Formation and Function of N-Acetylglucosaminyl-linked Phosphoryl-
and PyrophosphorylUndecaprenols in Membranes from Bacillus cereus*

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Membranes from Bacillus cereus AHU 1356 incorporated radioactivity from UDP-N-acetyl[14C]glucosamine into three alkaline-stable and acid-labile lipids which were extracted into chloroform:methanol (2:1) and separated from each other by thin layer chromatography on silica gel plates. The major labeled lipid (Lipid 1) and a minor one (Lipid 2) were identified as N-acetylglucosaminyl phosphorylUndecaprenol from several analytical criteria involving mass spectral data and from reversal of their formation by UDP. These two lipids appear to differ in geometry of their polyprenol moieties. The third labeled lipid (Lipid 3) was identified as N-acetylglucosaminyl pyrophosphorylUndecaprenol. Antibiotic 24010, a tunicamycin-like antibiotic, at 1 μg/ml was found to inhibit almost completely the formation of Lipid 3, whereas it inhibited the formation of Lipid 1 at much more weakly and rather enhanced the formation of Lipid 2. Radioactivity was also incorporated into a polymer from UDP-GlcNAc and from Lipid 3, UDP-N-acetylmannosamine, UDP-N-acetylgalactosamine, and UDP-glucose supported the incorporation. Antibiotic 24010 strongly inhibited the incorporation of radioactivity from UDP-GlcNAc into polymer, whereas it did not affect the incorporation from Lipid 3. Thus, it is concluded that N-acetylglucosaminyl pyrophosphorylUndecaprenol serves as a precursor in the synthesis of a polymer presumed as the cell wall polysaccharide of this bacterial strain.

It has been well documented that saccharide-linked pyrophosphorylpolyprenols serve as intermediates in the biosynthesis of polysaccharides and glycoproteins. N-Acetyl-d-glucosaminyl pyrophosphorylpolichol and N-acetyl-d-glucosaminyl pyrophosphorylUndecaprenol have been shown to participate in the biosynthesis of glycoproteins (1-5) and teichoic acids (6), respectively. In addition, phosphorylpolyprenols linked to monosaccharides such as mannose (1, 7, 8), glucose (9-11), galactose (12), and N-acetylmuramic acid (13) were shown to function as donors of the sugar residues in various organisms.

In the course of studies on enzymatic synthesis of peptidoglycan in Bacillus cereus, we found incorporation of N-acetylglucosamine from UDP-GlcNAc into lipid compounds. Since this reaction occurred in the absence of exogenous UDP-N-acetylmuramyl-pentapeptide, the lipid compounds were supposed to be related to synthesis of some polymers other than peptidoglycan. B. cereus AHU 1356 has a cell wall polysaccharide composed of repeating heptasaccharide units which contain glucosamine, galactosamine, mannosamine, and glucose in a molar ratio of 4:1:1:1 (14). Therefore, we examined the possibility that the GlcNAc-linked lipids may participate in the biosynthesis of the cell wall polysaccharide in this strain.

This paper reports the isolation and characterization of three GlcNAc-linked lipids obtained from incubation of B. cereus membranes with UDP-GlcNAc. One of the isolated lipids was identified as N-acetylglucosaminyl pyrophosphorylUndecaprenol, while the second one appeared to differ from the first one in the polyprenol moiety. The third lipid was identified as N-acetylglucosaminyl pyrophosphorylUndecaprenol. The functions of these lipids in the biosynthesis of the cell wall polysaccharide and the effect of antibiotic 24010, a tunicamycin-like antibiotic from a strain of Streptomyces, on the formation of the lipids and polysaccharide are also described.

EXPERIMENTAL PROCEDURES

RESULTS

Incorporation of Radioactivity from UDP-[14C]GlcNAc into Lipids and Polymer—When the membrane fraction from B. cereus AHU 1356 was incubated with low concentrations (0.01 to 0.1 mM) of UDP-GlcNAc labeled in either the acetyl or the glucosamine moiety, the radioactivity was rapidly incorporated into material soluble in chloroform:methanol (2:1), but only negligible amounts of radioactivity were incorporated into the polymer fraction. When incubation was carried out with UDP-[14C]GlcNAc at higher concentrations (0.2 to 0.6 mM), considerable amounts of radioactivity were also incorporated into the polymer fraction (Fig. 1).

