JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 424–429
0021-9193/89/010424-06$02.00/0

Vol. 171, No. 1

Structure and Glycosylation of Lipoteichoic Acids in *Bacillus* Strains

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Received 27 June 1988/Accepted 7 October 1988

The occurrence, structure, and glycosylation of lipoteichoic acids were studied in 15 *Bacillus* strains, including *Bacillus cereus* (4 strains), *Bacillus subtilis* (5 strains), *Bacillus licheniformis* (1 strain), *Bacillus polymyxa* (2 strains), and *Bacillus circulans* (3 strains). Whereas in the cells of *B. polymyxa* and *B. circulans* neither lipoteichoic acid nor related amphipathic polymer could be detected, the cells of other *Bacillus* strains were shown to contain lipoteichoic acids built up of poly(glycerol phosphate) backbone chains and hydrophobic anchors [gentiobiosyl(β1→1/3)diacylglycerol or monoacylglycerol]. The lipoteichoic acid chains of the *B. licheniformis* strain and three of the *B. subtilis* strains had *N*-acetylglucosamine side branches, but those of the *B. cereus* strains and the remaining two *B. subtilis* strains did not. The membranes of the *B. licheniformis* strain and the first three *B. subtilis* strains exhibited enzyme activities for the synthesis of β-*N*-acetylglucosamine-P-polyprenol and for the transfer of *N*-acetylglucosamine from this glycolipid to endogenous acceptors presumed to be lipoteichoic acid precursors. In contrast, the membranes of the other strains lacked both or either of these two enzyme activities. The correlation between the occurrence of *N*-acetylglucosamine-linked lipoteichoic acids and the distribution of these enzymes is consistent with the previously proposed function of β-*N*-acetylglucosamine-P-polyprenol as a glycosyl donor in the introduction of α-*N*-acetylglucosamine branches to lipoteichoic acid backbone chains.

Most gram-positive bacteria possess lipoteichoic acids (LTAs) as some of the major components in their membranes (17, 18, 27). Several investigators believe that LTA or other surface amphipathic membrane polymers are indispensable for cell growth and play roles in particular cellular functions, e.g. binding of cations and regulation of autolysis (9, 13). *Bacillus* species have been reported to be divided into two groups, A and B, on the basis of the structural characteristics of their membrane LTAs (14). The poly(glycerol phosphate) chains of LTAs in group A strains, namely *B. subtilis*, *B. licheniformis*, and *B. pumilus* strains, are substituted to different extents at their repeating units with α-*N*-acetylglucosamine and D-alanine residues and are covalently linked to lipid anchors having a common structure, β-gentiobiosyldiacylglycerol, whereas the backbone chains of LTAs in *B. coagulans* and *B. megaterium* (group B) are partially substituted with α-galactose residues and are joined to nonglycosylated lipid anchors, such as diacylglycerol.

It has been reported that the membrane preparation obtained from *B. cereus* AHU 1356 contains the enzymes which catalyze the synthesis of three different *N*-acetylglucosamine-linked lipids, α-*N*-acetylglucosaminyl pyrophosphorylundecaprenol (α-GlcNAc-PP-undecaprenol) and α- and β-GlcNAc-P-undecaprenols (23, 28). α-GlcNAc-PP-undecaprenol has been shown to function as an essential intermediate in the synthesis of some cell wall polysaccharides and linkage units for various teichoic acids (2, 3, 21, 22, 30, 31). Recently, β-GlcNAc-P-undecaprenol was indicated to serve as a glycosyl donor in the introduction of α-*N*-acetylglucosamine side branches to polymer presumed to be an LTA precursor in the membrane systems of group A strains (25a). However, the species and strains of bacteria examined in these studies are limited. In addition, a possibility that *N*-acetylglucosamine branches are introduced by direct transfer of the sugar from UDP-GlcNAc to LTAs could not be excluded in the examined membrane systems. In spite of the presence of a strong enzyme activity for β-GlcNAc-P-undecaprenol synthesis in the *B. cereus* AHU 1356 membranes, virtually no activity for the transfer of *N*-acetylglucosamine from this glycolipid to LTA was detected in this strain (25a). Moreover, in a preliminary experiment, a membrane preparation from a certain *B. subtilis* strain was shown to have no enzyme activity for β-GlcNAc-P-polyprenol synthesis. To establish the function of this glycolipid, further studies on the structure and biosynthesis of LTAs in a wide variety of bacterial strains seemed to be necessary. Here we report the results of studies of the isolation, structure, and enzymatic glycosylation of LTAs in 15 *Bacillus* strains, including *B. cereus* strains.

