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Phytanyl-Pyrophosphate-Linked Substrate for a Bacterial α-Mannosyltransferase

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The biochemical characterization of bacterial glycosyltransferases involved in the assembly of cell-wall-associated polysaccharides is often hindered by the lack of the appropriate undecaprenyl-pyrophosphate-linked acceptor substrate. In order to find a suitable synthetic substrate for the α1,3-mannosyltransferase AceA from *Acetobacter xylinum,* phytanyl-pyrophosphate-linked cellobiose was prepared. In the presence of GDP-[^{14}C]mannose and recombinant AceA, the phytanyl-pyrophosphate-linked cellobiose afforded a ^{14}C-labeled trisaccharide that was sensitive to α-mannosidase degradation in a fashion analogous to the natural undecaprenyl-pyrophosphate-linked cellobiose substrate. These results suggest that phytanyl-pyrophosphate-linked oligosaccharides may be useful substrates for other important bacterial glycosyltransferases.

Key Words: **mannosyltransferase; exopolysaccharide; undecaprenol.**

Many cell-surface-associated polysaccharides of gram-negative bacteria, such as peptidoglycan, exopolysaccharides, and the *O*-antigen portion of lipopolysaccharides, are assembled from an undecaprenyl-pyrophosphate-linked oligosaccharide intermediate (1). In most cases the oligosaccharide moiety is thought to be biosynthesized directly upon the undecaprenyl-pyrophosphate anchor by the action of specific glycosyltransferases. Recent studies of recombinant bacterial glycosyltransferases involved in exopolysaccharide biosynthesis have revealed that some of these enzymes will not transfer a sugar residue to a free oligosaccharide acceptor but rather appear to require the presence of some portion of the lipid-pyrophosphate anchor for transferase activity *in vitro* (2, 3). This substrate requirement makes the assay of such glycosyltransferases particularly difficult because the natural bacterial substrates exist in minute quantities and are laborious to isolate, while synthetic approaches to undecaprenyl-pyrophosphate-linked oligosaccharides have been hampered by the limited availability and high cost of undecaprenol-like polyisoprene analogues.

One recombinant bacterial glycosyltransferase of recent interest is the α1,3 mannosyltransferase AceA from *Acetobacter xylinum*. AceA catalyzes the transfer of mannose from GDP-mannose to an undecaprenyl-pyrophosphate-linked cellobiose acceptor during the assembly of the lipid-linked heptasaccharide intermediate of acetan (see Fig. 1) (4). AceA is similar to a number of other important bacterial mannosyltransferases notably WbaU, WbaW, and WbaZ, which are involved in *Salmonella O*-antigen biosynthesis, and GumH, which is involved in *Xanthamonas campestris* xanthan gum biosynthesis (5). We have previously shown that recombinant AceA is active with a native lipid-linked substrate, but will not transfer mannose from GDP mannose to free cellobiose (3). Here we report that synthetic phytanyl-pyrophosphate-linked cellobiose functions as an acceptor for the AceA mannosyltransferase reaction *in vitro.* Phytanyl-pyrophosphate has previously been shown to be an efficient substitute for dolichyl-pyrophosphate in the preparation of synthetic substrates of the yeast β1,4 mannosyltransferase, ALG1, that is involved in the early steps of *N*-glycan assembly (6–8). As phytanyl-phosphate can be readily prepared on a gram scale from the relatively inexpensive precursor phytol, this finding may provide a reasonable basis for the production of synthetic substrates or inhibitors for many types of important bacterial glycosyltransferases.

