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Biosynthesis of Linkage Units for Teichoic Acids in Gram-Positive Bacteria: Distribution of Related Enzymes and Their Specificities for UDP-Sugars and Lipid-Linked Intermediates

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The distribution and substrate specificities of enzymes involved in the formation of linkage units which contain *N*-acetylglucosamine (GlcNAc) and *N*-acetylmannosamine (ManNAc) or glucose and join teichoic acid chains to peptidoglycan were studied among membrane systems obtained from the following two groups of gram-positive bacteria: group A, including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Staphylococcus aureus*, and *Lactobacillus plantarum*; group B, *Bacillus coagulans*. All the membrane preparations tested catalyzed the synthesis of *N*-acetylglucosaminyl pyrophosphorylpolyprenol (GlcNAc-PP-polyprenol). The enzymes transferring glycosyl residues to GlcNAc-PP-polyprenol were specific to either UDP-ManNAc (group A strains) or UDP-glucose (group B strains). In the synthesis of the disaccharide-bound lipids, GlcNAc-PP-dolichol could substitute for GlcNAc-PP-undecaprenol. ManNAc-GlcNAc-PP-undecaprenol, ManNAc-GlcNAc-PP-dolichol, Glc-GlcNAc-PP-undecaprenol, Glc-GlcNAc-PP-dolichol, and GlcNAc-GlcNAc-PP-undecaprenol were more or less efficiently converted to glycerol phosphate-containing lipid intermediates and polymers in the membrane systems of *B. subtilis* W23 and *B. coagulans* AHU 1366. However, GlcNAc-GlcNAc-PP-dolichol could not serve as an intermediate in either of these membrane systems. Further studies on the exchangeability of ManNAc-GlcNAc-PP-undecaprenol and Glc-GlcNAc-PP-undecaprenol revealed that in the membrane systems of *S. aureus* strains and other *B. coagulans* strains both disaccharide-linked lipids served almost equally as intermediates in the synthesis of polymers. In the membrane systems of other *B. subtilis* strains as well as *B. licheniformis* and *B. pumilus* strains, however, the replacement of ManNAc-GlcNAc-PP-undecaprenol by Glc-GlcNAc-PP-undecaprenol led to a great accumulation of (glycerol phosphate)-Glc-GlcNAc-PP-undecaprenol accompanied by a decrease in the formation of polymers.

In the cell walls of gram-positive bacteria, various types of teichoic acids, such as ribitol teichoic acids (8, 12–14), glycerol teichoic acids (11, 31), poly(galactosylglycerol phosphate) (9–11, 16), and poly(*N*-acetylglucosamine 1-phosphate) (15), have been reported to be attached to muramic acid 6-phosphate residues of peptidoglycan through special linkage units which commonly consist of a linkage disaccharide, *N*-acetylmannosaminyl-β(1→4)*N*-acetylglucosamine (ManNAc-GlcNAc) or glucosyl-β(1→4)*N*-acetylglucosamine (Glc-GlcNAc), and glycerol phosphate (Gro-P)-containing parts. The latter parts are somewhat different in the number of Gro-P units and also in their constitution, depending on the bacterial species. For example, the ribitol teichoic acids of *Lactobacillus plantarum* AHU 1413 (14), *Bacillus subtilis* W23, and *Staphylococcus aureus* H (12, 13) contain one, two, and three Gro-P residues, respectively. Moreover, an anomalously great number, seven, of Gro-P units has been shown to be involved in the linkage unit for the poly(*N*-acetylglucosamine 1-phosphate) of *Bacillus pumilus* AHU 1650 (15). In addition, a saccharide, Glc-β(1→3)Glc-β(1→3)Gro-P, has been found in the linkage unit for the ribitol teichoic acid of *Listeria monocytogenes* EGD (8).

On the basis of the above structural characteristics, teichoic acids are presumed to be synthesized through the prior formation of the linkage units. This presumption is strongly supported by biosynthetic evidence obtained with membrane systems of several bacterial species (1, 3, 26, 34, 35). However, stepwise conversion of lipid intermediates involved in the synthesis of the linkage units has been proved only in a few cases (34, 35). In addition, we can not exclude the possibility that *N*-acetylglucosaminyl pyrophosphoryl-polyprenol (GlcNAc-PP-polyprenol) may be able to serve as a direct acceptor for Gro-P units from CDP-glycerol with the formation of an *N*-acetylglucosamine type of linkage unit (1, 4, 20–22, 29). Thus, it is necessary to detect and characterize a set of enzymes participating in the de novo synthesis of teichoic acids, especially in the reaction steps of the linkage unit formation, in a wide variety of gram-positive bacteria.