MATERIALS AND METHODS

Bacteria. The following *Bacillus* strains were given to us by S. Takao (Hokkaido University, Hokkaido, Japan): *B. cereus* AHU 1030, AHU 1355, and AHU 1356; *B. subtilis* AHU 1031, AHU 1035, AHU 1219, AHU 1392, and AHU 1616; *B. licheniformis* AHU 1372; *B. polymyxa* AHU 1231 and AHU 1385; and *B. circulans* AHU 1363, AHU 1365, and AHU 1646. *B. cereus* T was given to us by J. L. Strominger (Harvard University, Cambridge, Mass.). Cells were grown to the late log phase as described previously (14).

Isolation of LTAs. The procedures for extraction and purification of LTAs were essentially the same as those described previously (8, 14). Wet cells (about 20 g) were defatted by successive extraction with $CHCl_3$-CH_3OH (1:2, vol/vol) and $CHCl_3$-CH_3OH-H_2O (5:10:4, vol/vol/vol). The defatted cells, suspended at a concentration of 0.2 g of wet weight per ml in 20 mM sodium acetate buffer, pH 4.6 (buffer A), were mixed with an equal volume of 80% phenol. After the mixture was stirred at 68°C for 10 min, the aqueous layer was removed. The phenol layer combined with the insoluble residue was extracted three times with an equal volume of the same buffer. The aqueous layers were pooled, concen-

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trated, and dialyzed in the cold room against 20 mM sodium acetate buffer, pH 4.6, containing 0.1% Triton X-100 (buffer B). Insoluble material was removed by centrifugation, and the supernatant was treated with DNase (10 mg/mmol of phosphorus) and RNase (20 mg/mmol of phosphorus) at 25°C for 3 h.

The digest was concentrated by filtration with a Diaflo PM 10 membrane filter and subjected to chromatography on a Sepharose CL-6B column (2.2 by 60 cm) in buffer B. Except for *B. polymyxa* and *B. circulans*, major phosphorus-containing materials emerged as two peaks; the first peak contained LTAs, and the second peak contained nucleic acid fragments. The LTA fraction was applied on a DEAE-Sephacel column (1.8 by 4 cm) equilibrated with buffer B, and the column was eluted with the same buffer followed by a linear gradient of NaCl from 0 to 0.75 M in the same buffer. Phosphorus-containing fractions were pooled, concentrated by filtration with a Diaflo YM 2 membrane filter, dialyzed against deionized water, and lyophilized. To remove residual Triton X-100, phosphorus-containing polymer was precipitated by the addition of acetone.

The precipitate was dissolved in buffer A and subjected to hydrophobic interaction chromatography on an Octyl-Sepharose CL-4B column (about 1 ml of resin per μmol of phosphorus) equilibrated with buffer A. The column was eluted with the same buffer followed by a linear gradient of propan-1-ol from 0 to 60% (vol/vol) in the same buffer. Fractions under peaks of phosphorus-containing materials were separately pooled, concentrated, extensively dialyzed against deionized water, and lyophilized. In some cases, the phenol extraction was carried out after disruption of cells in a sonic disintegrator.

Preparation of membranes. The cells were suspended in 20 mM Tris-(hydroxymethyl)aminomethane-hydrochloric acid buffer (Tris hydrochloride buffer), pH 7.8 (1 g of wet cells per 10 ml) and treated in a sonic disintegrator for 5 min, and membranes were separated by differential centrifugation as described previously (28). After being washed twice with the same buffer, the resulting pellet was suspended in 20 mM Tris hydrochloride buffer, pH 7.5, to give a final protein concentration of about 40 mg/ml.

