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N-Acetylmannosaminyl(1→4)*N*-Acetylglucosamine, a Linkage Unit Between Glycerol Teichoic Acid and Peptidoglycan in Cell Walls of Several *Bacillus* Strains

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The structure of teichoic acid-glycopeptide complexes isolated from lysozyme digests of cell walls of *Bacillus subtilis* (four strains) and *Bacillus licheniformis* (one strain) was studied to obtain information on the structural relationship between glycerol teichoic acids and their linkage saccharides. Each preparation of the complexes contained equimolar amounts of muramic acid 6-phosphate and mannosamine in addition to glycopeptide components and glycerol teichoic acid components characteristic of the strain. Upon treatment with 47% hydrogen fluoride, these preparations gave, in common, a hexosamine-containing disaccharide, which was identified as *N*-acetylmannosaminyl(1→4)*N*-acetylglucosamine, along with large amounts of glycosylglycerols presumed to be the dephosphorylated repeating units of teichoic acid chains. The glycosylglycerol obtained from each bacterial strain was identified as follows: *B. subtilis* AHU 1392, glucosylα(1→2)glycerol; *B. subtilis* AHU 1235, glucosylβ(1→2)glycerol; *B. subtilis* AHU 1035 and AHU 1037, glucosylα(1→6)galactosylα(1→1 or 3)glycerol; *B. licheniformis* AHU 1371, galactosylα(1→2)glycerol. By means of Smith degradation, the galactose residues in the teichoic acid-glycopeptide complexes from *B. subtilis* AHU 1035 and AHU 1037 and *B. licheniformis* AHU 1371 were shown to be involved in the backbone chains of the teichoic acid moieties. Thus, the glycerol teichoic acids in the cell walls of five bacterial strains seem to be joined to peptidoglycan through a common linkage disaccharide, *N*-acetylmannosaminyl(1→4)*N*-acetylglucosamine, irrespective of the structural diversity in the glycosidic branches and backbone chains.

Ribitol teichoic acids of *Staphylococcus aureus* H and *Bacillus subtilis* W-23, as well as poly(*N*-acetylglucosamine [GlcNAc]-1-phosphate) of *Micrococcus mutans*, have been reported to be linked to peptidoglycan through a common linkage unit, (glycerol phosphate)$_3$-GlcNAc (3, 4, 8). Recently, the glycerol teichoic acid of *Bacillus cereus* AHU 1030 was shown to be linked to peptidoglycan through a disaccharide, *N*-acetylmannosaminyl [ManNAc]β(1→4)GlcNAc (13), and the same disaccharide bound to tri(glycerol phosphate) was shown to occur in the cell walls of *S. aureus* H as a linkage unit between ribitol teichoic acid and peptidoglycan (10). However, the poly(galactosylglycerol phosphate) of *Bacillus coagulans* AHU 1366 was shown to be linked to peptidoglycan through a linkage saccharide of another type, glucosyl [Glc]β(1→4)GlcNAc (9).

In our previous study on the distribution of mannosamine and mannosaminuronic acid among cell walls of *Bacillus* species (18 strains) (17), it was shown that the strains can be classified into the following three groups on the basis of mannosamine content in the cell walls: strains containing no detectable mannosamine (2 strains), those containing only 10 to 35 nmol of this saccharide per mg of cell walls (10 strains), and those containing as much as 370 to 470 nmol of this saccharide per mg of cell walls (6 strains). Of the second group of strains, 6 strains seemed to contain glycerol teichoic acids in their cell walls. By analogy with the case of *B. cereus* AHU 1030 (13), it was inferred that the mannosamine in the cell walls of the second group of bacterial strains may be a component of linkage saccharides between peptidoglycan and particular polymers, such as glycerol teichoic acids. To determine the structural relationship between glycerol teichoic acids and their linkage saccharides, we studied the structure of polymer-linked glycopeptides obtained from the cell walls of this group of strains.

The present paper reports that mannosamine is involved in glycerol teichoic acid-glycopeptide complexes from four strains of *B. subtilis* and one strain of *Bacillus licheniformis* as a component of the disaccharide ManNAc(1→4)GlcNAc, which seems to join the teichoic acid chain to peptidoglycan. The paper also reports the structure of the teichoic acid moieties of the complexes.

MATERIALS AND METHODS

Bacteria. *B. subtilis* AHU 1035, AHU 1037, AHU 1235, and AHU 1392 and *B. licheniformis* AHU 1371, kindly supplied by S. Takao, Hokkaido University, were grown as described previously (17).

Isolation of teichoic acid-glycopeptide complexes. Cells were harvested at half-maximal growth and disrupted at 4°C for 5 min in a 10-kHz sonic oscillator. The cell walls were isolated from the cell homogenates in a procedure involving heating, digestion with RNase and trypsin, and treatment with sodium dodecyl sulfate (1, 17). *N*-Acetylation of cell walls was carried out with acetic anhydride in an $NaHCO_3$ solution (2). Each of the *N*-acetylated cell wall preparations (140 to 400 mg) was completely digested with lysozyme (EC 3.2.1.17) (30 μg/mg of cell walls) at 37°C for 36 h. After dialysis and gel filtration on Sephadex G-50, each of the resulting polymer fractions was subjected to chromatography on a DEAE-cellulose column (1.2 by 6 to 12 cm) equilibrated with 5 mM Tris-hydrochloride buffer, pH 7.2, as described previously (1, 13). The column was eluted with the same buffer, followed by a linear gradient of NaCl from 0 to 0.4 M in the same buffer. Fractions containing both hexose and phosphorus, eluted at NaCl concentrations between 0.10 and 0.38 M, were pooled, dialyzed, and lyophilized (acidic

