	24 ± 3	26 ± 3	0.9 ± 0.1	3.3 ± 0.4	11 ± 1	76 ± 2
PS1901	*dacF*::Cm^r	3	50 ± 1	26 ± 1	22 ± 2	1.7 ± 0.3	7.3 ± 0.5	12[f]	75 ± 1
PS2031	*ponA*::Cm^r	3	50[f]	21 ± 1	28 ± 1	1.5 ± 0.1	5.3 ± 0.3	10 ± 1	74 ± 1
PS2123	In-frame Δ*prfA*	3	50[f]	22 ± 1	27 ± 1	1.2 ± 0.2	4.3 ± 0.6	11 ± 1	74 ± 2
PS2022	*pbpD*::Erm^r	3	50[f]	24 ± 3	24 ± 3	1.6 ± 0.2	6.2 ± 1.5	11[f]	74 ± 1
PS1804	*pbpE*::Erm^r	2	50[f]	25 ± 1	24 ± 1	1.8 ± 0.1	6.8 ± 0.1	11 ± 1	74[f]
PS1838	*pbpF*::Cm^r	3	50[f]	25[f]	24[f]	1.3 ± 0.1	5.2 ± 0.3	11 ± 1	73 ± 1

[a] Data are averages of independent analyses with errors of 1 standard deviation indicated.
[b] This is the number of independent spore preparations analyzed.
[c] This is the total TriP moiety as a percentage of the total TriP-plus-TP moiety.
[d] This is the percentage of total muramic lactam residues found in TS moieties.
[e] The values in parentheses are for effective cross-links; the remaining cross-links involve a peptide that has been cleaved from the muramic acid by a muramic acid-L-alanine amidase.
[f] These values showed no significant deviation among the samples assayed.

Determination of the nanomoles of each muropeptide recovered from a cortex peptidoglycan muramidase digest allowed the calculation of a number of parameters of peptidoglycan structure, including the percentage of muramic acid in the lactam form and a measure of the distribution of these lactam residues; the percentage of muramic acid with Ala, TriP, and TP side chains; and the percentage of peptide side chains involved in cross-links. The results of analyses of eight different preparations of wild-type spores produced the results shown in Table 4. While the total pool of peptide chains in the spore peptidoglycan was cross-linked at a frequency of 11% ± 1%, these cross-links were not evenly distributed among the various muropeptides. DS muropeptides containing TriP or TP were cross-linked at a frequency of 18% ± 1%, whereas this value for TS-TP was 10% ± 1%. The data in Table 4 are incomplete because of our failure to quantify the larger cross-linked muropeptides, such as DS-TP-HS-TP, HS-TP-HS-TP, and TS-TP-TS-TP-TS-TP. If we assume with which HS-TP is cross-linked to DS-TP with the same frequency with which TS-TP is cross-linked to DS-TP and that HS-TP is cross-linked to HS-TP with the same frequency as that with which it is cross-linked to TS-TP, then we can make predictions about the abundance of the resulting compounds. The only significant outcome of these calculations is that the percentage of peptides found in cross-links increases from 11% ± 1% to 12% ± 1%.