Chromatography of GlcNAc-linked Lipids—The chloroform:methanol-soluble fraction obtained from incubation with 0.01 mM UDP-[14C]GlcNAc gave three labeled lipids, Lipids 1 to 3, upon chromatography on silica gel plates in Solvent F (Fig. 2A). The Rf values of Lipids 1, 2, and 3, respectively, were: 0.49, 0.43, 0.11 in Solvent F; 0.45, 0.45, 0.37 in Solvent G. Lipid 1 was the major product accounting for approximately 70% of the radioactivity in these lipids, while Lipids 2 and 3 accounted for 13% and 15%, respectively. On the other hand, the chloroform:methanol-soluble fraction, obtained from incubation with higher concentrations (0.2 to 0.6 mM) of UDP-GlcNAc, was found to give, along with the spots of Lipids 1 to 3, additional labeled spots at Rf of 0.05 and at the origin of thin layer chromatograms (Fig. 2B). The labeled material with Rf of 0.05 was referred to as the oligosaccharide-lipid.

* Portions of this paper (including “Experimental Procedures,” Figs. 1 to 12, Tables I to III, and References) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 76M-286, cite author(s), and include a check or money order for $3.90 per set of photocopies.
and that at the origin as the larger oligosaccharide-lipids (see below). When labeled Lipids 1 to 3 separated by thin layer chromatography were individually subjected to chromatography on a DEAE-cellulose (acetate form) column. Lipids 1 and 2 were eluted at 50 to 70 mM ammonium acetate, where glycosyl phospholipids were known to be eluted (36). On the other hand, Lipid 3 was eluted in about 180 mM ammonium acetate, suggesting that this lipid has a pyrophosphoryl linkage (36). On Sephadex LH-20 column chromatography in 99% methanol containing 0.1 mM ammonium acetate, each of these lipids was eluted at the position expected for a lipid with a molecular weight of about 1000 to 1100. A reference sample, C<sub>9</sub>M-dolichol (molecular weight of 1108) was eluted in the same fractions.

**Mild Alkaline and Mild Acid Hydrolysis of Labeled Lipids**—Labeled Lipids 1 to 3 were stable to mild alkaline hydrolysis under the conditions (0.1 N NaOH in 90% ethanol, 75°C) sufficient to cause cleavage of fatty acid esters (37), while the three lipids were all decomposed by 0.1 N HCl in chloroform:methanol (2:1) at 20°C (Fig. 4). From the kinetics of the release of the labeled moiety (50% release in 10 to 15 min), the sugar in each lipid appears to be glycosidically bound to the lipid moiety through a phosphate or a pyrophosphate bridge. The labeled products, recovered from the aqueous phase after hydrolysis of the lipids with 0.01 N HCl in 25% 1-propanol at 100°C for 15 min, were coincident with authentic N-acetylglucosamine on paper chromatography (Solvents A to D), paper electrophoresis (Buffers A and B), and Bio-Gel P-2 column chromatography. In addition, on hydrolysis in 4 N HCl at 100°C for 4 h, the labeled lipids gave only glucosamine as a labeled product detectable on paper chromatograms in several solvents.

**Properties of Enzyme System Catalyzing Transfer of N-Acetylglucosamine to Lipids**—Fig. 5 shows the time course of incorporation of radioactivity into the lipids from UDP-GlcN<sup>[14C]</sup>Ac or [1-<sup>14</sup>P]UDP-GlcNAc. In contrast to the parallel incorporation of the sugar residues and phosphorus into Lipid 3, no phosphorus was incorporated into Lipid 1 or Lipid 2. As shown in Fig. 6, the formation of Lipids 1 and 2 appears to be reversed by the addition of UDP but not UMP, whereas the Lipid 3 formation was reversed by UMP. The formation of labeled UDP-GlcNAc during incubation of GlcN<sup>[14C]</sup>Ac-linked Lipid 1 or Lipid 2 with UDP was also demonstrated (Fig. 7). In contrast, UMP appears to be more effective in the formation of UDP-GlcNAc from Lipid 3. These results are consistent, on the one hand, with transfer of N-acetylglucosamine from UDP-GlcNAc to polysaccharidephosphate in the formation of Lipids 1 and 2 and, on the other hand, with transfer of GlcNAc-1-phosphate in the formation of Lipid 3. These transferase activities required bivalent cations. The optimum concentration of Mg<sup>2+</sup> was 40 mM for the formation of each of Lipids 1 to 3. The pH optima for the formation of Lipids 1, 2, and 3 were 7.6, 8.2, and 7.9, respectively. In the presence of 0.1% Nonidet P-40, the formation of the three lipids increased about 60%.