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polymer fraction). Each of the acidic polymer fractions was dissolved in a small volume of 0.05 M $(NH_4)_2CO_3$ and chromatographed on a Sephacryl S-200 column (1.5 by 100 cm) in the same salt solution. The major acidic polymers emerged as two peaks of material containing both hexose and phosphorus. The larger and smaller acidic polymers were separately rechromatographed on the same column and denoted as the teichoic acid-glycopeptide complexes I (TA-GP-I) and II (TA-GP-II), respectively.

Hydrolysis of teichoic acid-glycopeptide complexes with hydrogen fluoride. Each of the TA-GP-II preparations (10 to 40 mg) was treated in 0.5 to 1 ml of 47% hydrogen fluoride (HF) at 25°C for 12 h. After removal of HF by evaporation in an air flash and of anionic material by passage through a Dowex 2 column (acetate form), the products were subjected to gel filtration on a Sephadex G-25 column (1 by 147 cm, superfine) in 0.05 M $(NH_4)_2CO_3$ at a flow rate of 9 ml/h. Fractions (1 ml) were collected and assayed for hexose, total hexosamine, and reducing groups.

Proton magnetic resonance spectroscopy (400-MHz). For proton magnetic resonance spectroscopic analysis, the sample was dissolved in ^2H_2O after repeatedly dissolving in ^2H_2O and lyophilization. The 400-MHz magnetic resonance spectra were recorded on a Jeol NX-500 spectrometer, operating in the Fourier transform mode at a probe temperature of 25°C. Chemical shifts were given relative to an internal standard, 3-methylsilylpropane sulfonate.

Methylation analysis. Dephosphorylated repeating units were permethylated by the method of Hakomori (7) with some modifications; then the permethylated oligosaccharides were extracted with chloroform. A sample of each of the permethylated oligosaccharides was subjected to two-step hydrolysis in 0.05 M H_2SO_4-containing acetic acid as described by Stellner et al. (16). The resulting monosaccharide derivatives were converted to alditol acetates and analyzed by gas-liquid chromatography as described by Lindberg (11), using a glass column (3.1 mm by 2 m) packed with Gas-Chrom Q coated with ECNSS-M (3%) at 180°C. Standards, 2,3,4-tri- and 2,3,4,6-tetra-*O*-methyl alditol acetates of D-glucose and D-galactose, were prepared from amygdalin and stachyose, respectively, as described above.

Smith degradation. TA-GP-II (10 to 20 μmol of phosphorus) was oxidized with 0.05 M $NaIO_4$ in 0.5 ml of 0.1 M sodium acetate buffer, pH 5.0, for 24 h in the dark at 4°C. After the addition of 100 μmol of ethylene glycol, the product was reduced with $NaBH_4$ in 0.1 M borate buffer, pH 9.0, and dialyzed. The nondialyzable material was treated in 0.1 M HCl at 25°C for 16 h. After lyophilization, the sample was subjected to gel filtration on a Sephacryl S-200 column (1 by 100 cm) in 0.05 M $(NH_4)_2CO_3$. Fractions (1 ml) were collected and assayed for phosphorus and hexose.

Analytical methods and materials. Unless otherwise indicated, the analytical methods and materials were the same as those described in previous papers (13, 17). Monosaccharide components and dephosphorylated repeating units were analyzed, after trimethylsilylation, by gas-liquid chromatography on a glass column (3.1 mm by 2 m) containing Chromosorb WAW-DMCD coated with silicone SE-52 (5%), with temperature programming from either 130 to 220°C at 4°C/min after locking for 2 min at 130°C (for analysis of monosaccharide components) or 130 to 290°C at 6°C/min after locking for 2 min at 130°C (for analysis of dephosphorylated repeating units). The flow rate of carrier gas (N_2) was 40 ml/min. Paper chromatography was carried out by the descending method on 15 mM borate-treated or nontreated Toyo no. 50 filter paper in 1-butanol–pyridine–water (6:4:3, vol/vol/vol). Amygdalin and stachyose were purchased from Sigma Chemical Co. Exo-α-*N*-acetylglucosaminidase (EC 3.2.1.50) was prepared from human urine as described by Figura (5). Disaccharides, ManNAcβ(1→4)GlcNAc and Glcβ(1→4)GlcNAc, were isolated as the linkage saccharides from TA-GP-II of *B. cereus* AHU 1030 and *B. coagulans* AHU 1366, respectively, by the method described in the previous papers (9, 13).

RESULTS

Isolation of teichoic acid-glycopeptide complexes from cell walls. As reported previously (17), the cell walls of *B. subtilis* AHU 1035, AHU 1037, AHU 1235, and AHU 1392 and *B. licheniformis* AHU 1371 were presumed to have teichoic acids because of their high contents of glycerol, phosphorus, and hexose(s). Each of the acidic polymer fractions, obtained from lysozyme digests of these cell walls by gel filtration on Sephadex G-50, followed by chromatography on a DEAE-cellulose column, was separated into two major fractions, TA-GP-I and TA-GP-II, by gel filtration on a Sephacryl S-200 column. Figure 1 shows the result obtained with the preparation from *B. subtilis* AHU 1392 as a representative. In some cases, TA-GP-I and TA-GP-II were partly separated in the step of ion-exchange chromatography. The yield, the concentration of NaCl by which the acidic polymer was eluted from the DEAE-cellulose column, and the apparent molecular weight of each complex are summarized (Table 1).