Analysis of cortex peptidoglycan structure in mutant spores. Peptidoglycan was extracted from the spores produced by a variety of mutant strains (in all cases the recovery of muropeptides appeared to be quantitative) and analyzed by HPLC. We have previously demonstrated a significant increase in peptidoglycan cross-linking in the spores produced by a *dacB* mutant (PS2066) (17) which lacks the sporulation-specific penicillin-binding protein 5* (3). Here we have analyzed the structure of this altered spore peptidoglycan in much greater detail (Fig. 2B and D; Table 4). The spore peptidoglycan of this mutant had a slightly reduced amount of muramic lactam, a reduced amount of muramic acid-Ala, and an increased amount of muramic acid-peptide (Table 4). The peptide side chains were cross-linked at a frequency three times greater than that found in the wild type; however, not all these cross-links can be considered effective. A significant number of molecules (peaks 4 and 11 [Fig. 2; Tables 2 and 3]) which contained cross-linked peptides in which one peptide had been cleaved from the saccharide moiety by a muramic acid-alanine amidase were detected. However, the percentage of peptides found in effective cross-links was still over twofold greater than that found in the wild type; in combination with the increased number of peptides, this results in a greater than fourfold increase in cross-linking. This increase in cross-linking for TS-TP (42 versus 10% in the wild type) was greater than that for DS-TriP and DS-TP (36 versus 18% in the wild type). The fact that we never observed the muramic acid-alanine amidase digestion products in wild-type spore peptidoglycan preparations indicates that they are not an artifact of our preparation techniques but probably represent the true situation found in *dacB* mutant spores.

Mutations in two additional genes in the *dacB* operon, *spmA* and *spmB*, were found to result in a slight decrease in spore peptidoglycan cross-linking (Table 4). The decreases in cross-linking for DS and TS muropeptides were similar. Interestingly, a strain lacking expression of all three genes of the *dacB* operon because of a polar insertion in *dacB* (PS1899) exhibited a combination of the effects produced by the single *dacB* and *spm* mutations. There was a large increase in cross-linking, but only to 86% of the level seen in the *dacB* single mutant. Again, the increase in cross-linking for TS was greater than that for DS muropeptides. A *dacA* mutant strain which lacks PBP5 (28) produced spore peptidoglycan with a twofold reduction in the amount of TriP (Table 4). Spores produced by strains lacking the *prfA* or *ponA* (PBP1) gene product had an altered ratio of muramic acid residues with Ala and TP side chains (Table 4). We also analyzed the spore peptidoglycan structures of four other mutant strains (PS1804, PS1838, PS1901, and PS2022) and found no significant changes from the wild type (Table 4).

DISCUSSION

We have applied the high-resolution techniques of reversed-phase HPLC and MS to the analysis of spore cortex peptidoglycan structure. Whereas the previous method of cortex structure analysis required days or weeks (30, 32), the HLPC-based analysis can be completed in hours. This technique also

provides information that was not derived from the original analysis technique, such as the types and arrangements of peptide cross-links produced, i.e., TriP-TP versus TP-TP and DS-TP-DS-TP versus DS-TP-TS-TP versus TS-TP-TS-TP.

The structural parameters we have determined for wild-type spore peptidoglycan are in basic agreement with those determined previously. We found the muramic lactam/muramic acid-Ala/muramic acid-peptide ratio to be 50:25:25. This ratio was previously determined to be 47:18:35 (32). We were also able to quantify the small amount of DS-TriP previously identified in a muramidase digest of spore peptidoglycan (32). Four lines of evidence suggest that muramic lactam (and thus TS) and TriP can be associated preferentially with the cortex and germ cell wall, respectively. (i) We found no evidence for any TS-TriP, indicating that TriP is produced only in a part of the peptidoglycan in which there is no muramic lactam. (ii) DS units containing peptides were cross-linked at a frequency significantly higher than that of TS-TP, suggesting that these two types of structures are segregated to some degree within the spore peptidoglycan. (iii) PBP5* is expressed only in the mother cell compartment of the sporangium (4), probably giving it greater access to the cortex peptidoglycan than to the germ cell wall, and we found that a mutation eliminating PBP5* resulted in a greater change in the cross-linking of TS-TP than in that of DS-TP. The slight increase in TriP seen in the *dacB* mutant might have been due to the decrease in muramic lactam, resulting in a greater percentage of the spore peptidoglycan maintaining a germ cell wall structure. (iv) Analysis of changes in peptidoglycan structure during spore germination revealed increases in DS-peptide relative to TS-TP (24). This could result from degradation of a cortex containing a high percentage of TS units. We have evidence that muramic lactam serves as a major specificity determinant for spore germination lytic enzymes (24), a situation which could protect the germ cell wall from degradation during germination.