Assay of glycolipid formation from UDP-[^{14}C]GlcNAc. The assay of glycolipid formation from UDP-[^{14}C]GlcNAc was essentially the same as that described in a previous paper (28). The standard reaction mixture contained 0.1 mM UDP-[^{14}C]GlcNAc (100,000 cpm), 50 mM Tris hydrochloride buffer, pH 8.0, 60 mM $MgCl_2$, 2 mM EDTA, and membranes (1 mg of protein) in a total volume of 100 μl. After incubation at 25°C for 30 min, glycolipids were extracted with 2 ml of $CHCl_3$-CH_3OH (2:1, vol/vol), and separated by thin-layer chromatography on silica gel 60G plates in $CHCl_3$-CH_3OH-NH_3-H_2O (65:25:0.5:3.6, vol/vol/vol/vol). Gel at the areas corresponding to the bands of α-GlcNAc-PP-polyprenol (R_f = 0.11), β-GlcNAc-P-polyprenol (R_f = 0.43), and α-GlcNAc-P-polyprenol (R_f = 0.49) was scraped into a vial, and the radioactivity was measured in a liquid scintillation counter.

Assay of incorporation of radioactivity from β-[^{14}C]GlcNAc-P-polyprenol into LTAs. The standard reaction mixture contained 1 μM β-[^{14}C]GlcNAc-P-polyprenol (2,000 cpm), 0.1 M ammonium acetate buffer, pH 6.0, 40 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EDTA, and membranes (0.2 to 0.5 mg of protein) in a total volume of 100 μl. After incubation at 25°C for 30 min, the mixture was subjected to paper chromatography in isobutyric acid-0.5 M NH_4OH (5:3, vol/vol), and the radioactivity of polymer remaining at the origin of the chromatograms was measured in a liquid scintillation counter.

Characterization of radioactive polymer produced by incubation of membranes with β-[^{14}C]GlcNAc-P-polyprenol. The isolation of the radioactive polymer produced by incubation of the membrane fraction with β-[^{14}C]GlcNAc-P-polyprenol was carried out as described elsewhere (25a). The standard reaction mixture for the labeled LTA formation was scaled up fourfold, and 2.5 μM β-[^{14}C]GlcNAc-P-polyprenol (30,000 cpm) and the membrane fraction (3 mg of protein) were used. Water-soluble polymer was extracted with 40% phenol and characterized by chromatography on Sepharose CL-6B, DEAE-Sephacel, and Octyl-Sepharose CL-4B and by hydrogen fluoride (HF) treatment (25a).

Materials and analytical methods. UDP-[*acetyl*-^{14}C]GlcNAc (1.85 GBq/mmol) was prepared enzymatically as described previously (16). β-[^{14}C]GlcNAc-P-undecaprenol was prepared by large-scale incubation of the membrane fraction of *B. cereus* AHU 1356 with UDP-[^{14}C]GlcNAc (25a). Diglucosyldiacylglycerol was prepared by HF treatment of diacyl LTA of *B. subtilis* AHU 1235 (14). Glycerol was determined by using glycerokinase and L-glycerol 3-phosphate dehydrogenase after acid hydrolysis (2 M HCl, 120°C, 48 h) of samples (4); D-glucose was determined by using D-glucose oxidase after acid hydrolysis (2 M HCl, 100°C, 3 h) of samples (15); D-alanine was determined by using D-amino acid oxidase after alkaline hydrolysis (0.1 M NaOH, 30°C, 2 h) (8). Fatty acid ester was determined by the method of Novák (24); hexosamine was determined by the Morgan-Elson method (6) or by gas-liquid chromatography after acid hydrolysis (4 M HCl, 100°C, 4 h or 47% HF, 100°C, 20 min) of samples; total hexose was determined by the orcinol-H_2SO_4 method (10); phosphorus was determined by the method of Lowry et al. (19); and formaldehyde was determined by the method of Bok and Demain (5). Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as a standard. Gas-liquid chromatography for analysis of monosaccharides, dephosphorylated repeating units of LTA, and fatty acids was carried out as described previously (14).

To obtain hydrophilic and hydrophobic fragments from LTAs, each sample was treated with 47% HF at 25°C for 18 h. After removal of HF by evaporation, the product was partitioned with an equal volume of water and $CHCl_3$-CH_3OH (2:1, vol/vol). The water-soluble fragments, separated by subsequent column chromatography on Cellulofine GCL-25-m, and the organic solvent-soluble fragments, separated by thin-layer chromatography in $CHCl_3$-CH_3OH-H_2O (65:25:4, vol/vol/vol), were individually analyzed as described previously (14).

