*STRUCTURE OF THE PEPTIDOGLYCAN OF BACTERIAL SPORES: OCCURRENCE OF THE LACTAM OF MURAMIC ACID**

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Abstract.—Six major oligosaccharides were released from the peptidoglycan of spores of *Bacillus subtilis* by lysozyme treatment. They were isolated and characterized as a disaccharide, tetrasaccharide, and hexasaccharide composed of equal amounts of muramic acid and glucosamine and containing two, three, and four acetyl groups, respectively. Three of the compounds were substituted by a single L-alanine residue, and the other three by a single tetrapeptide substituent on the acetylmuramic acid residue at the reducing end of each compound. The other muramic acid residue in the tetrasaccharides (and two of the three in the hexasaccharides) were shown to be present as muramic lactams, a sugar not previously found in nature and, hence, a unique spore constituent. Other features of the structure of spore peptidoglycan are discussed.

The integument of bacterial spores contains peptidoglycan, in both a middle layer (the cortex) and in the innermost layer (the germ cell wall). The purpose of the present investigation was to elucidate the structure of the peptidoglycan in bacterial spore walls and to compare it to peptidoglycan in the walls of vegetative cells as a contribution to the problem of differentiation in the conversion of bacterial cells to spores. A number of important differences between the two structures has been found. The most interesting of these, the occurrence of the lactam of muramic acid (Fig. 1) in the peptidoglycan of bacterial spore walls, is described in the present paper. The occurrence of this sugar in natural materials has not previously been described, although *N*-acetylmuramic acid (Fig. 1) is an invariable constituent of the peptidoglycan of bacterial cell walls.

Materials and Methods.—B. subtilis (Porton strain) was grown for 24 hr at 37°C on antibiotic medium no. 3 (Difco) supplemented with a mixture of salts. Spores were freed from sporangia and cells by autolysis at 4°C, washed with 0.2 *M* sodium phosphate, pH 6.5, and purified by two-phase centrifugation.[1] Autolytic enzymes in the spores were inactivated by autoclaving (120°C, 30 min). After disruption of the spores with glass beads in the Nossal disintegrator, spore integuments were sedimented at 20,000 × *g* for 20 min, washed with 0.2 *M* sodium phosphate buffer and water, and then digested with trypsin (0.5 mg/ml).

Preparation of spore peptidoglycan: Integuments (from 50 to 100 mg spores/ml) were resuspended in 0.02 *M* Tris, pH 7.0, by brief sonication and then digested with lysozyme (0.3 mg/ml) for 16 hr at 37°C. Spore coats were sedimented at 20,000 × *g* and the soluble peptidoglycan was deproteinized with chloroform and amyl alcohol.

Isolation of oligosaccharides from the lysozyme digest: The digest was fractionated on a small scale by gel filtration on a column (100 × 0.8 cm) of Sephadex G-25. Individual fractions were subjected to preparative paper electrophoresis at pH 3.9. Oligosaccharides were detected on guide strips using the fluorescence procedure of Sharon and Seifter[2] and eluted. The larger amounts required for nuclear magnetic resonance (NMR) spectroscopy and analyses were obtained by passage of the lysozyme digest through a column

of Dowex 50-H^+ × 4. The fractions eluted with water from Dowex 50 were separated by gel filtration on Sephadex G-25 (75 × 2.2 cm) yielding three oligosaccharides, HS-Ala, TS-Ala, and DS-Ala. Gel filtration of the material eluted from Dowex 50 with 1 *M* pyridine separated TS-TP (a tetrasaccharide) and DS-TP (a disaccharide). DS and TS were obtained by treatment of DS-TP and TS-TP with *Streptomyces* muramyl-L-alanine amidase[3] in 0.02 *M* pyridine acetate, pH 5.4. All oligosaccharides were then converted to their acid form by treatment with Dowex 50-H^+ and lyophilized.