MATERIALS AND METHODS

General materials and methods. All chemicals used were reagent grade and purchased from Sigma-Aldrich or Fluka (L'isle d'Abeau Chesnes, France). AceA extracts were prepared from *E. coli* strain BL21(DE3) harbouring pCrGR2 (3) grown at 37°C in Terrific Broth

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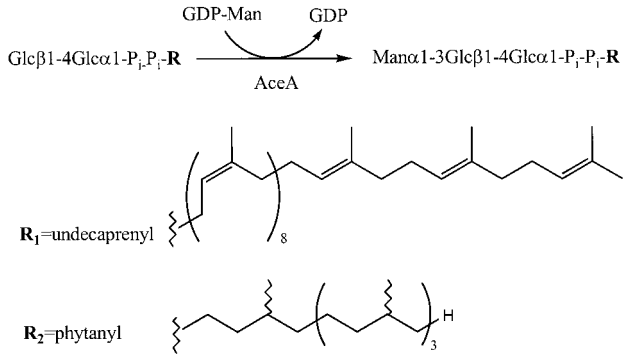

FIG. 1. Reaction catalyzed by AceA.

in the presence of kanamycin, 30 μg/mL. HPLC purification was performed with Waters equipment incorporating a Phenomenex Sphereclone semipreparative 5 μ ODS column. GDP-[U^{14}C]mannose was from Amersham-Pharmacia Biotech (Les Ulis, France). NMR spectra were obtained using a Varian INOVA 600 MHz spectrometer.

Preparation of P$_1$-Phytanyl-P$_2$-[β-D-glucopyranosyl-(1-4)-α-D-glucopyranosyl]-pyrophosphate. In a 50 mL round bottom flask, heptaacetyl-cellobiosyl-α-1-phosphate (132 mg, 0.13 mmol) (prepared essentially as described in (9)) was dissolved in anhydrous dimethylformamide (2 mL) under an argon atmosphere. *N,N′*-Carbonyldiimidazole (110 mg, 0.67 mmol) in DMF (1.5 mL) was transferred to the reaction flask and the reaction stirred for 4.5 h at room temperature. Meanwhile, phytanyl phosphate *t*-butyl ester (84 mg, 0.17 mmol) (prepared as described in (6)) was treated with trifluoracetic acid (4 mL) and stirred at room temperature for 10 min. The TFA was removed by evaporation to give phytanyl phosphate, which was taken up in anhydrous dichloromethane (2 mL) and transferred to the flask containing the heptaacetyl cellobiose-α-1-phosphate-imidazole conjugate. The coupling reaction was left to stir at room temperature for 48 h, at which time TLC (65:35:8 chloroform/methanol/1 M ammonium hydrogen carbonate) revealed the appearance of a major new product. The solvent was subsequently removed under vacuum and the residue further dried by repeated (three times) evaporation from toluene.

Reverse-phase HPLC using eluents A, 10 mM aqueous triethylammonium acetate, and B, methanol, and a 25 min linear gradient from 30:70 (A/B) to 0:100 (A/B) gave P$_1$-phytanyl-P$_2$-[2,3,4,6,-tetra-*O*-acetyl-β-D-glucopyranosyl-(1-4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl]-pyrophosphate (48 mg) in 34% yield; electrospray MS 1075.5 [M+H+] ^{1}H NMR (600 MHz) (1:1 $CDCl_3$:CD_3OD): δ 0.74–1.42 (phytanyl), 1.72, 1.76, 1.78, 1.81, 1.82, 1.84, 1.89 (7s, 21H, 7x-$COCH_3$), 3.48 (m, 1H, H5′), 3.59 (dd, 1H, H4), 3.70 (m, 2H, -OCH_{2-}), 3.80 (dd, 1H, H6a′), 3.90 (dd, 1H, H6a), 4.0 (d, 1H, H5), 4.16 (dd, 1H, H6b′), 4.31 (d, 1H, $J_{1,2}$ 8 Hz, H1′), 4.37 (dd, 1H, H6b), 4.60 (dd, 1H, H2), 4.62 (dd, 1H, H2′), 4.80 (dd, 1H, H4′), 4.91 (dd, 1H, H3′), 5.16 (dd, 1H, H3), 5.46 (d, 1H, $J_{1,2}$ 4 Hz, H1).