**Analysis of Isolated GlcNAc-linked Lipids**—Further characterization of the GlcNAc-linked lipids was carried out with the material isolated in the large scale preparation. Each preparation of Lipids 1 to 3 obtained as described under "Experimental Procedures" gave single, overlapping spots of radioactivity, phosphate, and iodine staining on the thin layer chromatograms in Solvents F and F<sub>1</sub>. Hydrolysis of the three lipids in 4 N HCl at 100°C for 4 h yielded a single ninhydrin-positive product which was coincident with glucosamine and distinguishable from galactosamine, mannosamine, or muramic acid in an amino acid analyzer (14). Analysis of Lipids 1, 2, and 3 revealed molar ratios of phosphorus to glucosamine (based on the measurement in an amino acid analyzer) of 0.95, 0.83, and 2.31, respectively. As shown in Fig. 8, the mass spectra of the lipid components from Lipids 1 to 3 gave the fragmentation pattern of undecaprenol with a weak molecular ion (M<sup>+</sup> = 706) and a stronger ion corresponding to the molecular ion less water (M<sup>+</sup> = 18 = 748). In each spectrum, a sequential pattern with peaks regularly distributed 68 mass unit apart (38).

The nuclear magnetic resonance of the lipid moieties of purified Lipids 1 and 2 showed a pattern of peaks characteristic of isoprenols (38); the peaks for the internal repeating isoprenyl unit at 4.94, 7.96, 7.98, 8.28, 8.34, and 8.42 <T>h</T> the analysis of the lipid moiety from Lipid 1 showed that the ratio of methyl protons (8.28 and 8.34 <T>h</T> in the cis-configuration versus methyl protons (8.42 <T>h</T> in the trans-configuration was 3:1. These data in conjunction with the mass spectral data indicate that the compound contains nine methyl groups in the cis-configuration and three methyl groups in the trans-configuration. On the other hand, the analysis of the lipid portion from Lipid 2 showed a ratio of methyl protons in cis:trans-configuration close to 2:1 rather than 3:1, although a precise value of this ratio could not be determined because of the small amount available for the analysis.

When chromatographed on silica gel plates in Solvent H, the petroleum ether-soluble fraction, obtained from acid hydrolysis of each of Lipids 1 to 3 in 0.01 N HCl at 100°C for 10 min, gave spots of a hydrocarbon and tertiary and primary alcohols at the positions reported by Higashi et al. (38) for hydrolysates of the undecaprenol derivatives from S. aureus and M. lysodeikticus. In this experiment, Lipid 2 was found to yield a much greater proportion of the tertiary alcohols as compared with the cases of the other two glycolipids. C<sub>9</sub>M-faciprenol with the ratio of cis- to trans-methyl protons of 2:1 was reported to run just behind the undecaprenol from S. aureus in reversed-phase thin layer chromatography on a Kieselguhr G plate (38), while a similar procedure did not effect appreciable separation between the primary alcohols obtained from Lipids 1 to 3.

From the above results, it is concluded that Lipids 1 and 2 were both N-acetylglucosaminyl phosphorylunodecaprenol, and that Lipid 3 was N-acetylglucosaminyl pyrophosphorylunodecaprenol. Lipid 2 seems to differ from Lipid 1 probably in the arrangement or the proportion of cis- and trans-isoprene units in the prenol moiety. However, it seemed also possible that Lipids 1 and 2 might differ in the anomeric configuration of the N-acetylglucosamine residues linked to the lipid moieties through the phosphate groups.