Analytical methods: Methods for determination of reducing power, Morgan-Elson color value, D- and L-alanine, and carboxyl terminal amino acids were as described by Ghuysen *et al.*[4] Amino acids and amino sugars were determined on a Beckman Spinco model 120C amino acid analyzer after hydrolysis in 6 *N* NCl at 105°C for 16 hr. Amino sugar contents were corrected for hydrolytic loss by multiplication of the glucosamine values by 1.32 and the muramic acid values by 1.38. Acetyl groups were determined by a small scale modification of the method of Ludowieg and Dorfman.[5] Paper electrophoresis was carried out using a Gilson model D apparatus for 2 to 3 hr at 30–40 v/cm. Buffers used were 7% acetic acid:2.5% formic acid, pH 1.0, and acetic acid:pyridine:water, 50:15:1935, pH 3.9, or 8:100:896, pH 6.5.

Results.—The spore peptidoglycan preparation obtained by lysozyme digestion of spore integuments from autoclaved spores of *B. subtilis* contained 94 per cent of the Dap and muramic acid originally present in the spore. Analyses indicated that walls of vegetative cells contained 1.0 acetyl residue per amino sugar and one peptide substituent per muramic acid residue, while the preparations from spores contained only 0.7 acetyl residue per amino sugar and about 0.3 peptide substituent per muramic acid residue. In the spores, the peptide substituent was a tetrapeptide, L-Ala-D-γ-Glu-meso-Dap-D-Ala. The spore preparations contained 47 per cent more L-alanine than required for the tetrapeptide substituent, i.e., an excess of 0.15 L-alanine per muramic acid residue. Thus, only 45 per cent of the muramic acid residues could be substituted by L-alanine, either alone or at the amino end of the tetrapeptide.

Fractionation of the lysozyme digest of spore integuments by gel filtration and high voltage paper electrophoresis yielded six major oligosaccharides, which in a large scale preparation were obtained in amounts of 20 to 100 mg. Higher molecular-weight materials and several minor compounds (cross-linked dimers) were also present. Three of the purified oligosaccharides were a disaccharide DS-TP, a tetrasaccharide TS-TP, and a hexasaccharide (HS-TP), each substi-

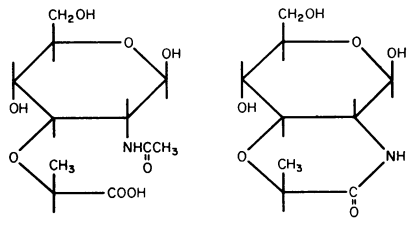

FIG. 1.

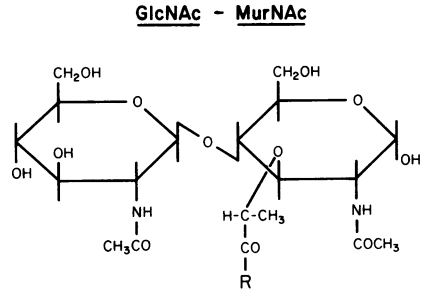

FIG. 2.—Spore disaccharides: in DS-Ala, R = L-alanine; in DS-TP, R = tetrapeptide and in DS, R = OH.

tuted with a *single* tetrapeptide. The other three oligosaccharides (DS-Ala, TS-Ala, HS-Ala) contained a single L-alanine residue instead of the tetrapeptide.

Structure of the disaccharides (Fig. 2): DS-Ala and DS-TP each contained one residue of acetylglucosamine and acetylmuramic acid and either L-alanine or the tetrapeptide substituent (Table 1). In each, a muramic acid was at the reducing end as shown by $NaBH_4$ reduction, and *N*-acetylglucosamine was at the nonreducing end as shown by removal with an exo-β-*N*-acetylglucosaminidase. Treatment of DS-TP with muramyl-L-alanine amidase removed the tetrapeptide substituent of DS-TP (but not the L-Ala substituent of DS-Ala). The disaccharide obtained was identical by various criteria to GlcNAc-β-1,4-MurNAc previously obtained from *Micrococcus lysodeikticus*[6] and other bacteria. Other analyses indicated that the L-alanine residue of DS-Ala was linked by an amide bond to the carboxyl group of muramic acid in this compound.

Structure of the tetrasaccharides—TS-Ala and TS-TP (Fig. 3): Analysis of TS-Ala showed the presence of two glucosamine, two muramic acid, and three acetyl groups for each alanine residue (Table 1). Reduction with ^3H-$NaBH_4$ indicated that a muramic acid residue was at the reducing end. Reducing power and Morgan-Elson color values were similar to the tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc from *M. lysodeikticus*[6, 7] and typical for a tetrasaccharide having a 4-*O*-substituted acetylmuramic acid residue at the reducing end. Exo-β-*N*-acetylglucosaminidase cleaved a residue of *N*-acetylglucosamine from the nonreducing end.