The present paper reports the results of studies on the distribution of such enzymes among various gram-positive bacteria. Moreover, the substrate specificity of the enzymes for sugar-linked lipid intermediates was investigated in detail with membrane systems, including those from *B. subtilis* W23 and *Bacillus coagulans* AHU 1366, used in previous studies (34, 35).

MATERIALS AND METHODS

Materials. UDP-*N*-acetyl[U-^{14}C]glucosamine (UDP-[^{14}C]-GlcNAc; 7.88 GBq/mmol) and UDP-[U-^{14}C]glucose (11.3 GBq/mmol) were purchased from Amersham International. Unlabeled nucleotide sugars, adenosine 5′-(α,β-methylene) triphosphate (AMP-CPP), dolichylphosphate (chemically phosphorylated dolichol [C_{80-105}]), and bacterial alkaline phosphatase were obtained from Sigma Chemical Co. UDP-*N*-acetylmannosamine (UDP-ManNAc) and UDP-[^{14}C]ManNAc were prepared by the method of Kawamura et al. (7). CDP-ribitol was prepared by the method of Moffatt (24). α-[^{14}C]GlcNAc-PP-undecaprenol (5×10^4 cpm/nmol) and α- and β-[^{14}C]GlcNAc-P-undecaprenols were prepared by the

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method of Yamamori et al. by using *Bacillus cereus* AHU 1356 membranes (33). [^{14}C]GlcNAc-PP-dolichol (5×10^4 cpm/nmol) was prepared from UDP-[^{14}C]GlcNAc and dolichylphosphate by the method of Nakayama et al. (28) by using *Saccharomyces cerevisiae* AHU 3027 membranes. Standard disaccharides, ManNAc-β(1→4)GlcNAc and Glc-β(1→4)GlcNAc, were isolated as the linkage saccharides from the teichoic acid-glycopeptide complexes of *B. cereus* AHU 1030 and *B. coagulans* AHU 1366, respectively, according to the methods described in previous papers (9, 31). *N*-Acetylglucosaminyl -β(1 → 4) *N* - acetylglucosamine (GlcNAc-GlcNAc) and the trimer and tetramer of GlcNAc isolated from acid hydrolysates of chitin (30). Antibiotic 24010 was a gift of M. Mizuno (Asashi Chemical Corp.) (23).

Microorganisms and preparation of their membranes. *S. aureus* 209P, D. Gale, and Copenhagen were given by J. L. Strominger (Harvard Medical School); *B. subtilis* W23 was a gift from S. Yamazato (Institute of Applied Microbiology of Tokyo University); *B. coagulans* AHU 1366 and *Saccharomyces cerevisiae* AHU 3027 were given by S. Takao (Hokkaido University). Other bacterial strains were the same as those described in previous studies (11, 13, 14, 36). Cells were grown and harvested at the exponential phase. Cell suspensions (2 g [wet weight] of cells per 10 ml of 20 mM Tris hydrochloride buffer, pH 7.8) were treated at 0°C in a sonic disintegrator for 5 to 20 min. Membranes were isolated from the cell homogenates by differential centrifugation as described previously (35). The 105,000 × *g* precipitates were suspended in the same buffer to give final protein concentrations of 30 to 50 mg/ml and stocked at −20°C until used. Cells of *Saccharomyces cerevisiae* AHU 3027 were disrupted with an X press, and the membranes were isolated by differential centrifugation as described previously (28).

Assay of GlcNAc-PP-polyprenol formation. The assay of GlcNAc-PP-polyprenol formation from UDP-[^{14}C]GlcNAc was essentially the same as that described in previous reports (33–35). The standard reaction mixture contained 40 μM UDP-[^{14}C]GlcNAc (8×10^4 cpm), 50 mM Tris hydrochloride buffer, pH 8.2, 60 mM $MgCl_2$, 2 mM EDTA, 0.78 mM AMP-CPP, and membranes (1 mg or less as protein) in a final volume of 50 μl. After incubation at 25°C for 30 min, glycolipids were extracted with $CHCl_3$-CH_3OH (2:1, vol/vol) and separated by thin-layer chromatography as described previously (33). The radioactivity of the gel at the area corresponding to the spot of GlcNAc-PP-polyprenol (R_f = 0.11) was measured in a liquid scintillation counter.