**Anomeric Configuration of GlcNAc-linked Lipids**—In order to gain information about the anomeric configuration of the three GlcNAc-linked lipids, water-soluble components released from these lipids by the treatment at pH 4.5 for 1 h at 100°C were analyzed. As shown in Fig. 9, paper chromatography of the hydrolysates of GlcN<sup>[14C]</sup>Ac-linked Lipids 1 and 2 yielded, in addition to labeled N-acetylglucosamine, a labeled product (Compound 1) with the same mobility as that of GlcNAc-1-phosphate. Under the same conditions, Lipid 3 gave another product (Compound 2) besides N-acetylglucosamine and Compound 1. Compound 1 was in agreement with GlcNAc-1-phosphate in its chromatographic and electrophoretic properties and its sensitivity to acid hydrolysis and alkaline phosphatase digestion. Compound 2 was provisionally characterized as GlcNAc-1-pyrophosphate, described by Brooks et al. (39). This compound showed a greater mobility than authentic GlcNAc-1-phosphate in paper electrophoresis in Buffer B. The treatment of this compound either in 0.01 N HCl (100°C, 15 min) or with alkaline phosphatase led to complete liberation of N-acetylglucosamine. This result fur-
ther supports the structure of Lipid 3 with its N-acetylglucosamine moiety linked via a pyrophosphate bridge to the prenol moiety.

When labeled GlcN~1-phosphate obtained from each of the labeled lipids was incubated with excess of UTP and UDP-N-acetylglucosamine pyrophosphorylase, all the radioactivity was recovered as a compound coincident with UDP-GlcNAc on paper chromatograms. This indicates that the N-acetylglucosamine residues were linked to the phosphate groups with α-configuration in the GlcNAc-1-phosphate fragments and probably in the corresponding moieties of Lipids 1 to 3.

Synthesis of GlcNAc-linked Lipids in Membranes from Various Bacteria—The possible occurrence of the activity for the formation of GlcNAc-linked lipids was investigated in the membrane preparations from various bacterial strains. As shown in Table I, the activity of Lipid 3 synthesis was detected in the membranes from most strains examined. On the other hand, marked formation of Lipids 1 and 2 was observed only with preparations from three B. cereus strains.

Analysis of Labeled Components in Oligosaccharide-lipid and Polymer—The labeled polymer and oligosaccharide-lipids produced in the membrane system from B. cereus AHU 1356 were analyzed for labeled sugar constituents after hydrolysis in 4 N HCl at 100°C for 4 h. As described in Table II, the polymer fraction from incubation with 0.6 mM UDP-[14C]GlcNAc and 0.2 mM UDP-[14C]glucose contained labeled glucosamine, galactosamine, mannosamine, and glucose residues in a molar ratio of about 2:1:1:1. However, if incubation was carried out with UDP-[14C]glucose alone as substrate, there was no incorporation of glucose into the polymer (data not shown). When 0.6 mM UDP-[14C]GlcNAc alone was used as substrate, the radioactivity incorporated into the polymer was recovered as glucosamine, galactosamine, and mannosamine in a ratio of 2:1:1. From this result, it appears that the labeled polymer formation observed is related to the process of the cell wall polysaccharide synthesis, involving the 2- and 4-epimerization of glucosamine residues.

The radioactive galactosamine and mannosamine residues were also found in the chloroform:methanol-soluble fraction obtained from incubation with 0.6 mM UDP-[14C]GlcNAc. Analysis of the oligosaccharide-lipid (Rf of 0.05) and the larger oligosaccharide-lipids (origin), separated from the chloroform:methanol-soluble fraction by thin layer chromatography (Fig. 2B), gave approximate ratios of glucosamine:galactosamine:mannosamine of 1:1:1 and 2:1:1, respectively (Table II). When the former lipid was hydrolyzed in 0.01 N HCl at 100°C for 15 min, the radioactivity was completely released as water-soluble material. On chromatography with a Bio-Gel P-2 column, the water-soluble product emerged as a single peak of radioactivity at the position corresponding to a trisaccharide (Fig. 10). Therefore, it is most likely that the labeled material with Rf of 0.05 on the chromatogram in Fig. 2B was a lipid compound linked through an acid-labile bond to a trisaccharide composed of the three hexosamines.