RESULTS

Extraction and isolation of LTAs. The extraction and chromatography of LTAs were carried out under mild acidic conditions of pH 4.6 to prevent the release of ester-linked alanine (8). Defatted cell preparations obtained from 15 *Bacillus* strains, namely, *B. subtilis* (5 strains), *B. cereus* (4 strains), *B. licheniformis* (1 strain), *B. polymyxa* (2 strains), and *B. circulans* (3 strains), were treated with 40% phenol at 68°C for 10 min. Each of the resulting crude extract preparations (80 to 200 μmol of phosphorus per g of dry cells) was subjected to Sepharose CL-6B gel chromatography after nuclease digestion. Figure 1 shows the results obtained with the preparations from *B. cereus* AHU 1356 and *B. circulans*

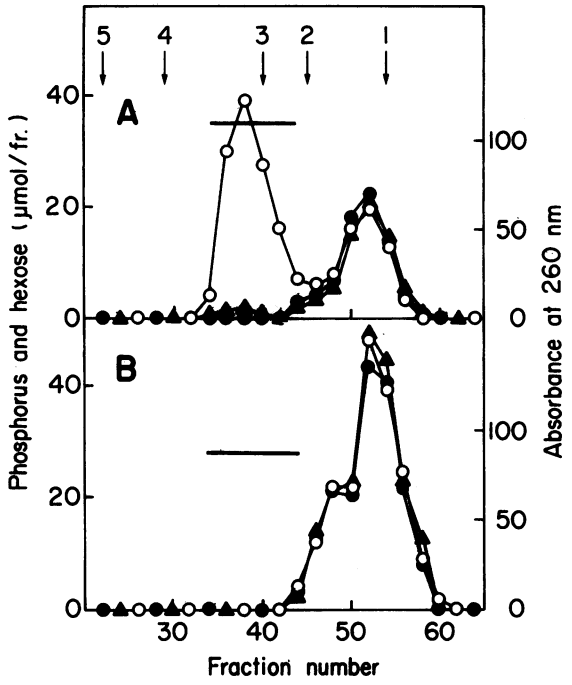

FIG. 1. Chromatography on Sepharose CL-6B of phosphorus-containing polymer extracted from cells. (A) Phosphorus-containing material (530 μmol of phosphorus) extracted from defatted cells of *B. cereus* AHU 1356 by phenol treatment was subjected to chromatography on a Sepharose CL-6B column (2.2 by 60 cm) in buffer B. Fractions (3.5 ml) were collected and analyzed for phosphorus (○), hexose (▲), and A_{260} (●). Fractions indicated by a bar were pooled and used as the crude LTA fraction. Arrows 1, 2, 3, 4, and 5 indicate the elution positions of glucose; dextrans T-9.7, T-40, and T-500; and blue dextran, respectively. (B) Material extracted from defatted cells of *B. circulans* AHU 1646 with phenol was subjected to chromatography as described for panel A.

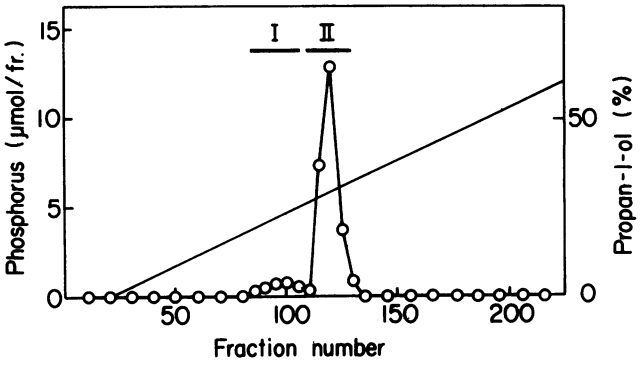

FIG. 2. Separation of LTA by Octyl-Sepharose chromatography. The LTA preparation of *B. cereus* AHU 1356 obtained by DEAE-Sephacel chromatography was loaded on a column of Octyl-Sepharose CL-4B (3 by 15 cm) in buffer A, and the column was eluted with a 0-to-60% gradient of propan-1-ol in buffer A. Fractions (6.5 ml) were collected and analyzed for phosphorus (○). The solid diagonal line indicates propan-1-ol concentration. Fractions indicated by bars I and II were pooled. Similar results were obtained with LTA preparations from other strains.

AHU 1646 as representatives. The preparations from *B. subtilis*, *B. licheniformis*, and the other *B. cereus* strains exhibited elution patterns similar to the one obtained with the preparation from *B. cereus* AHU 1356 (Fig. 1A). The polymer fractions obtained from these strains were used as the crude LTA preparations.