For deacetylation, typically, the foregoing compound (5 mg) of was converted to the triethylamine salt before being dissolved in anhydrous 4:1 dichloromethane/methanol (2.5 mL) under an atmosphere of argon, cooling to 4°C and addition of a freshly prepared 7% solution of NaOMe in methanol (50 μL). After 15 min the reaction was judged to be complete by TLC (65:35:8 chloroform:methanol:1 M ammonium hydrogen carbonate) and was applied to a 0.5 mL column of Dowex Ag50Wx8 (H^+ form) equilibrated in MeOH. The column was washed with MeOH (5 × 1.0 mL). The filtrate and washings were pooled, concentrated and co-evaporated three times from toluene and the product was subsequently reconverted into the triethylamine salt. ^{1}H NMR of the product in a 10:10:1 mixture of $CDCl_3$/CD_3OD/D_2O confirmed complete deacetylation; the product was used in the enzyme assay without further characterization or purification.

Preparation of AceA extracts. Cultures of the *E. coli* strain BL21(DE3) harboring the AceA containing expression vector, pCrGC2, were grown to an OD_{600} of 0.8, at which time IPTG was added to a final concentration of 5 μM. After 3 h at 37°C, cells were collected, rinsed once with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and passed twice through a French press. The lysed cells were spun at 100,000*g* for 1 h at 4°C and the supernatant was collected and used in the assay without further treatment.

Mannosyltransferase assay. The reaction mixture (100 μl) contained: crude AceA containing supernatant (total protein = 20 μg), 1% Triton X-100, 6 mM $MgCl_2$, 0.5 mM substrate, either phytanyl-pyrophosphate cellobiose (50 nmol) or the native cellobiose pyrophosphate lipid (extracted from *X. campestris* NRRL B-1459 permeabilized cells as described previously (3)), and 0.25 μCi GDP-[U^{14}C]-mannose (specific activity 301 Ci/mol). The reaction was carried out for 45 min at 37°C and stopped by the addition of water (100 μl) and of a 1:1 mixture of chloroform and methanol (200 μl). When using the synthetic substrate, the lipid-linked oligosaccharides contained in the organic phase typically contained radioactivity of 30,000 cpm. The oligosaccharide moiety was released from the lipid anchor as described previously (3), and where necessary, treated with jackbean α-mannosidase (0.2 U) in 0.1 M citrate buffer, pH 4.5 at 20°C for 20 h. The reaction products (5000–10,000 cpm in 5–10 μl) were supplemented with malto-oligosaccharides (50 μg), and analyzed by TLC (silica gel 60, 0.25 mm Sigma, run twice in 50:20:20 1-propanol: nitromethane:water) using mannose, cellobiose, maltose and malto-oligosaccharides as standards. The standards were revealed by charring after treatment with 5% H_2SO_4 in ethanol. Radioactivity was detected by exposure of the TLC plate to Kodak Biomax MS film using a Kodak Biomax Transcreen LE amplifying screen.

RESULTS AND DISCUSSION

The phytanyl-pyrophosphate linked cellobiose was obtained by coupling phytanyl phosphate to fully acetylated cellobiose–α-1-phosphate using carbonyldiimidazole as the coupling agent to form the pyrophosphate linkage as outlined in Fig. 2. The desired product was isolated by HPLC and deprotected just prior to use by