Since only three acetyl groups were present, the amino group of one of the two nonterminal hexosamines had to be free or substituted with an unidentified group. The following properties showed that the amino group was not free. TS-Ala had no cationic mobility at pH 1.9 and was not absorbed to Dowex 50-H^+. It was unchanged after treatment with acetic anhydride in aqueous bicarbonate and did not give dinitrophenyl-hexosamine derivatives on reaction with fluorodinitrobenzene. Partial acid hydrolysis (6 *N* HCl, 100°C, 30 min.) gave the disaccharide muramyl-β-1,4-glucosamine in high yield (50%) from TS-Ala and other spore tetrasaccharides; *none* was obtained from authentic GlcNAc-MurNAc-GlcNAc-MurNAc. This established the sequence GlcNAc-MurN-β-1,4-GlcNAc-MurNAc in TS-Ala. The third acetyl residue is on the internal

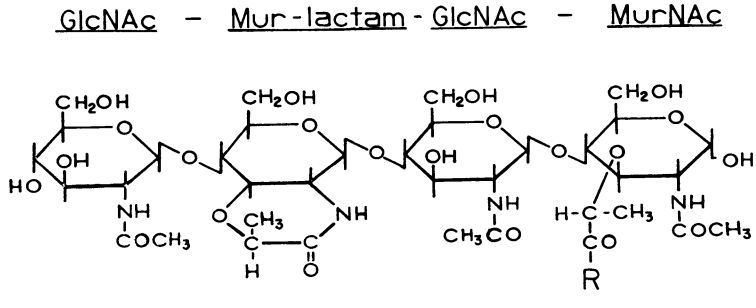

FIG. 3.—Spore tetrasaccharides: in TS-Ala, R = L-alanine; in TS-TP, R = tetrapeptide; and TS, R = OH.

TABLE 1. *Analyses of some spore oligosaccharides.*

	DS	DS-Ala	TS (μmoles/mg)	TS-Ala	HS-Ala[1]
Alanine	...	1.85	...	0.99	0.72
Glucosamine	1.99	1.71	2.10	2.00	2.09
Muramic acid	1.98	1.73	2.09	1.98	2.00
Acetyl	3.78	3.59	2.86	2.94	2.82
Calculated[2]	2.01	1.76	1.09	1.01	0.71
Calculated MW	496	567	915	986	1404

[1] Other data obtained for HS-Ala and HS-TP, not discussed in the text, indicated that these compounds differed from the corresponding tetrasaccharides only in containing an additional acetyl-glucosamine and muramic lactam at the nonreducing end.

[2] The calculation for one residue per mole assumes the presence of no lactams in DS and DS-Ala, one in TS and TS-Ala, and two in HS-Ala.

glucosamine and the internal muramic acid residue is present as the lactam. A positive amino group stabilizes an adjacent glycosidic bond to acid hydrolysis and the rapid opening of the lactam ring during acid hydrolysis, creating such a group, accounts for the high yield of the disaccharide, muramyl-glucosamine.

Data obtained for TS-TP were similar to those for TS-Ala. In addition, treatment of TS-TP with alkali[8] eliminated D-lactyltetrapeptide in 87 per cent yield, which indicates that the peptide was substituted on the reducing-terminal muramic acid residue (since the alkaline elimination reaction occurs only on a muramic acid residue with a free aldehyde). In addition, treatment of TS-TP with muramyl-L-alanine amidase yielded the spore tetrasaccharide, TS. TS-Ala was unaffected by treatment with this enzyme.

Absence of a free-carboxyl group in the internal muramic acid residue of TS-Ala, TS-TP, and TS: At neutral pH, each of these spore tetrasaccharides had an anionic mobility only two thirds that of the corresponding disaccharide. By contrast, the reference tetrasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc, had a slightly greater mobility than the homologous disaccharide, GlcNAc-MurNAc. GlcNAc-MurNAc-GlcNAc-MurNAc has two carboxyl groups and had twice the mobility of TS. Since TS and TS-Ala contained no positive charges, they must have half the negative charge or only one carboxyl group. TS-Ala contained carboxyl-terminal alanine and TS had a free muramic acid carboxyl group from which the tetrapeptide had been cleaved by the amidase. The carboxyl group on the other muramic residue in these compounds cannot be free.