Assay of formation of disaccharide-linked lipids, Gro-P-containing lipids, and polymer. For the assay of conversion of GlcNAc-PP-undecaprenol (or GlcNAc-PP-dolichol) into disaccharide-linked lipids, the reaction mixture contained 0.6 μM [^{14}C]GlcNAc-PP-undecaprenol (or [^{14}C]GlcNAc-PP-dolichol) (3,000 cpm), 0.2 mM UDP-ManNAc or UDP-glucose, 50 mM Tris hydrochloride buffer, pH 8.2, 30 mM $MgCl_2$, 2 mM EDTA, 0.2% Nonidet P-40, 0.78 mM AMP-CPP, antibiotic 24010 (20 μg/ml), and membranes (group A strains, 50 μg; group B strains, 5 μg) in a final volume of 100 μl. After incubation at 25°C for 10 min, glycolipids were extracted and separated by thin-layer chromatography as described previously (34, 35). The radioactivity of the gel at the areas corresponding to the spots of ManNAc-GlcNAc-linked lipids (R_f = 0.05 to 0.06) and Glc-GlcNAc-linked lipids (R_f = 0.04 to 0.05) was measured.

Conversion of GlcNAc-PP-dolichol or GlcNAc-PP-undecaprenol to GlcNAc-GlcNAc-linked lipid was assayed in the yeast membrane system as described previously (28). The reaction mixture contained 0.6 μM [^{14}C]GlcNAc-PP-dolichol (or [^{14}C]GlcNAc-PP-undecaprenol) (3,000 cpm), 2 mM UDP-GlcNAc, 50 mM Tris-maleate buffer, pH 9.2, 40 mM $MgCl_2$, 2 mM EDTA, 0.78 mM AMP-CPP, antibiotic 24010 (20 μg/ml), 0.2% sodium deoxycholate, and membranes (100 μg) in a final volume of 100 μl.

The assay of conversion of disaccharide-linked lipids into Gro-P-containing lipids or polymer was carried out under the same conditions as those in previous studies (34, 35), except that various disaccharide-linked lipids (2,500 cpm) at 0.5 μM, 0.5 mM CDP-glycerol, and membranes (group A strains, 200 μg; group B strains, 5 μg) were used. In some experiments, CDP-ribitol, UDP-galactose, or UDP-GlcNAc was added to the above mixtures at a concentration of 1 mM. Gro-P-containing lipids were first separated from polymer and the substrate lipids by paper chromatography in solvent A and then hydrolyzed in 50% phenol at 68°C for 2 h. The resulting water-soluble materials were digested with alkaline phosphatase and further fractionated into disaccharide derivatives containing one (G1), two (G2), three (G3), and four to eight (G4 to G8) Gro-P units by paper chromatography in solvent B, as previously described (34).

Preparation of various disaccharide-linked lipids. To obtain large amounts of ManNAc-[^{14}C]GlcNAc-PP-undecaprenol (or dolichol), Glc-[^{14}C]GlcNAc-PP-undecaprenol (or dolichol), and GlcNAc-[^{14}C]-GlcNAc-PP-dolichol (or undecaprenol), the incubation described above for the assay of conversion of [^{14}C]GlcNAc-PP-undecaprenol or [^{14}C]-GlcNAc-PP-dolichol to disaccharide-linked lipids was scaled up 50- to 200-fold. After incubation for 2 h, glycolipids were extracted and separated by thin-layer chromatography. Radioactive disaccharide-linked lipids were obtained in the following amounts: ManNAc-[^{14}C]GlcNAc-PP-undecaprenol (3.2×10^5 cpm) and ManNAc-[^{14}C]GlcNAc-PP-dolichol (6.9×10^4 cpm), 66 and 53% yields, respectively, in the membrane system of *B. subtilis* W23; Glc-[^{14}C]GlcNAc-PP-undecaprenol (5×10^5 cpm) and Glc-[^{14}C]GlcNAc-PP-dolichol (1.1×10^5 cpm), 55 and 50% yields, respectively, in that of *B. coagulans* AHU 1366; GlcNAc-[^{14}C]GlcNAc-PP-dolichol (6.2×10^4 cpm) and GlcNAc-[^{14}C]GlcNAc-PP-undecaprenol (6.9×10^4 cpm), 18 and 8% yields, respectively, in that of *S. cerevisiae* AHU 3027.

Analytical methods. Unless otherwise indicated, analytical methods were the same as those in previous studies (27, 34, 35). Descending paper chromatography was carried out in the following solvents: solvent A, isobutyric acid–0.5 M NH_3 (5:3, vol/vol); solvent B, 95% ethanol–1 M ammonium acetate, pH 3.8 (5:2, vol/vol); solvent C, 1-butanol–pyridine–water (6:4:3, vol/vol/vol). Thin-layer chromatography was performed on silica gel G plates in $CHCl_3$-CH_3OH-NH_3-H_2O (65:25:0.5:3.6, vol/vol/vol/vol).