TA-GP-II from each bacterial strain contained nearly equimolar amounts of mannosamine and muramic acid 6-phosphate, together with large amounts of presumable components of glycerol teichoic acid, namely, phosphorus, glyc-

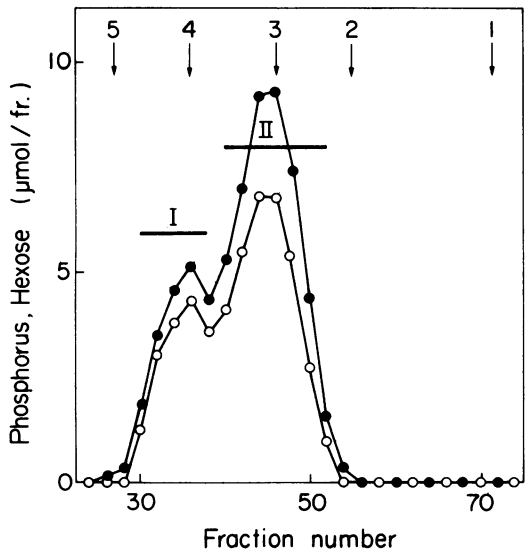

FIG. 1. Chromatography of acidic polymer fraction from *B. subtilis* AHU 1392 on Sephacryl S-200. The acidic polymer fraction obtained from lysozyme digests of *B. subtilis* AHU 1392 cell walls (113 mg) by DEAE-cellulose column chromatography was subjected to gel filtration on a Sephacryl S-200 column. Fractions (1.5 ml) were collected and analyzed for hexose (○) and phosphorus (●). Pooled fractions are indicated by bars. Larger (I) and smaller (II) acidic polymers were separately purified by rechromatrography on the same column and used as the teichoic acid-glycopeptide complexes, TA-GP-I and TA-GP-II, respectively. Similar results were obtained with other bacterial strains. Arrows 1, 2, 3, 4, and 5 indicate the elution positions of standards glucose, dextrans T-10, T-20, and T-40, and blue dextran, respectively.

TABLE 1. Yields, salt concentrations for elution from a DEAE-cellulose column, and apparent molecular weights of teichoic acid-glycopeptide complexes

Strain	Complex	Yield[a] (mg)	Salt conc[b] (M)	Apparent mol wt[c]
B. subtilis AHU 1035	TA-GP-I	12.4	0.25–0.38	43,000
	TA-GP-II	26.1	0.25–0.38	19,000
B. subtilis AHU 1037	TA-GP-I	19.3	0.16–0.34	44,000
	TA-GP-II	13.3	0.10–0.16	20,000
B. subtilis AHU 1235	TA-GP-I	20.0	0.22–0.35	45,000
	TA-GP-II	14.2	0.15–0.22	20,000
B. subtilis AHU 1392	TA-GP-I	15.5	0.17–0.34	54,000
	TA-GP-II	19.4	0.17–0.34	24,000
B. licheniformis AHU 1371	TA-GP-I	8.7	0.22–0.34	50,000
	TA-GP-II	21.8	0.22–0.34	22,000

[a] The yield is expressed by the weight of each complex recovered from 100 mg of cell walls.
[b] This value is the concentration of NaCl by which the acidic polymer was eluted from a DEAE-cellulose column.
[c] The apparent molecular weight was estimated by gel filtration on Sephacryl S-200 or S-300.

erol, and hexose(s) (Table 2). TA-GP-I from each bacterial strain had the same molar ratios of glycerol, phosphorus, hexose(s), and muramic acid 6-phosphate as did TA-GP-II from the same strain. For example, the ratio for TA-GP-I from *B. subtilis* AHU 1035 was 50:52:50:1 as compared with the value 51:54:52:1 for TA-GP-II from this strain. Thus, both complexes seem to have identical teichoic acid chains. By analogy with the case of *B. cereus* AHU 1030 (13), the smaller teichoic acid-glycopeptide complex (TA-GP-II) obtained from each bacterial strain seems to have one teichoic acid chain; however, the larger one (TA-GP-I) seems to have two identical teichoic acid chains, which may be attached to either a single glycan chain or separate glycan chains joined by a peptide cross-linkage. The average numbers of the repeating units in the teichoic acid chains from cell walls of *B. subtilis* AHU 1035, AHU 1037, AHU 1235, and AHU 1392 and *B. licheniformis* AHU 1371, respectively, were 52, 50, 59, 60, and 46, as calculated from the analytical data on the assumption that each teichoic acid chain is linked to the peptidoglycan moiety at a muramic acid 6-phosphate residue. The major portions (40 to 50%) of the mannosamine residues of the cell wall preparations were recovered in the isolated complexes, and comparable portions of teichoic acid components were also recovered in the isolated complexes. Thus, the mannosamine residues in the cell walls seem to be at least predominantly present linked to the teichoic acid moiety. The smaller teichoic acid-glycopeptide complex, TA-GP-II, from each bacterial strain mainly was used for further studies.

