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BIOSYNTHESIS OF GLYCEROL TEICHOIC ACID IN BACILLUS CEREUS: FORMATION OF LINKAGE UNIT DISACCHARIDE ON A LIPID INTERMEDIATE

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Summary: A tunicamycin-like antibiotic 24010 at a concentration of 1 μg/ml selectively inhibited the in vivo synthesis of glycerol teichoic acid of cell walls in Bacillus cereus AHU 1030. Incubation of membranes of this strain with N-acetylglucosaminyl pyrophosphorylundecaprenol and UDP-N-acetylmannosamine led to formation of a glycolipid having a saccharide moiety identical with the cell wall teichoic acid linkage unit, N-acetylmannosaminylβ(1→4)-N-acetylglucosamine. The membranes also catalyzed transfer of glycerol phosphate units from CDP-glycerol to this disaccharide-linked lipid. Thus the biosynthesis of the cell wall glycerol teichoic acid in this strain seems to involve the disaccharide-linked lipid as an intermediate.

Some bacterial wall polymers, such as poly(ribitol phosphate) teichoic acid, poly(GlcNAc-1-phosphate), and teichuronic acid, have been found to be attached to peptidoglycan through specialyzed linkage units (1,2). It has also been reported that the linkage units are synthesized from nucleotide sugars via lipid-linked intermediates (3-5). Our recent structural study on cell walls of Bacillus cereus AHU 1030 indicated that ManNAcβ(1→4)GlcNAc is contained in the cell walls as a linkage unit between glucosylated poly(glycerol phosphate) teichoic acid and peptidoglycan (6). This paper reports the enzymatic formation of disaccharide-linked lipid presumed as an intermediate in the biosynthesis of cell wall glycerol teichoic acid.

MATERIALS AND METHODS

Microorganism---The culture conditions of Bacillus cereus AHU 1030 were the same as in a previous paper (7), and cell growth was monitored turbidimetrically at 570 nm. The absorbance was about 4.0 for the equilibrated cultures.

Abbreviations: GlcNAc, N-acetylglucosamine; ManNAc, N-acetylmannosamine; GalNAc, N-acetylgalactosamine; GlcUA, glucuronic acid.

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Preparation of Saccharide-Linked Lipids---The procedures for preparation of the membrane fraction from B. cereus AHU 1030 cells and incubation of this fraction with UDP-[^{14}C]GlcNAc followed by separation of labeled glycolipids were essentially the same as those described previously (8). Only N-acetylglucosaminyl pyrophosphorylundecaprenol was yielded as the labeled GlcNAc-linked lipid. For preparation of disaccharide-linked lipid, the purified GlcNAc-linked lipid (150,000 cpm, 6 nmol) was incubated with the membrane fraction (8 mg of protein) at 25°C for 30 min in a reaction mixture containing 50 mM Tris-HCl, pH 8.2, 30 mM $MgCl_2$, 0.3% Nonidet P-40, and 0.12 mM UDP-ManNAc in a final volume of 0.5 ml. Lipids extracted from the reaction mixture with chloroform/methanol (2:1) were subjected to thin layer chromatography on silica gel G in chloroform/methanol/water (65:25:4). The radioactive product with mobility lower than that of the substrate lipid was extracted and used as the disaccharide-linked lipid (30,000 cpm, 1.2 nmol). To prepare glycerol-linked lipids, a reaction mixture, containing 50 mM Tris-HCl, pH 8.2, 30 mM $MgCl_2$, disaccharide-linked lipid labeled in N-acetylglucosamine (12,000 cpm, 0.48 nmol, dissolved in 15 μl of 2% Nonidet P-40), 0.1 mM CDP-glycerol, and the membrane fraction (2 mg of protein) in a final volume of 0.1 ml, was incubated at 25°C for 30 min, and labeled lipids were then extracted and separated by thin layer chromatography as described above. The residue resulting from the chloroform/methanol extraction of the reaction mixture was brought to dryness and extracted twice with 1 ml of 1 M NH_4OH. The NH_4OH extracts pooled were subjected to paper chromatography in isobutyric acid/0.5 M NH_4OH (5:3). The origin of the paper was cut out and counted for radioactivity to measure the polymer formation.