RESULTS

Formation of GlcNAc-PP-polyprenol. The cell walls of gram-positive bacteria newly tested in this study contain various types of teichoic acids, and on the basis of the types of their linkage saccharides these bacteria are classifiable into two groups, A and B. Group A strains, including *B. subtilis* W23 (13), AHU 1035, AHU 1037, AHU 1235, and AHU 1392, *Bacillus licheniformis* AHU 1371 (11), *L. plantarum* AHU 1413 (14), *B. pumilus* AHU 1650 (15), and *S. aureus* H (13), 209P, Copenhagen, and D. Gale (unpublished data), have a ManNAc-GlcNAc type of linkage saccharide for the attachment of teichoic acids, while group B strains, *B. coagulans* AHU 1366 (9), AHU 1631, AHU 1634, and AHU 1638 (16), have another type of linkage saccharide, Glc-GlcNAc.

TABLE 1. Formation of GlcNAc-PP-polyprenol[a] and conversion of [^{14}C]GlcNAc-PP-undecaprenol to disaccharide-linked lipids[b]

Membrane source	Formation of GlcNAc-PP-polyprenol	Disaccharide-linked lipid formation in the presence of:		Type of linkage saccharide (reference)
		UDP-ManNAc	UDP-glucose	
Group A				
B. licheniformis AHU 1371	1,200[c,d]	400	0	ManNAc-GlcNAc (11)
B. pumilus AHU 1650	910	350	0	ManNAc-GlcNAc (15)
B. subtilis AHU 1035	810	1,850	0	ManNAc-GlcNAc (11)
B. subtilis AHU 1037	650[c]	300	0	ManNAc-GlcNAc (11)
B. subtilis AHU 1235	850[c]	1,170	0	ManNAc-GlcNAc (11)
B. subtilis AHU 1392	550	300	0	ManNAc-GlcNAc (11)
L. plantarum AHU 1413	350	430	0	ManNAc-GlcN (14)
S. aureus 209P	100	250	0	ManNAc-GlcNAc[e]
S. aureus D. Gale	110	300	0	ManNAc-GlcNAc[e]
S. aureus Copenhagen	90	280	0	ManNAc-GlcNAc[e]
Group B				
B. coagulans AHU 1631	1,130	0	1,130	Glc-GlcNAc (16)
B. coagulans AHU 1634	1,400	0	630	Glc-GlcNAc (16)
B. coagulans AHU 1638	1,080	0	1,200	Glc-GlcNAc (16)

[a] UDP-[^{14}C]GlcNAc was incubated with each membrane preparation under the standard assay conditions, and the amounts of [^{14}C]GlcNAc-PP-polyprenol are shown in counts per minute.

[b] [^{14}C]GlcNAc-PP-undecaprenol was incubated with each membrane preparation under the standard assay conditions, and the amounts of radioactive disaccharide-linked lipids are shown in counts per minute.

[c] Similar results were reported previously (32).

[d] This lipid fraction contained GlcNAc-PP-polyprenol and GalNAc-PP-polyprenol (38) in a ratio of 2:1.

[e] Unpublished data.

In a biosynthetic study of GlcNAc-linked lipoteichoic acids of *Bacillus* species, GlcNAc-PP-polyprenol was shown to be synthesized in *B. subtilis* AHU 1037 and AHU 1235 and *B. licheniformis* AHU 1371, as described previously (32). In the present work, the membrane preparations of 10 other bacterial strains were also tested for the ability to synthesize GlcNAc-linked lipids. Under the standard assay conditions, GlcNAc-PP-polyprenol was synthesized in all the tested bacterial strains (Table 1), while GlcNAc-P-polyprenols were synthesized in a rather limited number of bacterial strains, including four *B. subtilis* strains, *B. licheniformis* AHU 1371, and *B. pumilus* AHU 1650.

Formation of linkage-disaccharide-bound lipids. The formation of linkage-disaccharide-bound lipids was studied using [^{14}C]GlcNAc-PP-undecaprenol, obtained from the *B. cereus* AHU 1356 membrane system (33), as a glycosyl acceptor. Incubation of [^{14}C]GlcNAc-PP-undecaprenol with UDP-ManNAc and membranes from group A strains consistently resulted in the formation of a radioactive disaccharide-linked lipid (Table 1), which was identical to ManNAc-GlcNAc-PP-undecaprenol in its chromatographic behavior. However, when UDP-ManNAc was replaced by UDP-glucose in the reaction with these membranes, no appreciable amount of radioactive disaccharide-linked lipid was produced. In contrast, membranes from *B. coagulans* (group B) catalyzed the conversion of [^{14}C]GlcNAc-PP-undecaprenol to Glc-[^{14}C]GlcNAc-PP-undecaprenol in the presence of UDP-glucose. In this reaction, UDP-ManNAc could not substitute for UDP-glucose.