Chromatography of Solubilized Polymer—The labeled polymer, obtained from incubation of the B. cereus membranes with 0.6 mM UDP-[14C]GlcNAc and 0.2 mM UDP-glucose, was solubilized by water extraction repeated 3 times at 100°C for 15 min, and subjected to chromatography on a Sephadex G-200 column as shown in Fig. 11. The labeled material was eluted in two peaks, the major one emerging at the void volume of the column and the minor one (molecular weight, about 4.9 × 106) near the position of the T 40 marker. Incubation without UDP-glucose gave similar material which appeared to have a smaller molecular weight.

Effects of Antibiotic 24010 on the Synthesis of Lipids and Polymer—It has been reported that the formation of GlcNAC-linked polypropenol is preferentially inhibited by tunicamycin (40-43). Antibiotic 24010, which was found in Streptomyces No. 24010 and partially characterized by Mizuno et al. (22), closely resembles tunicamycin in chemical, physical, and biological properties. In this experiment, effects of antibiotic 24010 on the synthesis of the labeled lipids and polymer were studied in the membrane system from B. cereus AHU 1356. As shown in Fig. 12, the antibiotic at a concentration of 1 μg/ml almost completely inhibited the incorporation of radioactivity from 0.6 mM UDP-GlcN[14C]Ac not only into Lipid 3 but also into the oligosaccharide-lipid and polymer, while the same antibiotic at this concentration inhibited the Lipid 1 formation only partly (about 10%). A complete inhibition of the Lipid 1 formation required an antibiotic concentration of 500 μg/ml. In contrast, this drug rather enhanced the Lipid 2 formation. This result strongly suggests that the polymer synthesis involved the formation of Lipid 3 and probably of the oligosaccharide-lipid as obligatory intermediate steps, although this does not necessarily exclude the possibility that Lipids 1 and 2 might participate in the polymer synthesis. The formation of the GlcNAc-linked lipids in the membranes from other bacteria was also affected by antibiotic 24010 as in the membranes of B. cereus AHU 1356. Thus, the Lipid 3 formation in every membrane system shown in Table I was completely inhibited by this antibiotic at less than 10 μg/ml. In addition, as indicated in Fig. 6, antibiotic 24010 affects the reversal of the GlcNAc-linked lipid synthesis in the manner characteristic of each reaction.

Incorporation of Radioactivity from GlcNAc-linked Lipids into Polymer—When Lipid 3 labeled in the N-acetylglucosamine moiety was incubated with the membranes in the presence of UDP-GalNAc and UDP-ManNAc, the radioactivity was incorporated into the polymer, whereas there was no incorporation from labeled Lipid 1 or labeled Lipid 2 (Table III, Experiment I). The incorporation of radioactivity was enhanced by UDP-GlcNAc and UDP-glucose. It is noted that the transfer of radioactivity from Lipid 3 to the polymer was not influenced by antibiotic 24010. On the other hand, incorporation of radioactivity from UDP-GlcN[14C]Ac into the polymer, observed in the presence of UDP-GlcNAc and UDP-ManNAc, was completely inhibited by the antibiotic (Table III, Experiment II). This evidence excludes the possibility that the incorporation of radioactivity observed in Experiment I might be through intermediate formation of UDP-GlcNAc from labeled Lipid 3. Thus, the above results are consistent with the function of Lipid 3 as an intermediate in the biosynthesis of the cell wall polysaccharide of B. cereus.