Smith Degradation---Smith degradation was performed according to Spiro (9). The disaccharide (labeled in N-acetylglucosamine, 2,800 cpm, 0.11 nmol), obtained from the disaccharide-linked lipid by hydrolysis in 0.01 N HCl at 100°C for 15 min, was reduced in 2 ml of 0.2 M $NaBH_4$ at pH 8.2 for 16 h at 0°C in the presence of 500 nmol each of GlcNAcβ(1→4)-GlcNAc and GlcUAβ(1→3)-GlcNAc added as carriers. After desalting, the product was oxidized with 35 mM $NaIO_4$ in 1.5 ml of 35 mM sodium acetate, pH 4.5, for 12 h at 4°C, then reduced with $NaBH_4$ as described above, and hydrolyzed in 4 N HCl at 100°C for 4 h. The resulting amino polyols were separated by chromatography on an Aminex A-10 column (0.3 x 100 cm) in 0.35 M sodium citrate buffer, pH 5.4, and detected by the ninhydrin reaction and by the measurement of radioactivity.

Preparation of Cell Walls from Antibiotic-Inhibited Cells---B. cereus cells grown in 2 liter of a culture medium were collected at the early exponential phase (absorbance, 1.0) and resuspended in 1 liter of a fresh medium. Portions of the cell suspension (250 ml) were incubated in the absence and presence of antibiotic 24010 at concentrations of 0.2, 1, and 10 μg/ml. After 2 h, the cells were harvested, and cell walls were prepared as described previously (7).

Other Materials and Methods---Unless otherwise indicated, analytical methods and materials were the same as those in previous papers (6-8). Labeled and unlabeled UDP-ManNAc were prepared as described by Kawamura et al. (10). GlcNAcβ(1→4)GlcNAc and GlcUAβ(1→3)GlcNAc were prepared from chitin and hyaluronic acid, respectively. CDP-[^{14}C]glycerol was prepared using crude enzyme of B. cereus AHU 1030 according to the method of Shaw (11). Antibiotic 24010 was supplied by Dr. M. Mizuno, Asahi Chemical Industry Co., Nobeoka, Japan (12). UDP-[^{14}C]GlcNAc, [^{14}C]glucosamine, and [^{14}C]lysine purchased from Radiochemical Centre Amersham, [^{14}C]glycerol 3-phosphate from New England Nuclear, hyaluronic acid from Nakarai Chemicals, and hyaluronidase (type I) and CDP-glycerol from Sigma Chemical Corp. were used.

Table 1. Composition of Cell Walls from Antibiotic-Treated Cells. Cell walls were prepared from antibiotic-treated and control cells as described in Materials and Methods. The amounts of cell walls were 26.7 mg and 25.8 mg for cells treated with antibiotic 24010 at 0.2 μg/ml and 1.0 μg/ml, respectively, as compared to the value 32.7 mg for control cells. Total muramic acid is the sum of muramic acid and muramic acid 6-phosphate.

Component	Content in cell walls (nmol/mg)		
	Control cells	Cells treated with antibiotic	
		0.2 μg/ml	1.0 μg/ml
Phosphorus	1880	1067	940
Glycerol	1780	1050	908
Glucose	1280	785	653
Mannosamine	35	21	20
Muramic acid 6-phosphate	36	48	57
Total muramic acid	399	559	587
Glucosamine	568	659	690
Alanine	660	819	913
Glutamic acid	392	532	598
Diaminopimelic acid	404	538	595