The above results are consistent with the presence of ManNAc-β(1→4)GlcNAc and Glc-β(1→4)GlcNAc as the linkage saccharides of teichoic acids in the cell walls of group A (8, 11–15) and group B (9, 10, 16) strains, respectively.

Exchangeability of various lipids in reactions for formation of Gro-P-containing lipids and polymer. Plausible pathways for the biosynthesis of ribitol teichoic acid and poly(galactosylglycerol phosphate) in membrane systems from *B. subtilis* W23 and *B. coagulans* AHU 1366 have previously been proposed (34, 35). Therefore, the above systems were used as the representatives of the membrane systems of the groups A and B strains for determination of efficiency of several glycolipids as intermediates in the linkage unit formation. [^{14}C]GlcNAc-PP-dolichol, just as [^{14}C]GlcNAc-PP-undecaprenol, was efficiently converted into disaccharide-linked lipid, ManNAc-[^{14}C]GlcNAc-PP-dolichol or Glc-[^{14}C]GlcNAc-PP-dolichol, by incubation with membranes of *B. subtilis* W23 and UDP-ManNAc or with membranes of *B. coagulans* AHU 1366 and UDP-glucose. Thus, the structure of the lipid moiety had little influence on the formation of disaccharide-linked lipids from GlcNAc-linked lipids. Previously, dolichylphosphate and ficaprenylphosphate were reported to be exchangeable for each other as glycosyl acceptors in several glycosyl transfer reactions (6, 17).

Furthermore, six kinds of radioactive disaccharide-linked lipids prepared as described in Materials and Methods were tested for the ability to serve as Gro-P acceptors. In the membrane system of *B. subtilis* W23, ManNAc-GlcNAc-PP-dolichol, Glc-GlcNAc-PP-undecaprenol, and Glc-GlcNAc-PP-dolichol, just as ManNAc-GlcNAc-PP-undecaprenol, were converted to Gro-P-containing lipid intermediates (Table 2). In the presence of CDP-glycerol and CDP-ribitol, the radioactivity from these four glycolipids was incorporated into polymer in the membrane system of *B. subtilis* W23. However, GlcNAc-GlcNAc-PP-undecaprenol hardly reacted, and GlcNAc-GlcNAc-PP-dolichol did not react at all.

In the membrane system of *B. coagulans* AHU 1366, ManNAc-GlcNAc-PP-undecaprenol and Glc-GlcNAc-PP-dolichol as well as Glc-GlcNAc-PP-undecaprenol were converted to Gro-P-containing lipid intermediates and polymer (Table 2). ManNAc-GlcNAc-PP-dolichol and GlcNAc-GlcNAc-PP-undecaprenol could only partly substitute for Glc-GlcNAc-PP-undecaprenol as a Gro-P acceptor in this membrane system, while GlcNAc-GlcNAc-PP-dolichol could not serve as a Gro-P acceptor.

TABLE 2. Relative abilities of various disaccharide-linked lipids to serve as the acceptor in the formation of Gro-P-containing lipids and polymer[a]

Acceptor disaccharide-linked lipid	Formation in *B. subtilis* W23 of:		Formation in *B. coagulans* AHU 1366 of:	
	Gro-P-containing lipids[b]	Polymer[c]	Gro-P-containing lipids[b]	Polymer[d]
ManNAc-[^{14}C]GlcNAc-PP-undecaprenol	1.00	1.00	0.90	1.00
ManNAc-[^{14}C]GlcNAc-PP-dolichol	0.97	0.95	0.51	0.45
Glc-[^{14}C]GlcNAc-PP-undecaprenol	0.97	0.80	1.00	1.00
Glc-[^{14}C]GlcNAc-PP-dolichol	0.96	0.70	0.95	0.96
GlcNAc-[^{14}C]GlcNAc-PP-undecaprenol	0.10	0.09	0.49	0.46
GlcNAc-[^{14}C]GlcNAc-PP-dolichol	0	0	0	0

[a] Incubation of each membrane preparation with various types of disaccharide-linked lipids was carried out under standard conditions. The incorporation of the radioactivity into Gro-P-containing lipids and polymer was measured. The values show relative incorporation of the radioactivity recovered in the respective products and were calculated by taking values for the reaction products from ManNAc-GlcNAc-PP-undecaprenol (in the *B. subtilis* W23 system) and Glc-GlcNAc-PP-undecaprenol (in the *B. coagulans* AHU 1366 system) as 1.00.
[b] Radioactivity of Gro-P-containing intermediates produced in the absence of CDP-ribitol or UDP-galactose.
[c] Radioactivity of polymer produced in the presence of CDP-ribitol.
[d] Radioactivity of polymer produced in the presence of UDP-galactose.