Isolation of linkage saccharides and dephosphorylated repeating units from teichoic acid-glycopeptide complexes. As reported previously (9), upon HF treatment, the teichoic acid-glycopeptide complex obtained from *B. coagulans* AHU 1366, as well as that from *B. cereus* AHU 1030, gave the linkage disaccharide and dephosphorylated repeating units of teichoic acid chain in a quantitative yield. Thus, HF treatment seems to provide a simple procedure for the isolation of the linkage saccharide and dephosphorylated repeating units from the teichoic acid-glycopeptide complexes. HF treatment of TA-GP-II of *B. licheniformis* AHU 1371 gave a hexosamine-containing disaccharide (peak A-1), in addition to a large amount of nonreducing, hexose-containing material presumed to be dephosphorylated repeating units of the teichoic acid chain (peak A-2) (Fig. 2A). The products from TA-GP-II obtained from *B. subtilis* AHU 1235 and AHU 1392 also gave similar elution patterns on gel filtration. The hexosamine-containing disaccharide in the first peak and the nonreducing, hexose-containing material in the second peak were separately purified by rechromatography on the same column and used as the linkage saccharide and dephosphorylated repeating units, respectively. However, on gel filtration of HF hydrolysates of the complexes from *B. subtilis* AHU 1035 and AHU 1037, a hexosamine-containing disaccharide and nonreducing, hexose-containing material were eluted as overlapping peaks at the position of standard chitobiose (Fig. 2B). The two materials could be separated from each other by subsequent paper chromatography in 1-butanol–pyridine–water (6:4:3, vol/vol/vol). The values of mobility relative to that of *N*-acetylglucosamine were 0.66 and 0.22 for the hexosamine-containing disaccharide and the hexose-containing material, respectively, as compared with 0.65 and 1.38 for the standards chitobiose and glycerol. The separated materials were individually purified by gel filtration on Sephadex G-25 and used as the linkage saccharide and dephosphorylated repeating units.

Table 3 shows the yield and composition of the linkage saccharide and dephosphorylated repeating units obtained from the TA-GP-II preparations. The linkage saccharide

TABLE 2. Contents of characteristic components of teichoic acid-glycopeptide complexes

TA-GP-II from	nmol/mg of complex						
	Muramic acid 6-phosphate	Mannosamine	Excess glucosamine[a]	Phosphorus	Glycerol	Hexose	Glutamic acid[b]
B. subtilis AHU 1035	32.1	35.1	42.1	1,740	1,640	1,670	128
B. subtilis AHU 1037	35.1	42.5	53.9	1,800	1,690	1,430	134
B. subtilis AHU 1235	33.0	34.7	94.5	1,950	1,900	1,800	174
B. subtilis AHU 1392	32.0	37.5	44.6	2,300	2,120	1,870	131
B. licheniformis AHU 1371	53.4	51.1	77.2	2,540	2,030	1,670	130

[a] Excess glucosamine is the difference between the amount of glucosamine and the total amount of muramic acid derivatives.
[b] Glutamic acid is shown as a representative component of glycopeptide moiety.

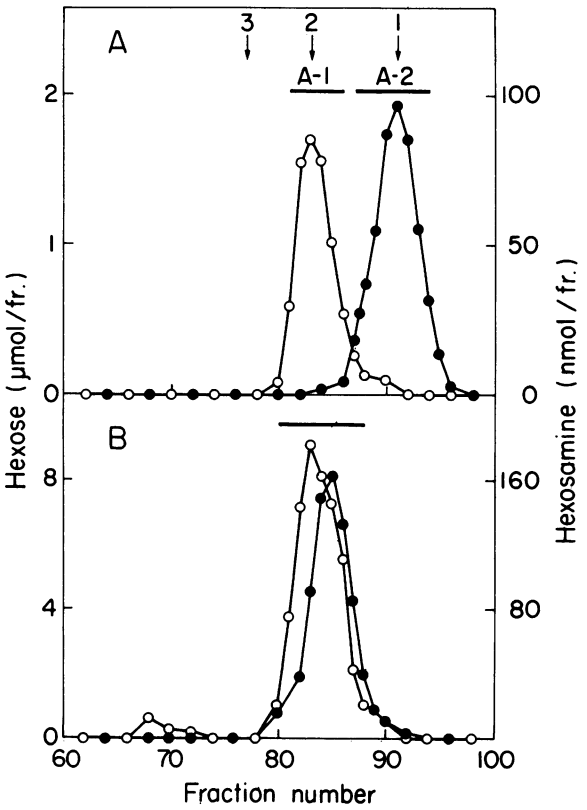

FIG. 2. Gel filtration of neutral products from HF hydrolysis of teichoic acid-glycopeptide complexes. TA-GP-II preparations from *B. licheniformis* AHU 1371 (8.7 mg) and *B. subtilis* AHU 1035 (24 mg) were treated in 47% HF. The neutral products were subjected to gel filtration on a Sephadex G-25 column. Fractions (1 ml) were collected and analyzed for total hexosamine (○) and hexose (●). (A) Elution pattern of products from *B. licheniformis* AHU 1371. Hexosamine-containing disaccharide (peak A-1) and hexose-containing material (peak A-2) were pooled as shown by bars. Similar results were obtained with TA-GP-II preparations from *B. subtilis* AHU 1235 and AHU 1392. (B) Elution pattern of products from *B. subtilis* AHU 1035. Hexosamine-containing disaccharide and hexose-containing material were eluted as overlapping peaks and pooled as shown by a bar. A similar result was obtained with the TA-GP-II preparation from *B. subtilis* AHU 1037. Arrows 1, 2, and 3 indicate the elution positions of the monomer, dimer, and trimer of *N*-acetylglucosamine, respectively.