In contrast, neither phosphorus-containing polymer nor hexose-containing polymer was obtained from the extract of *B. polymyxa* or *B. circulans* (Fig. 1B). When the fractions corresponding to the polymer fraction (indicated by a bar in Fig. 1B) were pooled and analyzed, significant amounts of carbohydrate or amino acids were not detected. Direct analysis of the nondialyzable material in the crude phenol extract from *B. circulans* AHU 1646 also failed to give appreciable amounts of carbohydrate or phosphate except for nucleic acid components. In a previous paper (12), it was reported that prior disruption of cells leads to complete extraction of LTAs. However, an attempt to extract LTAs from *B. circulans* and *B. polymyxa* cells after disruption in a sonic disintegrator was unsuccessful. Therefore, this group of strains seem to possess neither LTA nor related amphipathic polymer and are classified as being in group C.

The crude LTA preparations obtained from the 10 *Bacillus* strains other than *B. polymyxa* and *B. circulans* were subjected to successive column chromatography on DEAE-Sephacel and Octyl-Sepharose CL-4B. Upon chromatography on DEAE-Sephacel, the LTAs from these strains were eluted as single peaks of phosphorus-containing material at the following NaCl concentrations, which probably differed depending on the degree of D-alanine substitution (14): *B. cereus* AHU 1356, 0.20 M; *B. subtilis* AHU 1035, AHU 1392, and AHU 1616 and *B. licheniformis* AHU 1372, 0.25 M; *B. cereus* AHU 1355 and *B. subtilis* AHU 1219, 0.30 M; *B. cereus* AHU 1030 and T, 0.40 M; and *B. subtilis* AHU 1031, 0.50 M. Hydrophobic interaction chromatography on Octyl-Sepharose CL-4B resulted in good separation of each LTA preparation into two fractions. As an example, the elution profile given by the LTA preparation from *B. cereus* AHU 1356 is shown in Fig. 2. The components of fractions I and II, respectively, are believed to be LTAs with a monoacylglycerol moiety (monoacyl LTA) and with a diacylglycerol moiety (diacyl LTA) on the basis of their fatty acid contents and by analogy with the data reported previously for LTAs of group A strains (14). Similar elution profiles were obtained with LTA preparations from other strains, indicating that in these strains the majority (more than 90%) of LTAs are in a diacyl form.

Composition of LTAs. The results of analyses of the major LTAs (diacyl LTAs) obtained from 10 *Bacillus* strains are summarized in Table 1. The analytical data account for more than 90% of the weights of the respective LTA preparations. These polymer preparations contained nearly equimolar amounts of glycerol and phosphorus, indicating that their backbone chains consisted of repeating glycerol phosphate units. In addition, the LTAs obtained from *B. subtilis* AHU 1035, AHU 1392, and AHU 1616 and *B. licheniformis* AHU 1372, like the LTAs from previously reported group A strains (14), contained considerable amounts of glucosamine as well as D-alanine. In contrast, the LTAs from four *B. cereus* strains and *B. subtilis* AHU 1219 contained D-alanine but no glucosamine, and the LTA from *B. subtilis* AHU 1031 contained neither glucosamine nor D-alanine.

The above LTA preparations contained D-glucose in small amounts, which is suggestive of the presence of diglucosyl units in their lipid anchor portions. Moreover, in the LTAs from *B. subtilis* AHU 1035, AHU 1392, and AHU 1616, the glycerol phosphate repeating units seemed to be partially substituted by D-glucose.

From the molar ratios of phosphorus to formaldehyde formed by periodate oxidation (14), the numbers of repeating

TABLE 1. Composition of diacyl LTAs

Diacyl LTA source	μmol of the following components per mg of dry LTA prepn:						
	Phosphorus	Glycerol	Fatty acids	Glucose	D-Alanine	Glucosamine	HCHO[a]
B. cereus AHU 1030	4.46	4.15	0.36	0.36	1.12	0	0.20
B. cereus AHU 1355	4.10	4.10	0.25	0.33	1.59	0	0.19
B. cereus AHU 1356	3.63	3.45	0.25	0.18	3.26	0	0.16
B. cereus T	4.53	4.48	0.26	0.41	0.82	0	0.20
B. subtilis AHU 1031	4.95	4.80	0.30	0.30	0	0	0.12
B. subtilis AHU 1035	3.20	3.20	0.32	0.45	1.89	0.67	0.11
B. subtilis AHU 1219	4.31	4.14	0.30	0.39	1.47	0	0.11
B. subtilis AHU 1392	3.06	3.25	0.24	0.39	1.65	0.92	0.11
B. subtilis AHU 1616	3.15	3.34	0.25	0.35	1.35	0.98	0.10
B. licheniformis AHU 1372	3.37	3.30	0.17	0.27	2.16	1.01	0.12