Presence of muramic lactam: Analyses of TS-Ala (Table 1) were those expected for a triacetyl-tetrasaccharide-alanine containing no other groups. Elemental analysis of TS-Ala gave values in satisfactory agreement with the proposed structures. Found: C = 47.4%, H = 6.59%, N = 7.01%, Ash = 0.83%. Calculated for $C_{39}H_{63}N_5O_{24}$: C = 47.51%, H = 6.44%, N = 7.10%. Any unidentified substituent present on the blocked amino or carboxyl groups would need to have a molecular weight of less than 50. No ester or amide was present. These data suggested that the blocked carboxyl and amino groups were mutually substituted to form a lactam.

The reduction of muramic lactam with sodium borohydride, the most novel aspect of its chemistry, provided a direct demonstration of its presence. When

spore oligosaccharides were treated under conditions (0.2 *M* $NaBH_4$, 25°C, 16 hr) which normally allow reduction only of the reducing terminal aldehyde or of uronic esters, nearly all of the muramic acid residues in each of the spore oligosaccharides was lost. Although lactams and amides are usually not reduced by this reagent, Cerutti and Miller[9] reported the reduction of a lactam, dihydrouridine, to form an acyclic amino alcohol. Reduction of muramic lactam (Fig. 1) with a complex metal hydride would be expected to give mainly the cyclic secondary amine (Fig. 4, I), together with the acyclic primary amino alcohol (Fig. 4, II).

Reduction of TS-Ala with ^3H-$NaBH_4$ gave a product containing 90 per cent of the glucosamine and alanine, but less than 0.6 per cent of the muramic acid of TS-Ala, i.e., both of the muramic acid residues of the tetrasaccharide were reduced. The reduction product was cationic on electrophoresis at pH 1.9 and neutral at pH 5.5. It therefore contained a new positively charged amino group. On treatment with acetic anhydride in aqueous bicarbonate, 16 per cent of the product was *N*-acetylated. The remainder retained its charged amino group even after repeated attempts at *N*-acetylation. Reduction of the spore disaccharides (which do not contain muramic lactam), GlcNAc-MurNAc, or GlcNAc-MurNAc-GlcNAc-MurNAc did not affect their electrophoretic mobility. In the latter compound, only the terminal muramic acid was reduced.

After acetylation, the two products of $NaBH_4$ reduction of TS-Ala could be separated easily on Dowex 50-H^+. Hydrolysis of the nonacetylatable reduction product (6 *N* HCl, 105°, 44 hr) yielded glucosamine, alanine, C-1 reduced muramic acid, and a basic compound I (M_{GlcN} = 0.89). Hydrolysis of the glycosidic bond adjacent to the positive charge was very slow (half life, 11 hr). The base I contained 1.8 moles of tritium per tritium in the C-1 reduced muramic acid. Treatment of the base with NaOH failed to liberate any radioactive propanediol (in contrast to II), nor was any formed after subsequent acidic or basic hydrolysis. The infrared spectrum showed no amide carboxyl absorption near 1650 cm^{-1}, and the NMR spectrum at 100 MHz was consistent with the structure I (Fig. 4).

The minor *N*-acetylated reduction product was acidic, having mobilities similar to TS-Ala. Acid hydrolysis (6 *N* HCl, 105°, 4 hr) gave alanine, glucosamine, C-1 reduced muramic acid, and a basic tritiated compound II (M_{GlcN} = 0.85). More extensive hydrolysis destroyed this compound. Treatment of this compound with 0.2 *N* NaOH eliminated nearly all of the radioactivity (86%) as

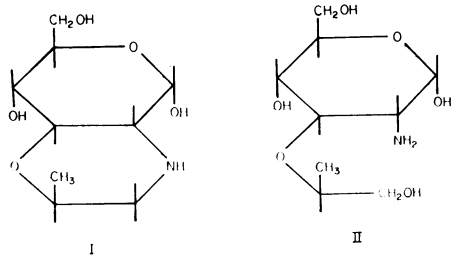

FIG. 4.—Compounds obtained on reduction of muramic lactam in the oligosaccharides with $NaBH_4$ followed by acid hydrolysis. I is the cyclic secondary amine (major product). II is the acrylic primary amino alcohol (minor product).