fraction from *B. subtilis* AHU 1235 contained mannosamine, glucosamine, and glycerol in a molar ratio of 1:2:0.5. The data suggest that this fraction contained some impurity presumed to be *N*-acetylglucosamine-linked glycerol. Attempts to separate this impurity from the linkage saccharide was unsuccessful. However, the treatment of this fraction with exo-α-*N*-acetylglucosaminidase from human urine, followed by gel filtration on Sephadex G-25, gave a disaccharide composed of equimolar amounts of mannosamine and glucosamine. The disaccharide was further purified and then used as the linkage saccharide of *B. subtilis* AHU 1235. The impurity was tentatively characterized as *N*-acetylglucosaminyl-α-*N*-acetylglucosaminyl-α-glycerol on the basis of its molecular weight (coelution with linkage disaccharide on Sephadex G-25) and behavior in the exo-α-*N*-acetylglucosaminidase treatment. On the above treatment, this material yielded free *N*-acetylglucosamine and free glycerol in a molar ratio of 2:1.

As calculated on the basis of the mannosamine contents of TA-GP-II (Table 2) and the yields of linkage disaccharide (Table 3), the amounts of mannosamine recovered in the linkage saccharide preparations account for 80 to 90% of the mannosamine residues contained in the teichoic acid-glycopeptide complexes. However, the amounts of hexose and glycerol residues recovered as components of dephosphorylated repeating units were 65 to 80% and 30 to 70%, respectively, relative to those in the starting complexes. The lower values for glycerol seem to be explained by the presence of unglycosylated glycerol residues in the teichoic acid chains. When the products from HF hydrolysis of the complexes were directly analyzed by gas-liquid chromatography, considerable amounts of glycerol were present along with small amounts of free hexose(s), whereas neither free *N*-acetylglucosamine nor free *N*-acetylmannosamine could be detected.

Identification of linkage saccharide. The linkage saccharide obtained from each TA-GP-II preparation gave equimolar amounts of *N*-acetylglucosamine and *N*-acetylmannosamine as analyzed by gas-liquid chromatography after acid hydrolysis (4 M HCl, 100°C, 4 h) followed by *N*-acetylation (Table 3). When each disaccharide preparation was analyzed after reduction with $NaBH_4$, equimolar amounts of *N*-acetylglucosaminitol and *N*-acetylmannosamine were given. In the modified Morgan-Elson reaction, each preparation gave a much lower color yield than did *N*-acetylglucosamine (molar color yields relative to that of *N*-acetylglucosamine, 0.02 to 0.04). The Smith degradation of the reduced disaccharides gave *N*-acetylxylosaminitol. Furthermore, on paper chromatography in 1-butanol–pyridine–water (6:4:3, vol/vol/vol) on 15 mM borate-treated filter paper, each of the linkage disaccharide preparations was coincident with standard ManNAcβ(1→4)GlcNAc (migration relative to that of chitobiose, 1.10) and distinguishable from standard Glcβ(1→4)GlcNAc (1.00). The above results indicate that the teichoic acid-glycopeptide complexes obtained from the five bacterial strains had a common disaccharide, ManNAc (1→4)GlcNAc, and that this disaccharide is probably involved in the linkage unit between each glycerol teichoic acid chain and peptidoglycan in the cell walls of these strains just as it is in the cell walls of *B. cereus* AHU 1030 (13).

Characterization of dephosphorylated repeating units of teichoic acids. On the basis of the composition shown in Table 3, the dephosphorylated repeating units obtained from *B. subtilis* AHU 1235 and AHU 1392 seem to be glucosylglycerol, and that from *B. licheniformis* AHU 1371 seems to be galactosylglycerol. The $NaIO_4$ oxidation of these compounds resulted in the degradation of the glycosyl residues without loss of the glycerol residues, indicating glycosylation of the glycerol residues at C-2. The anomeric configuration of the glycosidic linkages in each TA-GP-II preparation was directly investigated by 400-MHz proton magnetic resonance spectroscopy. The signals of anomeric protons of hexosyl residues were assigned from the chemical shifts (δ) and coupling constants (*J*) as follows: *B. subtilis* AHU 1392, α-glucoside (δ = 5.189 ppm, *J* = 3.41 Hz); *B. subtilis* AHU 1235, β-glucoside (δ = 4.657 ppm, *J* = 7.84 Hz); *B. licheniformis* AHU 1371, α-galactoside (δ = 5.179 ppm, *J* = 3.91 Hz). Thus, the repeating saccharide units of the teichoic acids obtained from *B. subtilis* AHU 1235 and AHU 1392 and *B. licheniformis* AHU 1371 were glucosylβ(1→2)glycerol, glucosylα(1→2)glycerol, and galactosylα(1→2)glycerol, respectively.