[a] μmol of HCHO released from LTA by $NaIO_4$ oxidation.

units in the poly(glycerol phosphate) chains of the LTA preparations were calculated to be 25 to 35. These values are coincident with those calculated from the fatty acid contents of respective diacyl LTAs on the assumption that each polymer chain was linked to a diacylglycerol residue. As shown in Table 2, the diacyl LTAs exhibited similar fatty acid compositions but differed characteristically in the contents of some fatty acids.

Characterization of dephosphorylated repeating units of LTAs. The diacyl LTA preparations were hydrolyzed with 47% HF at 25°C for 18 h, and the hydrolysates were partitioned between the aqueous and organic solvent phases. On paper electrophoresis in pyridine-acetic acid-water (10:30:960, vol/vol/vol, pH 4.1), the water-soluble products obtained from the LTAs of the four *B. cereus* strains and *B. subtilis* AHU 1219 gave two fractions, which were determined to be glycerol and 2-D-alanylglycerol by a procedure described in a previous paper (14). Thus, the hydrophilic polymer chains of LTAs from these strains seem to consist of glycerol phosphate and 2-D-alanylglycerol phosphate units in various proportions. The LTA preparation from *B. subtilis* AHU 1031 gave only glycerol as the water-soluble product, indicating that this LTA had unsubstituted poly(glycerol phosphate) chains.

The LTAs of *B. subtilis* AHU 1035, AHU 1392, and AHU 1616 gave several different fragments after HF hydrolysis. By the analytical procedure described in a previous paper (14), these fragments were identified as *N*-acetylglucosaminyl(α1→2)glycerol, glucosyl(α1→2)glycerol, glycerol, and their D-alanyl derivatives, indicating that the LTAs of these strains were similar to those of the previously reported group A strains (14) in the structure of their hydrophilic polymer parts. By the same procedure, the LTA of *B. licheniformis* AHU 1372 was shown to have a similar polymer chain, but it lacked glucosyl side branches.

Characterization of lipid anchor portions of LTAs. Thin-layer chromatography of the organic solvent-soluble products, obtained from the LTA of *B. cereus* AHU 1356 by HF treatment, gave four spots (R_fs = 0.40, 0.62, 0.70, and 0.78) of acylglycerol derivatives, which were located by I_2 staining and exhibited a positive reaction to the α-naphthol-H_2SO_4 reagent. Similar results were also obtained with LTA preparations from other strains. The main hydrophobic fragment (R_f = 0.62), which comigrated with the standard diglucosyldiacylglycerol and accounted for about 65% of the glucose residues in the organic phase, contained D-glucose, glycerol, and fatty acids in an approximate molar ratio of 2:1:2. This fragment was characterized to be gentiobiosyl(β1→1/3)diacylglycerol by a procedure involving methylation analysis, $NaIO_4$ oxidation, and β-glucosidase digestion, as described previously (14). The minor fragments with R_f values of 0.40, 0.70, and 0.78 were tentatively characterized as diglucosylmonoacylglycerol, monoglucosylmonoacylglycerol, and monoglucosyldiacylglycerol, respectively. These fragments probably arose from the main hydrophobic fragment, diglucosyldiacylglycerol, by secondary hydrolysis during the HF treatment. Thus, the lipid anchor portions of the major LTAs from the *Bacillus* strains tested were shown to have a common partial structure, glucosyl(β1→6)-glucosyl(β1→1/3)glycerol, coincident with the structure of the lipid anchor portions of the LTAs from the previously studied group A strains (14).