RESULTS AND DISCUSSION

Influence of Antibiotic 24010---The biosynthetic pathways involving GlcNAc-linked pyrophosphoryl polyprenols as precursors are known to be inhibited by the antibiotics of tunicamycin group. The synthesis of bacterial cell walls is also inhibited by tunicamycin with a somewhat lower sensitivity (13,14). When a tunicamycin-like antibiotic, 24010, was added at a concentration of 10 μg/ml to an exponentially growing culture of B. cereus AHU 1030, the cell growth was completely stopped, and in some cases cell lysis was observed. On the other hand, lower doses (0.2 or 1 μg/ml) of this drug caused only a small reduction of growth (10% or 31%). Under the influence of the antibiotic at these low concentrations, as shown in Table 1, the contents in cell walls of the teichoic acid components, namely glycerol, glucose, and phosphorus, as well as the content of mannosamine, which is a component of the linkage unit between teichoic acid and peptidoglycan, were decreased by about 50%, whereas the contents of the peptidoglycan components were rather increased. This result, in conjunction with our previous data concerning the effect of this antibiotic on the enzymatic formation of GlcNAc-pyrophosphorylundecaprenol (8), strongly suggests that the formation of this glycolipid is a key step in the

Table 2. Effects of Nucleotide Sugars on the Conversion of GlcNAc-Linked Lipid

[^{14}C]GlcNAc-pyrophosphorylundecaprenol (4,500 cpm, 0.15 nmol) was incubated with the membrane fraction (1 mg of protein) at 25°C for 30 min in a reaction mixture containing 50 mM Tris-HCl, pH 8.2, 20 mM $MgCl_2$, 0.5% Nonidet P-40, and the indicated nucleotide sugar (20 μM UDP-sugar and/or 0.1 mM CDP-glycerol), in a final volume of 100 μl. The lipids were extracted and separated into disaccharide-linked lipid and glycerol phosphate-containing lipids by thin layer chromatography. The NH_4OH extract of the residue resulting from the lipid-extaction was subjected to paper chromatography in isobutyric acid/0.5 M NH_4OH (5:3), and radioactivity at the origin of the paper was counted to measure the incorporation into polymer.

Nucleotide sugars	Radioactivity (cpm)		
	Disaccharide-linked lipid	Glycerol phosphate-linked lipids	Polymer
None	20	10	10
UDP-ManNAc	930	30	10
UDP-GlcNAc	40	10	10
UDP-GalNAc	60	10	10
UDP-glucose	10	10	10
CDP-glycerol	10	50	20
UDP-ManNAc, CDP-glycerol	280	230	150

biosynthesis of glycerol teichoic acid. The above suggestion is supported by the result of a study on the in vitro formation of the teichoic acid intermediates from this glycolipid.

Formation of Disaccharide-Linked Lipid---Incubation of [^{14}C]GlcNAc-pyrophosphorylundecaprenol with UDP-ManNAc and the membrane fraction of B. cereus resulted in formation of another saccharide-linked lipid which showed lower mobility (R_f = 0.27) than the substrate lipid (R_f = 0.37) upon thin layer chromatography on silica gel G in chloroform/methanol/water (65:25:4). Based on the data from paper chromatography and Sephadex G-25 column chromatography of a water-soluble fragment released from the reaction product by mild acid hydrolysis (0.01 N HCl, 100°C, 15 min), it was suggested that in this enzyme reaction the GlcNAc-linked lipid was converted to a disaccharide-linked lipid. As shown in Table 2, UDP-GlcNAc, UDP-GalNAc, UDP-glucose, or CDP-glycerol could not substitute for UDP-ManNAc in the enzymatic conversion of the labeled substrate lipid. On the other hand, incubation of [^{14}C]GlcNAc-linked lipid with both UDP-ManNAc and CDP-glycerol yielded radioactive products presumed as glycerol phosphate-linked lipids besides a polymer and the disaccharide-linked