However, the dephosphorylated repeating units obtained from *B. subtilis* AHU 1035 and AHU 1037 contained equimolar amounts of glucose and galactose (Table 3). The

TABLE 3. Yield and composition of linkage disaccharide and dephosphorylated repeating units

TA-GP-II from	Linkage disaccharide			Dephosphorylated repeating units			
	Yield[a] (nmol)	Composition		Yield[b] (nmol)	Composition		
		Mannosamine	Glucosamine		Glycerol	Glucose	Galactose
B. subtilis AHU 1035	31.3	1.00	0.95	590	1.00	1.04	0.96
B. subtilis AHU 1037	34.5	1.00	0.98	475	1.00	0.94	1.06
B. subtilis AHU 1235	28.8	1.00[c]	1.06[c]	1,300	1.00	0.96	0
B. subtilis AHU 1392	29.3	1.00	0.96	1,390	1.00	0.99	0
B. licheniformis AHU 1371	41.7	1.00	0.95	1,200	1.00	0	0.96

[a] Yield is expressed in nanomoles of disaccharide recovered from 1 mg of each TA-GP-II.
[b] Yield is expressed in nanomoles of glycerol recovered from 1 mg of each TA-GP-II.
[c] Data on the linkage disaccharide purified by the treatment with exo-α-*N*-acetylglucosaminidase, followed by gel filtration as described in the text.

$NaIO_4$ oxidation of either preparation led to the formation of formaldehyde and the degradation of hexose and glycerol residues. This result suggests glycosyl substitution of C-1 or C-3 of the glycerol residues in these preparations. Proton magnetic resonance spectroscopic data (δ = 5.004 ppm, J = 3.45 Hz; δ = 4.951 ppm, J = 3.67 Hz) of anomeric protons indicated that both glucosyl and galactosyl residues were in the α-configuration. Acid hydrolysates of the permethylated products from each preparation gave 2,3,4,6-tetra-*O*-methylglucitol acetate and 2,3,4-tri-*O*-methylgalactitol acetate, as analyzed by gas-liquid chromatography. Thus, the most probable structures for the dephosphorylated repeating units of the teichoic acids from *B. subtilis* AHU 1035 and AHU 1037 are glucosylα(1→6)galactosylα(1→1 or 3)glycerol.

Smith degradation of teichoic acid-glycopeptide complexes. Two types of backbone chains, poly(glycerol phosphate) and poly(glycosylglycerol phosphate), are known in wall glycerol teichoic acids. Upon Smith degradation, the teichoic acids with the former type of backbone chains are expected to give $NaIO_4$-resistant polymeric products, which are composed of phosphorus and glycerol, whereas those with the latter type of backbone chains are expected to give $NaIO_4$-oxidized fragments with small molecular weights, except for the case in which the glycosyl residues are substituted at C-3 by phosphoryl groups. The hexose residues in TA-GP-II preparations obtained from *B. subtilis* AHU 1235 and AHU 1392 were completely oxidized by $NaIO_4$. The Smith degradation products from both strains (Fig. 3A) gave the same elution patterns on gel filtration as did the products from *B. cereus* AHU 1030 (13). The molecular weights of the backbone chains were reduced to 5,000 to 6,000 from ca. 16,000 to 20,000, probably because of the loss of the glycosidic branches and because of some cleavage at phosphodiester bonds in the acid treatment step. This result indicates that the backbone chains of the teichoic acids from *B. subtilis* AHU 1235 and AHU 1392 were poly(glycerol phosphate).

In contrast, $NaIO_4$ treatment completely degraded the hexose residues in the TA-GP-II preparation obtained from *B. licheniformis* AHU 1371, and gel filtration of the Smith degradation products gave a large peak of small, phosphorus-containing fragments and a small peak of larger, phosphorus-containing material which seems to be derived from a teichuronic acid component contained in the TA-GP-II preparation as a contaminant (Fig. 3B). Upon gel filtration of the small fragments through Sephadex G-25, most of the phosphorus was recovered as a component of a compound eluted in front of standard chitobiose. This compound was shown to contain glycerol and phosphorus in a molar ratio of 2:1. From these results it seems most likely that the backbone chain of the teichoic acid of this strain is composed of repeating galactosylα(1→2)glycerol-3(1)phosphate units which are joined by phosphodiester bonds at C-6 of the galactose residues.

The $NaIO_4$ oxidation of the TA-GP-II preparations obtained from *B. subtilis* AHU 1035 and AHU 1037 resulted in the complete degradation of the glucosyl residues, whereas the galactosyl residues were undegraded. In addition, the galactosyl residues, as well as the phosphorus and glycerol groups, were recovered as components of polymeric material (Fig. 3C). These results indicate that the galactosyl residues were substituted also at C-3, probably by phosphoryl groups in the polymers. Thus, the teichoic acids of these strains are composed of repeating units, glucosylα(1→6)galactosylα(1→1 or 3)glycerol-3(1)phosphate, which are probably joined by phospodiester bonds at C-3 of the galactose residues. In view of the lower values of glycosyl substitution on the glycerol residues, the backbone chain seemed to be composed of galactosylglycerol-phosphate units and glycerol-phosphate units. The arrangement of these units in the backbone chain remains to be resolved.