Distribution of enzymes responsible for the formation of *N*-acetylglucosamine-linked lipids and the transfer of *N*-ace-

TABLE 2. Fatty acid composition of LTA

Fatty acid	mol% of total fatty acid in:				
	B. cereus AHU 1030	*B. cereus* AHU 1355[a]	*B. subtilis* AHU 1031	*B. subtilis* AHU 1219	*B. subtilis* AHU 1035[b]
$C_{14:0}$	0.5	1.2	1.9	5.4	2.9
Iso-$C_{15:0}$	1.9	0.7	0.8	2.2	2.7
Anteiso-$C_{15:0}$	23.7	19.6	53.3	29.2	33.3
Iso-$C_{16:0}$	5.4	15.0	5.3	15.7	4.2
$C_{16:0}$	8.1	23.8	12.6	21.7	17.3
Iso-$C_{17:0}$ and anteiso-$C_{17:0}$	45.1	34.5	23.9	17.7	21.6
$C_{18:0}$	1.3	2.6	1.3	6.1	4.8
Other fatty acids	14.0	2.6	0.9	2.0	13.2

[a] Similar compositions were shown in the LTA preparations from *B. cereus* AHU 1356 and T.
[b] Similar compositions were shown in the LTA preparations from *B. subtilis* AHU 1392 and AHU 1616 and *B. licheniformis* AHU 1372.

TABLE 3. Distribution of enzyme activities for the synthesis of β-GlcNAc-P-polyprenol and for the transfer of *N*-acetylglucosamine residue from this glycolipid to endogenous LTA precursor

Strain	Formation of β-[^{14}C]GlcNAc-P-polyprenol (cpm per mg of protein per 30 min)[a]	Incorporation of radioactivity from β-[^{14}C]GlcNAc-P-polyprenol into polymer (cpm per mg of protein per 30 min)[b]
B. subtilis AHU 1035	100	730
B. subtilis AHU 1392	90	670
B. subtilis AHU 1616	130	860
B. subtilis AHU 1031	0	0
B. subtilis AHU 1219	0	0
B. licheniformis AHU 1372	450	3,040
B. cereus AHU 1030	0	610
B. cereus AHU 1355	740	40
B. cereus AHU 1356	760	30
B. cereus T	480	30
B. circulans AHU 1646	0	0
B. polymyxa AHU 1385	0	0

[a] Membranes (1 mg of protein) prepared from the indicated bacteria were incubated for 30 min with 50 μM UDP-[^{14}C]GlcNAc (100,000 cpm). Radioactivity in β-GlcNAc-P-polyprenol separated by thin-layer chromatography was determined.

[b] Membranes (0.2 to 0.5 mg of protein) were incubated for 30 min with 1 μM β-[^{14}C]GlcNAc-P-polyprenol (2,000 cpm) at pH 6.0. Radioactivity in the polymer was determined.

tylglucosamine from β-GlcNAc-P-polyprenol to polymer. The enzyme activities for the synthesis of *N*-acetylglucosamine-linked lipids were assayed under the conditions described in Materials and Methods (Table 3). The enzyme for α-Glc NAc-PP-polyprenol synthesis was distributed among the membrane preparations from all the strains tested. The activity for α-GlcNAc-P-polyprenol synthesis is present in *B. cereus* AHU 1355, AHU 1356, and T as reported previously (23, 28). This activity was also detected in *B. subtilis* AHU 1219. A strong activity for β-GlcNAc-P-polyprenol synthesis is present in the three *B. cereus* strains other than AHU 1030 as reported previously (23, 28). This enzyme activity was also found in four other strains, *B. subtilis* AHU 1035, AHU 1392, and AHU 1616 and *B. licheniformis* AHU 1372, which were shown to have *N*-acetylglucosamine-linked LTAs in their membranes.

The membrane preparations of the last four strains also exhibited the activity for incorporation of *N*-acetylglucosamine from β-[^{14}C]GlcNAc-P-polyprenol into endogenous polymers. The radioactive polymers produced coincided with diacyl LTAs as determined by chromatography on Sepharose CL-6B, DEAE-Sephacel, and Octyl-Sepharose CL-4B and by HF treatment (14, 25a). In contrast, only a very low activity of transferring *N*-acetylglucosamine from this glycolipid to polymer was demonstrated in the three *B. cereus* strains other than AHU 1030. Upon chromatography on columns of DEAE-Sephacel and Octyl-Sepharose CL-4B, the radioactive polymers produced with the membranes of these three strains were distinguished from LTA. *B. cereus* AHU 1030 possessed a significant activity of transferring *N*-acetylglucosamine from this glycolipid to the polymer which coincided with LTA in chromatographic behavior, but it showed no appreciable activity for β-GlcNAc-P-polyprenol synthesis. The membranes of other strains which have no *N*-acetylglucosamine-linked LTA were shown to possess neither of these two enzyme activities.