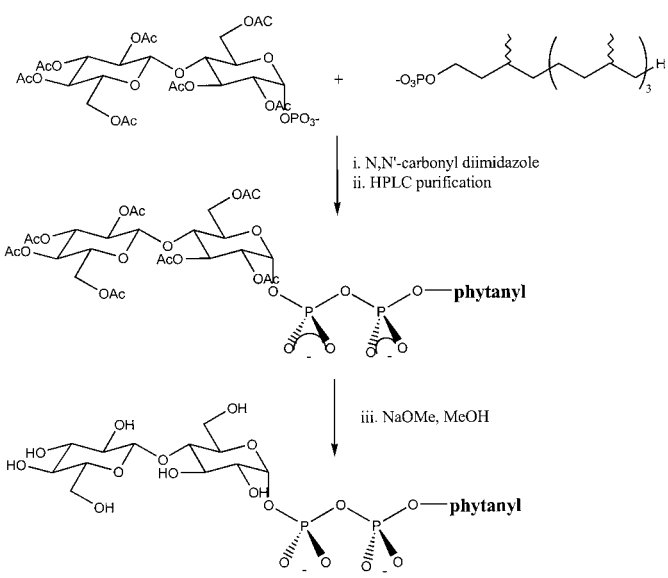

FIG. 2. Essential steps in the preparation of phytanyl-pyrophosphate-linked cellobiose.

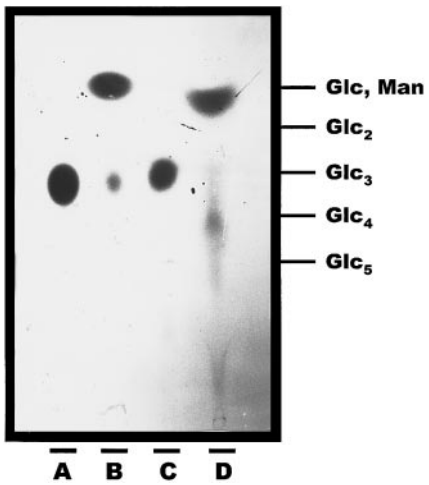

FIG. 3. Thin-layer chromatography of oligosaccharides released from the lipid anchor after the AceA reaction with GDP-[^{14}C]mannose. Lanes A and B: Using *X. campestris*-derived undecaprenyl-pyrophosphate cellobiose acceptor. Lanes C and D: Using phytanyl-pyrophosphate cellobiose acceptor. Lanes B and D: Released oligosaccharide after treatment with α-mannosidase. Standards: glucose, mannose, cellobiose, and oligomaltose.

saponification with sodium methoxide. We subsequently showed that the α1,3-mannosyltransferase AceA, will efficiently transfer ^{14}C-labeled mannose from GDP-[U^{14}C]-mannose to the synthetic acceptor phytanyl-cellobiosyl pyrophosphate. Cleavage of the lipid–pyrophosphate (3) and analysis by TLC of the released oligosaccharides of the transferase reaction reveals the formation of a trisaccharide that comigrates with the natural reaction product, Manα(1,3)-Glcβ(1,4)-Glc (Fig. 3, lanes A and C). α-Mannosidase treatment resulted in the release of ^{14}C-labeled mannose, which strongly suggests the formation of an α-glycosidic linkage (Fig. 3, lanes B and D).

The phytanyl portion of the synthetic structure reported here mimics a polyisoprene structure in its branched carbon backbone, but unlike the *cis–trans* polyisoprene structures undecaprenol or dolichol, this backbone is relatively short (C_{20}) and is completely saturated. Phytanol more closely mimics dolichol in that the first isoprene unit of dolichol is also saturated, while it is unsaturated in undecaprenol. Evidently, neither the chain length nor the saturation state, notably of the first isoprene unit, are critical to the AceA mannosyltransferase activity. However, the stereochemistry of the anomeric linkage or the presence of the phosphates may be critical, as AceA will not accept a cellobiosyl-β-1-butyl-*O*-nitrophenol derivative (R. A. Geremia, personal communication). Future studies employing other lipid anchor types will reveal whether AceA, like the yeast mannosyltransferase ALG1, requires a branched lipid or the presence of both the phosphate groups of the anchorage (7). The availability of the synthetic substrate facilitates the assay of AceA, and will thus greatly aid the purification of this enzyme as well as future kinetic studies. As phytanyl phosphate can be readily obtained, the synthesis of substrate analogs and inhibitors for mechanistic studies of AceA and other bacterial glycosyltransferases can now be realistically envisioned.

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