Gas-liquid chromatography of dephosphorylated repeating units. When each of the dephosphorylated repeating units of teichoic acids obtained from five bacterial strains was analyzed by gas-liquid chromatography under the conditions described above, each compound gave the following characteristic retention time (relative to that of the internal standard adonitol): glucosylα(1→2)glycerol (*B. subtilis* AHU 1392 and *B. cereus* AHU 1030), 1.715; glucosylβ(1→2)glycerol (*B. subtilis* AHU 1235), 1.768; galactosylα(1→2)glycerol (*B. licheniformis* AHU 1371), 1.703; glucosylα(1→6)galactosylα(1→1 or 3)glycerol (*B. subtilis* AHU 1035 and AHU 1037), 2.660. This gas chromatographic method seems to supply a simple method for the analysis of dephosphorylated repeating units resulting from HF hydrolysis of various types of glycerol teichoic acids.

DISCUSSION

The results described above indicate that mannosamine is involved in glycerol teichoic acid-glycopeptide complexes from four strains of *B. subtilis* and one strain of *B. licheniformis* as a component of the disaccharide ManNAc(1→4)GlcNAc. Since the *N*-acetylated cell wall preparations were used as the starting materials, there is a possibility that either hexosamine residue in the linkage saccharide may be *N*-unsubstituted. However, the *N*-acetylated and the native, non-*N*-acetylated preparations of *B. cereus* AHU 1030 cell walls gave the same linkage disaccharide (13). Therefore, both hexosamine residues in the linkage saccharides investigated here seem to be also *N*-acetylated.

After digestion of the cell walls with lysozyme, ca. 50% of

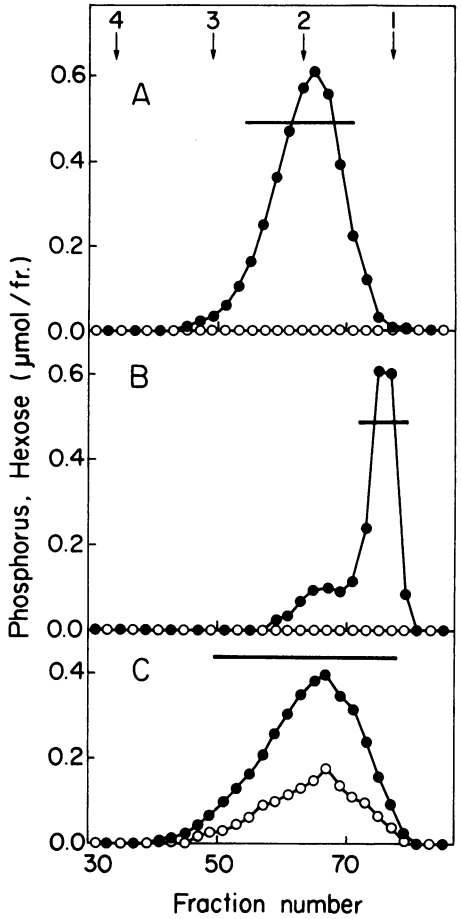

FIG. 3. Smith degradation of teichoic acid-glycopeptide complexes. Smith degradation products of TA-GP-II preparations obtained from *B. subtilis* AHU 1392 and AHU 1035 and *B. licheniformis* AHU 1371 were subjected to gel filtration on a Sephacryl S-200 column. Fractions (1 ml) were collected and analyzed for hexose (○) and phosphorus (●). (A) Preparation from *B. subtilis* AHU 1392. Phosphorus-containing materials were pooled as shown by a bar and further analyzed. A similar result was obtained with TA-GP-II from *B. subtilis* AHU 1235. (B) Preparation from *B. licheniformis* AHU 1371. (C) Preparation from *B. subtilis* AHU 1035. A similar result was obtained with TA-GP-II from *B. subtilis* AHU 1037. Arrows 1, 2, 3, and 4 indicate the elution positions of standards glucose, dextrans T-5 and T-20, and blue dextran, respectively.

the mannosamine residues were recovered in the teichoic acid-glycopeptide complexes. Since this value is comparable to the recovery of the teichoic acid components, phosphorus and glycerol, the mannosamine residues seem to be present in combination with teichoic acids in the cell walls. In each of the isolated complexes, the mannosamine residues were contained in amounts equimolar to muramic acid 6-phosphate, the presumptive site of attachment of teichoic acid chains to peptidoglycan. After HF treatment of the complexes, mannosamine was almost quantitatively recovered in the hexosamine-containing disaccharide fraction. Therefore, the cell walls seem to contain a unit of this disaccharide per teichoic acid chain. In a previous study of the cell walls of *B. cereus* AHU 1030 (13), we have shown that the disaccharide ManNAcβ(1→4)GlcNAc is glycosidically linked to the muramic acid 6-phosphate residue of glycopeptide and is also linked to the teichoic acid chain at the nonreducing terminal *N*-acetylmannosamine residue. Although in the present work there is no direct evidence of the binding of the disaccharide to either teichoic acid or glycopeptide, the above results, together with the previous finding of linkage saccharides in *B. cereus* AHU 1030 (13), *S. aureus* H (10), and *B. coagulans* AHU 1366 (9), led to a conclusion that the disaccharide most probably is involved in the linkage region between glycerol teichoic acids and peptidoglycan in the cell walls of the five bacterial strains studied.