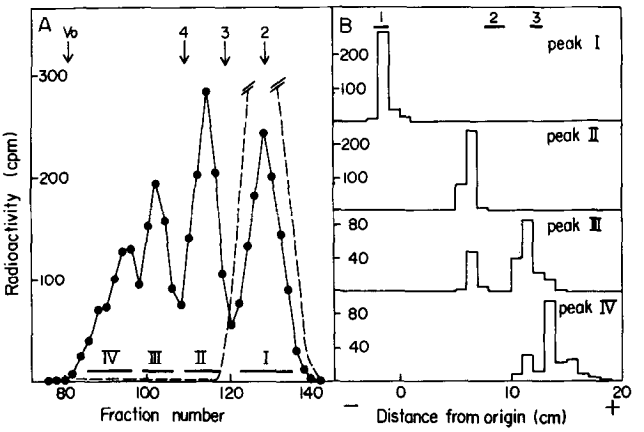

Fig. 1. Separation of Glycerol Phosphate-Containing Saccharides by Gel Filtration and Paper Electrophoresis.
A, the GlcNAc-labeled disaccharide-lipid was incubated with the membrane fraction in the presence (solid line) or absence (dashed line) of CDP-glycerol as described in Materials and Methods, and the lipids were extracted with chloroform/methanol (2:1) and hydrolyzed in 0.01 N HCl/25% n-propanol at 100°C for 15 min. After removal of lipid material by the extraction with chloroform/n-propanol (3:2), the saccharide fraction was subjected to gel filtration on a Sephadex G-25 column (1 x 75 cm) in 0.05 M ammonium bicarbonate. The four peaks, I to IV, were pooled as indicated. The arrows show the elution positions of standard saccharides: 2 to 4, dimer to tetramer of N-acetylglucosamine, Vo, blue dextran. B, labeled material in each peak from the gel filtration was subjected to paper electrophoresis at 40 V/cm for 90 min in pyridine/acetic acid/water (35:5:960, pH 5.8). Bars 1, 2, and 3 show the migration positions of N-acetylglucosamine, GlcNAc-1-phosphate, and glycerol 1-phosphate, respectively.

lipid. The glycolipid produced in the reaction of [^{14}C]GlcNAc-linked lipid with UDP-[^{14}C]ManNAc gave an equimolar amount of labeled glucosamine and labeled mannosamine on strong acid hydrolysis (6 N HCl, 100°C, 4 h). The disaccharide released from the glycolipid by mild acid hydrolysis gave an equimolar amount of glucosaminitol and mannosamine when hydrolyzed in strong acid after reduction with $NaBH_4$. The Smith degradation of the disaccharide, obtained from the lipid product labeled in the GlcNAc residue, yielded xylosaminitol as the major radioactive fragment, indicating that the reducing terminal GlcNAc residue in the disaccharide was substituted at C-4. In addition, the ManNAc residue in the disaccharide was shown to be completely destroyed by the chromic anhydride oxidation. These results indicate that the saccharide moiety in the lipid has the structure ManNAcβ(1→4)GlcNAc, which is identical with

the structure of the linkage unit between glycerol teichoic acid and peptidoglycan.

Formation of Glycerol Phosphate-Containing Lipids---When the labeled disaccharide-lipid was incubated with the membrane fraction in the presence of CDP-glycerol, radioactive lipids were formed which migrated with lower mobility (R_f=0-0.05) than the disaccharide-linked lipid on thin layer chromatography. On gel filtration, mild acid hydrolysates of the lipid fraction from the reaction mixture gave three peaks (peaks II-IV) of radioactive materials besides the peak of the disaccharide (peak I) (Fig. 1A). The radioactive materials in peaks II, III, and IV seem to possess one, two, and three or more of negatively charged groups, respectively, on the basis of their electrophoretic mobility (Fig. 1B). The labeled lipid products, prepared using the labeled disaccharide-linked lipid and CDP-[^{14}C]glycerol as substrates, were also analyzed after mild acid hydrolysis followed by gel filtration. On the treatment with 0.5 M NaOH at 37°C for 30 min (6), the labeled material in peak II gave an equimolar amount of glycerol phosphate and the disaccharide, while those in peaks III and IV gave compounds tentatively characterized as the dimer and oligomers of glycerol phosphate, together with the same disaccharide. The membranes also catalyzed the incorporation of radioactivity from the labeled disaccharide-linked lipid and CDP-[^{14}C]glycerol into the polymer fraction. Therefore, it seems most probable that in this enzyme system the glycerol phosphate units are successively transferred from CDP-glycerol to the disaccharide-linked lipid to form oligo- and poly(glycerol phosphate) chains which are bound to the disaccharide moiety of the lipid. Further studies on the enzymatic process of the teichoic acid synthesis and on the precise structure of the intermediates are in progress.

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