^{3}H-1,2-propanediol which was identified by chromatography and by codistillation with carrier propanediol.

Infrared and NMR spectra: Comparison of the infrared spectra of the spore and reference oligosaccharides and their salts revealed a systematic decrease in the relative intensity of the amide II band at 1540 cm^{-1} in compounds containing muramic lactam. This is consistent with the lack of amide II absorption in five- or six-membered lactam rings. NMR spectra at 100 MHz of TS, TS-Ala, and HS-Ala were in precise quantitative agreement with the proposed structures. The major difference between the spectra of the spore tetrasaccharide TS and authentic GlcNAc-MurNAc-Glc-NAc-MurNAc (Fig. 5) was in the number of acetamido groups. The NMR spectra confirmed the absence of other substituents and suggested that all of the glycosidic bonds were in the β configuration.

Presence of muramic lactam in other species: Most of the work reported here for *B. subtilis* was repeated with oligosaccharides from the spore peptidoglycan of *Clostridium sporogenes* with similar results. Lysozyme digests of peptidoglycan from spores of four other *Bacillus* species (*B. megaterium*, *B. cereus* T, *B. sphaericus*, and *B. stearothermophilus*) gave compounds corresponding in electrophoretic mobility and R_f to the six compounds isolated from *B. subtilis* and *Cl. sporogenes*. Reduction of all of these spore peptidoglycans (but not of cell wall peptidoglycans) with ^{3}H-$NaBH_4$ gave, after hydrolysis, basic products with the same mobilities as those obtained from reduced muramic lactam from *B. subtilis*.

In order to exclude the possibility that muramic lactams may have formed during autoclaving of the spores, reduction of peptidoglycan from nonautoclaved spores of *B. subtilis* with ^{3}H-$NaBH_4$ was carried out. It yielded 87 per cent of the amount of the basic reduction products obtained from an autoclaved control.

Discussion.—The occurrence of muramic lactam in significant amounts appears to be unique to spore peptidoglycan. Estimates based upon reduction with ^{3}H-$NaBH_4$, the composition of the peptidoglycan, or the recoveries of low molecular weight lysozyme digestion products all indicate that about 55 per cent of the muramic acid residues in *B. subtilis* spores have the lactam structure. The facile reduction of the lactam with ^{3}H-$NaBH_4$ provides a sensitive and specific test for its presence. No lactam could be detected in cell walls of *Bacillus* species.

Modification of the glycan chain is relatively rare in peptidoglycans. Previously reported modifications to the normal alternating β-1,4-linked chain of acetylglycosamine and acetylmuramic acid include acetylation or phosphorylation of the muramyl 6-hydroxyl and the occasional absence of a peptide or *N*-acetyl substituent.[10] On the other hand, a great many modifications of the peptide structure occur, especially in the gram-positive bacteria. By contrast, the peptides of spore peptidoglycans were remarkably uniform; all species examined had the same undifferentiated tetrapeptide originally isolated from cell walls of *Escherichia coli*.[11]

It is evident that the glycosidic bond adjacent to a muramic lactam is resistant to lysozyme. The major products of lysozyme digestion were tetra- and hexasaccharides in which all of the lactams were in nonterminal positions. Digestion of TS-TP with a high concentration of lysozyme (1 mg/ml) for 24 hr produced small amounts of DS-TP and HS-TP, but no hydrolysis was detected. The