Conversion of disaccharide-linked lipids into different Gro-P-containing lipid intermediates and polymer in membranes of various strains. To study in more detail the formation of Gro-P-containing intermediates and polymer, ManNAc-[^{14}C]GlcNAc-PP-undecaprenol and Glc-[^{14}C]GlcNAc-PP-undecaprenol were separately incubated with membranes from 13 bacterial strains in the presence of CDP-glycerol and the conversion of the labeled lipids into polymer and lipid intermediates containing one (G1), two (G2), three (G3), and four to eight (G4 to G8) Gro-P units was measured. In some experiments, CDP-ribitol, UDP-galactose, or UDP-GlcNAc was added to support the conversion of Gro-P-containing intermediates to polymers characteristic of the strains.

Incubation of Glc-[^{14}C]GlcNAc-PP-undecaprenol with CDP-glycerol and membranes from group B strains, as well as incubation of ManNAc-[^{14}C]GlcNAc-PP-undecaprenol with CDP-glycerol and membranes from group A strains, yielded a series of radioactive Gro-P-containing lipid intermediates and also radioactive polymer (Table 3). The major Gro-P-containing lipid intermediates, synthesized from disaccharide-linked lipids and CDP-glycerol in the membrane systems of *L. plantarum* AHU 1413, *S. aureus* (three strains), and *B. coagulans* (three strains), possessed unique numbers of Gro-P units, namely 1, 1 to 3, and 1 to 4. The numbers are roughly correlated with the Gro-P unit numbers in the linkage units, namely, 1, 3, and 2 (or 3), reported for the ribitol teichoic acid of *L. plantarum* AHU 1413 (14), that of *S. aureus* H (13) (also of strains 209P, D. Gale, and Copenhagen [unpublished data]), and the poly(galactosyl-glycerol phosphate) of *B. coagulans* AHU 1366 (9), respectively.

In the membrane systems of *L. plantarum* AHU 1413 and three *S. aureus* strains, the radioactivity from ManNAc-[^{14}C]GlcNAc-PP-undecaprenol was incorporated into polymers by incubation with CDP-glycerol and CDP-ribitol, indicating that the ribitol teichoic acid chains were probably

TABLE 3. Conversion of disaccharide-linked lipids to Gro-P-containing lipids

Membrane source	Addition	Conversion of[a]:									
		[^{14}C]GlcNAc-PP-undecaprenol	ManNAc-[^{14}C]GlcNAc-PP-undecaprenol					Glc-[^{14}C]GlcNAc-PP-undecaprenol				
		G1–G3	G1	G2	G3	G4–G8	Polymer	G1	G2	G3	G4–G8	Polymer
Group A												
L. plantarum AHU 1413	None	0	1,840	0	0	0	0	1,760	0	0	0	0
	CDP-ribitol	ND[b]	1,530	140	50	0	170	1,700	0	0	0	0
S. aureus 209P[c]	None	0	100	1,490	190	0	0	430	1,340	0	0	0
	CDP-ribitol	ND	50	100	20	0	1,660	430	500	0	0	820
B. subtilis AHU 1035	None[d]	0	80	140	120	200	1,080	1,550	40	90	20	140
B. subtilis AHU 1037	None[d]	0	130	150	130	190	1,290	1,610	0	0	40	200
B. subtilis AHU 1235	None	0	60	90	120	170	1,160	1,700	30	0	0	100
B. subtilis AHU 1392	None	0	60	70	110	160	630	1,470	0	0	0	200
B. licheniformis AHU 1371	None	0	1,050	400	180	180	100	1,930	30	0	0	0
	UDP-galactose	ND	830	340	170	220	290	1,900	40	0	0	0
B. pumilus AHU 1650	None[e]	0	450	110	130	400	680	1,800	20	0	0	0
Group B												
B. coagulans AHU 1631[f]	None	0	110	80	390	1,210	0	210	40	40	1,260	20
	UDP-galactose	ND	90	70	150	420	1,090	230	40	270	100	1,040

[a] The indicated, radioactive saccharide-linked lipids were individually incubated with membranes and CDP-glycerol. Data are shown in counts per minute after subtraction of control values. G1, G2, G3, and G4–G8 represent lipid intermediates which contained one, two, three, and four to eight Gro-P units, respectively.
[b] ND, Not determined.
[c] Similar results were obtained with membranes from *S. aureus* D. Gale and Copenhagen.
[d] Addition of UDP-galactose caused no change in the reaction products.
[e] Addition to UDP-GlcNAc caused no change in the reaction products.
[f] Similar results were obtained with membranes from *B. coagulans* AHU 1634 and AHU 1638.