DISCUSSION

The results described above indicate that the membranes from B. cereus catalyze the transfer of N-acetylglucosamine and N-acetylmuramylpentapeptide to prepolylipids. The major product, Lipid 1, was confirmed to be N-acetylglucosamine phosphoryldecaprenol by analysis of the purified preparation. The prenol from this glycolipid had a ratio of cis- to trans-double bonds of 3:1. The same structural feature was reported by Higashi et al. (38) and Scher et al. (44) for C50-isoprenoids which function in the synthesis of N-acetylglucosaminyl(pentapeptide)-P-pentol in S. aureus and of a mannosyl lipid in M. lysodeikticus. One of the minor products, Lipid 3, was identified as N-acetylglycosamine pyrophosphoryldecaprenol. Although this structure remains to be confirmed by nuclear magnetic resonance studies, the prenol moiety of Lipid 3 may be identical with that of Lipid 1, since the petroleum ether-soluble products obtained from Lipid 3 after the mild acid treatment were coincident...
with those from Lipid 1 as examined by reversed-phase thin layer chromatography and other chromatographic procedures. The other minor product, Lipid 2, was also characterized as an N-acetylglucosamine derivative of undecaprenyl phosphate. This lipid appears to differ from Lipid 1 in the geometrical structure of its prenol moiety. However, the possibility cannot be excluded that Lipid 2 had the same prenol moiety as Lipid 3. It is unlikely that Lipid 2 was an artifact derived from Lipid 1 during the assay procedure, because when each of the isolated preparatives of labeled Lipids 1 and 2 was separated again by thin layer chromatography after incubation in the standard reaction mixture with the boiled membranes and unlabeled UDP-GlcNAc, no appreciable interconversion of the labeled lipids was detected (data not shown).

In recent studies on the structure of the cell wall polysaccharide of B. cereus AHU 1356, Amano et al. (14) indicated that the polysaccharide has a backbone chain, composed of glucosamine, mannosamine, and galactosamine residues in a ratio of 1:1:1, and branches containing glucose and three-quarters of the total glucosamine residues. The finding of the labeled galactosamine and mannose residues in the polymer fraction obtained from incubation of the membranes with UDP-$[^{14}C]GlcNAc$ suggests that the membrane preparation catalyzes 2- and 4-epimerization of N-acetylgalactosamine probably in the state linked to UDP. In E. coli, Kawanura et al. have found UDP-N-acetylglucosamine 2-epimerase which catalyzes interconversion of UDP-GlcNAc and UDP-ManNAc (18). This enzyme was also detected together with UDP-N-acetylglucosamine 4-epimerase in the supernatant and membrane fractions of B. cereus AHU 1356 (45). The 2-epimerase is allosterically activated by UDP-GlcNAc, exhibiting only negligible activity at UDP-GlcNAc concentrations lower than about 0.1 mM (45). Thus, the failure of synthesizing the polymer at low UDP-GlcNAc concentrations seems to be accounted for by the lack of UDP-ManNAc supply. When UDP-ManNAc was added to the incubation mixture, incorporation of radioactivity was observed even at low concentrations of UDP-$[^{14}C]GlcNAc$ (Table III, Experiment II). The labeled polymer synthesis under these conditions was markedly stimulated by the addition of UDP-GalNAc. In addition, the incorporation of radioactivity from UDP-$[^{14}C]GlcNAc$-linked Lipid 3 into the polymer required both UDP-ManNAc and UDP-GalNAc (Table III, Experiment 1). Therefore, these nucleotides are believed to serve as donors of the N-acetylmannosamine and N-acetylgalactosamine residues in the polysaccharide synthesis.

The data in Table I and Fig. 11 suggest that glucose was incorporated into the polymer fraction to constitute the polysaccharide together with the three N-acetyhexosamines. However, since a polymer comprising the three N-acetylhexosamines could be synthesized even in the absence of UDP-glucose, it seems likely that the in vitro synthesis of the backbone chain of this polysaccharide, just as the synthesis of Salmonella typhimurium O-antigen polysaccharide, can proceed without prior formation of the complete repeating unit structure (46). The oligosaccharide-lipid, tentatively characterized as a trisaccharide-linked lipid, seems to function as an intermediate in the polysaccharide synthesis, because its saccharide moiety coincided in composition with the repeating unit of the polysaccharide backbone, and because its formation was inhibited by the antibiotic just as was the polysaccharide formation. The labeled polymer synthesis from UDP-$[^{14}C]GlcNAc$ linked Lipid 3 was stimulated by the addition of either UDP-glucose or UDP-GlcNAc (Table III, Experiment 1). This may be accounted for on the assumption that lipid-linked higher oligosaccharides which contain glucose and extra N-acetylgalactosamine residues are involved in the polysaccharide synthesis, serving as better substrates than the trisaccharide-linked lipid in the polymerization of the repeating units.