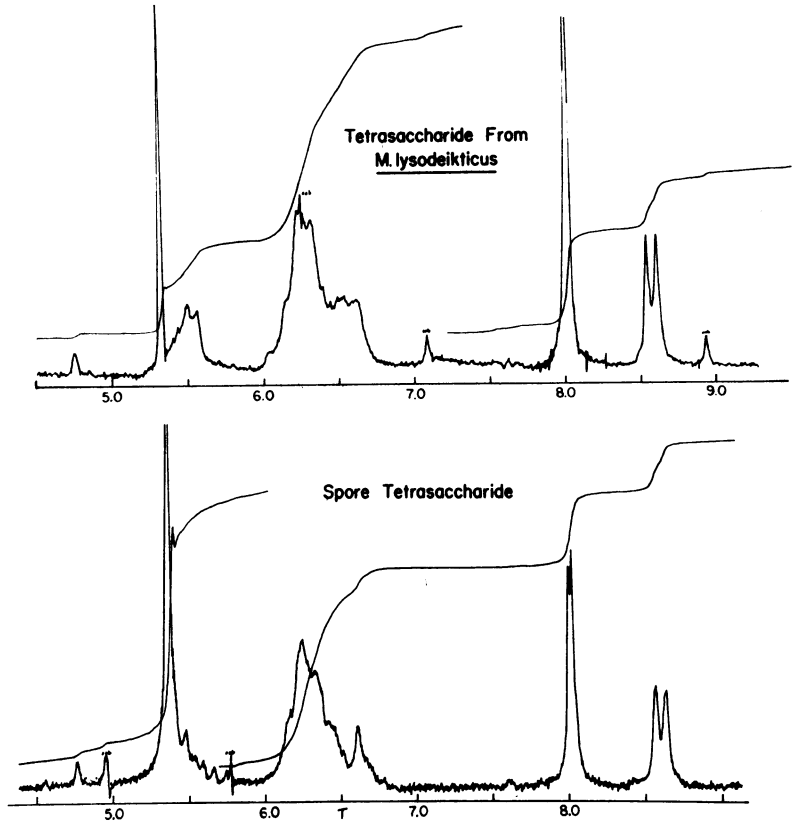

FIG. 5.—NMR spectra (100 MHz) of spore tetrasaccharide and tetrasaccharide from *M. lysodeikticus*. The ratio of methyl protons in the acetamido groups (8.0 τ) to those in the lactyl side chains of muramic acid (8.6 τ) was 12.1 to 6.0 in the tetrasaccharide from *M. lysodeikticus* and 8.8 to 6.0 in the spore tetrasaccharide. The amount of α-anomeric proton (4.8 τ) present in each case was about 0.6 and presumably represented that portion of the reducing terminal sugars present in that configuration. The remainder of the anomeric protons are β.

resistance of this bond to hydrolysis by lysozyme is evidently caused by the rigidity imposed upon the pyranose ring by the fused lactam ring. This rigidity would hinder the binding of the muramic residue in the "D" position and the formation of the planar intermediate in the model proposed by Blake *et al.*[12]

Since muramic lactam appears to be a major component of all spore peptidoglycans, it seems likely that this structure is specifically involved in a function of the spore cortex. Lewis *et al.*[13] proposed that the anhydrous state of the spore core, and hence probably its heat resistance, was brought about by contraction of the cortex. Warth *et al.*[14] suggested that the contraction could be caused by introduction of a polyvalent cation to the polyanionic cortical peptidoglycan. It is not immediately obvious how the lactam contributes to this function. Lactam formation reduces the amount of peptide cross-linking possible and therefore makes a more plastic structure. There is evidence for regularity in the

spacing of lactams along the glycan chain. Lysozyme digestion of a glycan having random distribution of lactams would give disaccharides in greater yield than tetra- and higher oligosaccharides. In fact, tetrasaccharides were isolated in three times the amount of the disaccharides. Perhaps some local structure formed by muramic lactams, and muramic acid residues substituted by L-alanine and tetrapeptide exists in the cortex.

In addition to the occurrence of lactam, the peptidoglycan of spores of *B. subtilis* differs from peptidoglycan of vegetative cell walls in other important respects.[15] (1) Peptide subunits in the vegetative cell wall were 41 per cent cross linked, whereas in the spores, the peptide cross linking was reduced to 19 per cent. (2) Peptide substituents in spores terminate in D-alanine residues, while in cell walls, meso-Dap residues were carboxyl terminal. (3) The meso-Dap residues in the cell wall were mostly amidated but were not amidated in the spore. (4) As shown above, many muramic acid residues are substituted by L-alanine in the spore, whereas all the residues are substituted by peptides in the cell wall. These changes are so extensive as to suggest that the synthesis of the spore peptidoglycan is carried out by an entirely different set of enzymes than that used for synthesis of the peptidoglycan in vegetative cell walls.

Abbreviations used: MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; Dap, α,ϵ-diaminopimelic acid. For products isolated from spores: DS, disaccharide; TS, tetrasaccharide; HS, hexasaccharide; TP, tetrapeptide. M_{GlcN} is the electrophoretic mobility relative to glucosamine at pH 3.9.

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