synthesized on Gro-P-containing lipid intermediates in the same fashion as that in the previously described *S. aureus* H and *B. subtilis* W23 systems (35). In the *L. plantarum* system, the level of the Gro-P-containing lipid intermediate with one Gro-P unit decreased with the polymer formation, while in the *S. aureus* systems, the level of the Gro-P-containing lipid intermediate with two and three Gro-P units mainly decreased. On the other hand, incubation of Glc-[^{14}C]GlcNAc-PP-undecaprenol with membranes of three *B. coagulans* strains in the presence of UDP-galactose led to polymer formation accompanied by a decrease in the amounts of lipid intermediates which contained three or more Gro-P units, indicating that the poly(galactosylglycerol phosphate) chains were synthesized on these Gro-P-containing lipid intermediates in the same fashion as that in the *B. coagulans* AHU 1366 system (34).

In contrast, in the membrane systems of four *B. subtilis* strains and *B. licheniformis* AHU 1371, large amounts of radioactivity were incorporated into polymer without supplementation of sugar nucleotides other than CDP-glycerol, suggesting that glycerol teichoic acids were formed under these conditions. Because the major teichoic acids in the cell walls of *B. subtilis* AHU 1235 and AHU 1392 have been reported to be α- and β-glucosylated glycerol teichoic acids (11), their backbone chains are probably synthesized on ManNAc-GlcNAc-PP-undecaprenol by successive transfer of Gro-P units from CDP-glycerol. On the other hand, the backbone chains of the major teichoic acids of *B. subtilis* AHU 1035 and AHU 1037 and *B. licheniformis* AHU 1371 have been reported to contain galactosylglycerol phosphate units as their repeating units (11). However, the addition of UDP-galactose caused no significant increase in the formation of radioactive polymer, and it seems that a substantial amount of poly(galactosylglycerol phosphate) chain could not be formed in these membrane systems.

In the membrane system of *B. pumilus* AHU 1650, lipid intermediates with four or more Gro-P units were formed from ManNAc-GlcNAc-PP-undecaprenol. However, the addition of UDP-GlcNAc, which is a possible precursor in the synthesis of the backbone chain (3), did not cause any increase in the polymer formation, suggesting that glycerol teichoic acid rather than poly(*N*-acetylglucosamine 1-phosphate) was preferentially synthesized under these conditions.

In contrast to the above finding that disaccharide-linked lipids served as the Gro-P acceptor in every membrane system tested, incubation of [^{14}C]GlcNAc-PP-undecaprenol or [^{14}C]GlcNAc-PP-dolichol with membranes from newly tested bacterial strains as well as from *B. subtilis* W23 and *B. coagulans* AHU 1366 did not give radioactive Gro-P-containing lipids or polymer in any appreciable amount, suggesting the inability of GlcNAc-linked lipids to accept Gro-P units from CDP-glycerol.

Effect of exchange between ManNAc-GlcNAc-PP-lipid and Glc-GlcNAc-PP-lipid on linkage unit formation. In the membrane systems of three *S. aureus* strains, Glc-GlcNAc-PP-undecaprenol served as a Gro-P acceptor at the same efficiency as did ManNAc-GlcNAc-PP-undecaprenol (Table 3). Moreover, in the presence of CDP-ribitol, radioactive polymer was formed from Glc-[^{14}C]GlcNAc-PP-undecaprenol. Similar results were obtained with the three *B. coagulans* systems; namely, ManNAc-GlcNAc-PP-undecaprenol could fully substitute for Glc-GlcNAc-PP-undecaprenol in the synthesis of Gro-P-containing lipid intermediates and polymer. However, in the membrane systems of *L. plantarum* AHU 1413, four *B. subtilis* strains, *B. licheniformis* AHU 1371, and *B. pumilus* AHU 1650, the replacement of ManNAc-GlcNAc-PP-undecaprenol with Glc-GlcNAc-PP-undecaprenol caused a great increase in the level of the lipid intermediate with one Gro-P unit and a marked decrease in the formation of the lipid intermediates with two or more Gro-P units, as well as in the polymer formation.

DISCUSSION

The results described above, together with data reported previously (26, 34, 35), lead to the conclusion that in almost all gram-positive bacteria, teichoic acids are synthesized through an essentially common pathway that includes three stages, the formation of linkage-disaccharide-bound lipid, the transfer of Gro-P units to this glycolipid, and the addition of different repeating units to the Gro-P-containing lipid intermediates. All the strains tested except for *B. coagulans* were shown to possess the enzymes required for the synthesis of ManNAc-β(1→4)GlcNAc-PP-polyprenol. *B. coagulans* strains are defective in UDP-GlcNAc 2-epimerase and *N*-acetylmannosaminyltransferase (34, 36). Therefore, it seems most likely that during evolution *B. coagulans* gained the ability to synthesize the Glc-β(1→4)GlcNAc type of linkage disaccharide unit in place of the common linkage disaccharide unit, ManNAc-β(1→4)GlcNAc.