The data on the analysis of the dephosphorylated repeating units of the teichoic acid moieties, together with the results of Smith degradation, led to the most probable structures for the teichoic acid-glycopeptide complexes (Fig. 4). These structures involve at least four types of glycerol teichoic acids which are different in the backbone chains and glycosidic branches. By analogy with the cases of ribitol teichoic acids reported by Coley et al. (3, 4) and by Kojima et al. (10), it is most probable that an oligo(glycerol phosphate) unit intervenes between each teichoic acid chain and the disaccharide unit as shown in the dashed line (Fig. 4). The uniformity of the linkage saccharide units indicated here for glycerol teichoic acids of various types may be related to the pathway of biosynthesis of these polymers. Actually, a preliminary study revealed that the membranes prepared from the five bacterial strains, just as those from *B. cereus* AHU 1030 (12), catalyze the synthesis of the disaccharide on a lipid and the transfer of glycerol phosphate units from CDP-glycerol to the disaccharide-linked lipid (unpublished data). This result also supports the conclusion that the disaccharide, together with glycerol phosphate units, is involved in the linkage region between teichoic acids and peptidoglycan in the five strains examined.

The result of the present work is consistent with the inference that mannosamine may be involved in the linkage region between peptidoglycan and glycerol teichoic acids in the cell walls of a wide variety of bacteria (17). In addition, preliminary studies on the ribitol teichoic acids in the cell walls of *S. aureus* 209P, *B. subtilis* W-23 and AHU 1390, *Listeria monocytogenes* (unpublished data), and *S. aureus* H (10) and on the poly(GlcNAc-1-phosphate) in the cell walls of *Bacillus pumilus* AHU 1650 (unpublished data) indicated that the linkage saccharide of the disaccharide form, ManNAc(1→4)GlcNAc, also occurs as the common linkage saccharide for the ribitol teichoic acids and some other acidic polysaccharides.

The distal part of teichoic acids is phylogenetically expected to undergo a rapid evolutionary change just as in the *O*-antigen part of lipopolysaccharides, whereas the proximal parts, including the linkage saccharide and oligo(glycerol phosphate) units, are expected to be more conservative structural parts, like the core and lipid A parts of lipopolysaccharides (14). Thus, the linkage region of teichoic acids may provide a valuable taxonomic marker in the evolution of bacteria. On the basis of the sequence homology in ribosomal 16S RNA, Stackebrandt and Woese proposed that the genus *Staphylococcus* is phylogenetically related to the genus *Bacillus* (15). The presence of the linkage saccharide of the ManNAc-GlcNAc type in both genera is in consonance with this proposal. However, the genus *Bacillus* encompasses a number of strains showing a great diversity in the DNA cytosine-plus-guanine content, and its taxonomy has been still unclear. According to the Gordon classification, which is based on the shape of the spore and swelling of the sporangium, the species belonging to this genus can be classified into three groups (6). As far as we have studied, the linkage saccharide and the ManNAc-GlcNAc type is present in *B. subtilis*, *B. cereus*, *B. licheniformis*, and *B.*

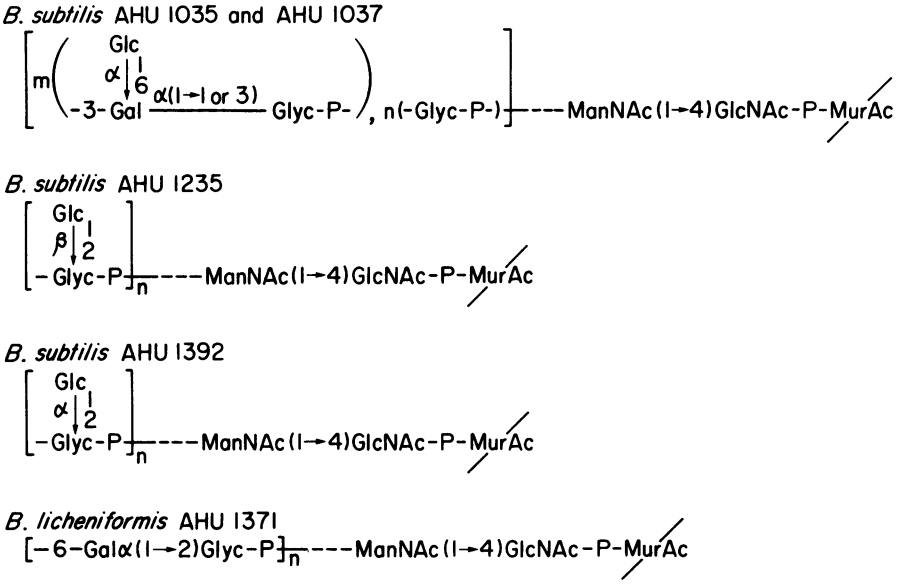

FIG. 4. Most probable structure of teichoic acid-glycopeptide complexes from five bacterial strains. Symbols: Glc, glucose; Gal, galactose; Glyc, glycerol; ManNAc, *N*-acetylmannosamine; GlcNAc, *N*-acetylglucosamine; MurAc, *N*-acetylmuramic acid; P, phosphate.

pumilus, which belong to group 1 (groups 1A and 1B) of the Gordon classification, whereas the linkage saccharide of the Glc-GlcNAc type seems to be restricted to *B. coagulans*, which is intermediate between groups 1 and 2. To precisely correlate the phylogenetic and taxonomic classification with the structure of the linkage region of teichoic acids, further studies on the cell walls of various bacterial strains must be done.

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