Our present estimate of 12% cross-linking is similar to the 16% cross-linking determined previously by chromatographic techniques (32) but differs significantly from the value (37%) we found previously by chemical techniques (19). Analysis of vegetative peptidoglycan cross-linking by HPLC (24) produced values similar to those found previously by chemical methods (19, 25, 31), indicating that the chemical method may simply be inadequate for an analysis of intact spore peptidoglycan. Perhaps the unique structure of spore peptidoglycan renders some free Dpm inaccessible to chemical modification, producing artificially high estimates of cross-linking. Our determinations that 26 and 43% of the muramic acid residues were replaced by peptides in wild-type and *dacB* mutant spores, respectively, differ from the values of 45 and 81% that we found previously (17). The differences in the two measurements for each strain are similar, and we believe that the discrepancies are due to underestimation of total spore hexosamine in the earlier assays (17).

A *dacB* mutation resulted in a greater-than-fourfold increase in peptide cross-links per unit of glycan strand. As we postulated earlier (17), this should result in a peptidoglycan with less flexibility than that found in wild-type spores. The fact that *dacB* mutant spores have a normal degree of spore core dehydration (17) argues against a mechanical activity of the cortex being required for spore core dehydration. However, we have measured only the degree of cross-linking in the final spore peptidoglycan structure. The possibility remains that cross-linking in this *dacB* mutant is normal at an early stage of spore peptidoglycan synthesis (allowing a mechanical activity of the cortex) and simply becomes abnormally high during late sporulation. We previously found that mutations affecting either of the other two genes in the *dacB* operon, *spmA* and *spmB*, had no major effect on spore peptidoglycan structure (17). The more detailed analytical techniques used here revealed a 27 to 36% decrease in spore peptidoglycan cross-linking in these mutants. It is difficult to predict whether this degree of change in the peptidoglycan structure could result in the dramatic defect in core dehydration observed in these mutant spores (17). Alternatively, the change in cross-linking could be a result of incomplete dehydration. An examination of the spore peptidoglycan structure at various stages during spore development will clarify the roles of these three gene products in spore maturation.

An examination of spore peptidoglycan in a number of other mutant strains lacking penicillin-binding proteins revealed only two other instances of structural changes. A *dacA* mutant lacking PBP5, the major D-alanine carboxypeptidase found in vegetative cells (2, 10), had a twofold reduction in the number of TriP moieties in its spore peptidoglycan. The PBP5 that remains in the sporulating cell during the latter stages of sporulation (26) must be competent to remove the D-alanine from some of the spore peptidoglycan peptides. An initial analysis of the spores produced by this mutant strain indicated that they were partially heat sensitive (28), but later analyses failed to reproduce this result (3, 17); the mutant spores were as resistant as the wild type. We believe that the relatively minor alteration in the peptidoglycan structure we detect in *dacA* mutant spores is most consistent with the latter results. Strains lacking the product of either of the genes of the *ponA* operon, *prfA* and *ponA* (PBP1), had a slightly altered ratio of muramic acid residues with L-Ala rather than TP in their spore peptidoglycan. Previous analyses of these mutant spores (23) have indicated that this structural alteration has no effect on spore heat resistance or germination.

The development of a technique for rapid quantitative analysis of spore peptidoglycan structure will allow progress on a number of fronts. The specific contributions of a large number of gene products to the synthesis and modification of this structure can be rapidly determined. The pool of potentially interesting gene products (penicillin-binding proteins, autolysins, etc.) is already large and is growing rapidly with the progression of the *B. subtilis* Genome Project (9). Mutations which affect spore peptidoglycan structure will reveal not only the gene products involved in synthesis but the pathways by which the various structural modifications are achieved. As mutations that affect these pathways are identified, it will become more clear which aspects of the spore peptidoglycan structure contribute to spore resistance and germination properties.

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