It has been well documented that saccharide-linked pyrophosphorylpolyprenols serve as essential intermediates in the biosynthesis of various bacterial polysaccharides and *N*-glycosidically linked saccharide chains of glycoproteins (18, 19, 25, 37). Various phosphorylated polyprenols different in chain length, saturation of double bonds, and *cis/trans* ratio have been reported to serve as carriers of reducing terminal saccharide residues, and the difference in the lipid moieties seems to influence their functions (19). On the other hand, subsequent glycosylation reactions seem to be dependent on the sugar moieties of sugar-bound lipid intermediates. The sugar moieties of the teichoic acid precursors, ManNAc-β(1→4)GlcNAc-PP-polyprenol and Glc-β(1→4)GlcNAc-PP-polyprenol, are different from the glycoprotein oligosaccharide intermediate, GlcNAc-β(1→4)GlcNAc-PP-dolichol, only in the substitution at C-2 of the distal sugar residues. The availability of these three types of disaccharides linked to pyrophosphorylundecaprenol and pyrophosphoryldolichol permitted us to examine the substrate specificities of the enzymes participating in the second and third stages of the teichoic acid synthesis. Each of the membrane systems from group A (*B. subtilis* W23) and group B strains (*B. coagulans* AHU 1366) was able to utilize both ManNAc-GlcNAc-linked and Glc-GlcNAc-linked pyrophosphoryldolichols as well as the corresponding pyrophosphorylundecaprenol derivatives for the polymer synthesis (Table 2). GlcNAc-GlcNAc-PP-undecaprenol could partially substitute for the above glycolipids in these membrane systems, but GlcNAc-GlcNAc-PP-dolichol could not at all. Therefore, it is concluded that the enzymes involved in the teichoic acid synthesis in these two membrane systems have broad specificities for the disaccharide-linked lipid intermediates with respect to the substitution at C-2 of their distal sugar residues and also with respect to the chain length and saturation at the terminal double bonds of their lipid moieties.

In more detailed experiments (Table 3), it was revealed that the exchange of ManNAc-GlcNAc-PP-undecaprenol and Glc-GlcNAc-PP-undecaprenol had a distinct influence on the formation of Gro-P-containing lipid intermediates in some bacterial species. Particularly in the membrane sys-

tems of *L. plantarum* AHU 1413, four *B. subtilis* strains, *B. licheniformis* AHU 1650, and *B. pumilus* AHU 1371, the use of Glc-GlcNAc-PP-undecaprenol led to a great decrease in the polymer formation accompanied by an accumulation of Gro-P-Glc-GlcNAc-PP-undecaprenol. In the membrane systems of *S. aureus* and *B. coagulans*, however, the exchange of the disaccharide-linked lipid intermediates had virtually no or a small influence on the formation of Gro-P-containing lipid intermediates and polymer. The above results are most likely explained by assuming the presence of two or more kinds of glycerophosphotransferases. The presumable glycerophosphotransferase which catalyzes the transfer of the first Gro-P unit seems to be common to various bacterial species and reactive to both of the acceptor disaccharide-linked lipids. In contrast, the enzymes catalyzing the transfer of the second Gro-P unit and the subsequent steps may be different in amounts and substrate specificity depending on the species of bacteria. This assumption also explains the formation of lipid intermediates with distinct numbers of Gro-P units in the membranes of the respective bacterial species.

In the membrane systems of *B. coagulans*, poly(galactosylglycerol phosphate) chains seem to be formed by the alternate addition of galactose and glycerol phosphate residues from UDP-galactose and CDP-glycerol to growing chains of intermediates (34; Table 3). However, the formation of poly(galactosylglycerol phosphate) could not be demonstrated in the membrane system of *B. licheniformis* AHU 1371 or *B. subtilis* AHU 1035 or AHU 1037. Therefore, it seems possible that in these species, the polymer chains are synthesized through repeating-unit-bound lipid intermediates similar to those reported previously by Baddiley and his co-workers (2, 5).

In none of the membrane systems tested, could GlcNAc-PP-undecaprenol serve as a Gro-P unit acceptor (Table 3). Thus, it is unlikely that the monosaccharide type of linkage unit is involved in the attachment of teichoic acids to peptidoglycan in gram-positive bacteria.

Further investigation involving solubilization and separation of enzymes participating in the formation of the linkage units and polymer chains are in progress.

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