DISCUSSION

The results described above indicate that LTAs in 10 of the 15 *Bacillus* strains tested are commonly made up of hydrophilic poly(glycerol phosphate) chains and hydrophobic gentiobiosyldiacylglycerol anchors. Therefore, the 10 strains seem to be included in group A. The group A strains are further divided into two groups on the basis of the presence or absence of *N*-acetylglucosamine branches in the backbone chains of their LTAs. The LTAs of *B. subtilis* AHU 1035, AHU 1392, and AHU 1616 and *B. licheniformis* AHU 1372, as well as six previously reported strains, *B. subtilis* AHU 1037, AHU 1235, AHU 1390, and W23, *B. licheniformis* AHU 1371, and *B. pumilus* AHU 1650, contain *N*-acetylglucosamine residues as their side branches, while the LTAs of *B. subtilis* AHU 1031 and AHU 1219 and four *B. cereus* strains are devoid of *N*-acetylglucosamine branches. On the other hand, neither LTA nor a closely related substance was detected in *B. circulans* or *B. polymyxa*, which may be designated group C strains.

The absence of α-*N*-acetylglucosamine branches from LTA is explained by the deficiency of β-GlcNAc-P-polyprenol synthetase (in *B. cereus* AHU 1030) or the enzyme catalyzing *N*-acetylglucosamine transfer from β-GlcNAc-P-polyprenol to LTA precursors (in the other *B. cereus* strains) or both of these enzymes (in *B. subtilis* AHU 1031 and AHU 1219). The deficiency of the above two enzymes in the group C strains is also consistent with the absence of LTA from this group of strains. In addition, none of the activities for β-GlcNAc-P-polyprenol formation and the transfer of *N*-acetylglucosamine residues from β-GlcNAc-P-polyprenol to polymer was detected in any of membrane preparations from *B. coagulans* AHU 1631 and AHU 1638, *B. megaterium* AHU 1240 and T, *Staphylococcus aureus* H, 209P, Copenhagen, and D. Gale, and *Lactobacillus plantarum* AHU 1413, which have LTAs devoid of *N*-acetylglucosamine branches (data not shown). Thus, these results support the previous proposal that β-GlcNAc-P-polyprenol serves as an *N*-acetylglucosaminyl donor in the introduction of the *N*-acetylglucosamine branches to LTAs in cells.

The synthesis of another GlcNAc-linked lipid, α-GlcNAc-P-polyprenol, was demonstrated in the membrane systems of a limited number of bacterial strains, including *B. subtilis* AHU 1219, *B. cereus* AHU 1355, AHU 1356, and T, and *B. megaterium* AHU 1373 (25a, 28). The *B. cereus* strains are known to produce neutral cell wall polysaccharides which have α- and β-*N*-acetylglucosamine branches (1, 32). Recently, *B. subtilis* AHU 1219 was also shown to contain a neutral cell wall polysaccharide which possesses β-*N*-acetylglucosamine branches (unpublished observation). Therefore, α-GlcNAc-P-polyprenol and β-GlcNAc-P-polyprenol may serve as *N*-acetylglucosaminyl donors for the β-*N*-acetylglucosamine and α-*N*-acetylglucosamine branches, respectively. Actually, each membrane system of *B. cereus* AHU 1355, AHU 1356, and T transferred a very small amount of *N*-acetylglucosamine residues from β-GlcNAc-P-polyprenol to a polymer, which seems to be a neutral cell wall polysaccharide. Studies of the function of these two glycolipids in the above *Bacillus* strains are in progress.

With respect to the biological role of LTAs, neither LTA nor related substances were found in the group C strains. If the LTAs are necessary for the growth of cells and play important cellular functions, some amphipathic polymers instead of LTAs may occur as the membrane components. In fact, some gram-positive bacteria lacking the typical LTAs have been reported to possess other types of amphi-

pathic substances, such as the succinylated lipomannan in *Micrococcus* species (25), the fatty acid-substituted heteropolysaccharides in *Bifidobacterium bifidum* (7), *Actinomyces viscosus* (26), and *Streptococcus sanguis* (29), and the Forssman antigen in *Streptococcus pneumoniae* (11). However, such amphipathic substances could not be detected in the group C strains. In conclusion, comparative studies of membrane LTAs as well as cell surface polymers seem to provide useful information on the classification of and taxonomic relationship between *Bacillus* species.

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