This is the first report on the isolation and identification of GlcNAc-linked polyisoprenyl monophosphates. Recently, Rohr et al. (20) indicated that a GlcNAc-linked lipid functions as an intermediate in the biosynthesis of teichuronic acid in M. lysodeikticus. This glycolipid has been presumed to have a phosphoryl linkage rather than a pyrophosphoryl linkage because of reversal of its formation by UDP. However, we could not detect the formation of Lipid 1 or Lipid 2 in a membrane preparation from the same bacteria (Table I). It is of interest to examine whether these lipids function in the teichuronic acid synthesis. The function of Lipids 1 and 2 is unknown in B. cereus. Whereas the native cell wall polysaccharide of B. cereus AHU 1356 contains 4.3 glucosamine residues per mannose residue, the enzymatically synthesized polymer contained only about 2 glucosamine residues per mannose residue. This discrepancy suggests that in the present incubation system, enzymes responsible for the formation of the N-acetylgalactosamine-linked lateral branches might not be working well. In addition, marked synthesis of Lipide 1 and 2 was detected only in membrane obtained from such strains of B. cereus that contain cell wall polysaccharides composed mainly of N-acetylhexosamines (24). Therefore, there is a possibility that these lipids may participate in the polysaccharide synthesis at the stage of branch formation following polymerization. The data on Lipid 3 formation in B. subtilis membranes (Table I) are consistent with the recent report on the formation of pyrophosphorylindividuals linked with N-acetylgalactosamine and its oligomers (47), but the significance of the Lipid 3 synthesis in the other bacteria remains obscure.

Tunicamycin (48, 49) has proved to be a useful diagnostic tool for examining involvement of a GlcNAc-linked (pyro)phosphorylisoprenol as an intermediate in a biosynthetic pathway (40-43). The present data indicate that antibiotics of this sort may also inhibit somewhat the formation of a GlcNAc-linked phosphorylisoprenol and may even stimulate the formation of some other GlcNAc-linked phosphoryl isoprenol. The marked difference in the sensitivity to the antibiotics may permit us to distinguish between pathways involving GlcNAc-linked phosphoryl lipids as intermediates and those involving GlcNAc-linked pyrophosphoryl lipids.

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REFERENCES

Rohr, 1 49 appear on p. 6522.
Table I

| Bacteria | Control (nmol/100 mg protein) | GlcNAc-P-125I (nmol/100 mg protein) | Specific radioactivity (cpm/nmol) |
|----------|------------------------------|-------------------------------------|-------------------------------|
| E. coli  | 0.00 871 1301 2090           | 0.00 420 420 420                  | 1500                           |
| L. casei | 0.00 871 1301 2090           | 0.00 420 420 420                  | 1500                           |
| B. cereus| 0.00 871 1301 2090           | 0.00 420 420 420                  | 1500                           |

Table II

| 21-23C | 24-26C | 27-29C | 30-32C | 33-35C | 36-38C | 39-41C | 42-44C | 45-47C | 48-50C |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| GlcNAc-P-125I | GlcNAc-P-P-125I | GlcNAc-P-125I | GlcNAc-P-P-125I | GlcNAc-P-125I | GlcNAc-P-P-125I | GlcNAc-P-125I | GlcNAc-P-P-125I | GlcNAc-P-125I | GlcNAc-P-P-125I |
| 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |

Fig. 1. Effects of antibiotics on incorporation of GlcNAc-P-125I and GlcNAc-P-P-125I into lipopolysaccharide. The antibiotic concentrations were adjusted to 100 μg/ml. The results are expressed as means ± SD. (1) GlcNAc-P-125I; (2) GlcNAc-P-P-125I; (3) control. The differences were significant at *p < 0.05. 

Fig. 2. Effects of antibiotics on sulfation of GlcNAc-P-125I and GlcNAc-P-P-125I into lipopolysaccharide. The antibiotic concentrations were adjusted to 100 μg/ml. The results are expressed as means ± SD. (1) GlcNAc-P-125I; (2) GlcNAc-P-P-125I; (3) control. The differences were significant at *p < 0.05.
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