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Lipid-bound Saccharides in *Rhizobium meliloti**

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Marcelo E. Tolmasky‡, Roberto J. Staneloni§, and Luis F. Leloir

From the Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina

The lipid-bound saccharides formed by incubation of uridine diphosphate glucose with a particulate enzyme of *Rhizobium meliloti* were studied. They behaved like polyprenyl diphosphate saccharides when treated with ammonia or hot phenol, when catalytically hydrogenated, and on DEAE-cellulose chromatography. The saccharide moieties obtained after heating at pH 2 for 10 min at 100 °C were separated with a gel filtration column. The following compounds were detected: galactose, glucosyl β1-3 galactose (Tolmasky, M. E., Staneloni, R. J., Ugalde, R. A., and Leloir, L. F. (1980) *Arch. Biochem. Biophys.* 203, 358-364), and some octasaccharides (I). These were compared by paper electrophoresis, thin layer and paper chromatography with an octasaccharide obtained from *Alcaligenes faecalis* var. myxogenes strain 22 (II). Furthermore, Compounds I and II were compared with the exopolysaccharide of *Rhizobium meliloti* (III) by partial acid hydrolysis and methylation analysis. The results were consistent with the identity of the repeating unit of Compound III with Compounds I and II except for differences in the substituents (acetyl or succinyl). Studies on the labeling of the lipid-bound saccharides have shown that the sequence is: first, galactose and glucosyl β1-3 glactose, then the rest of glucose residues, and finally, the substituents (acetyl and pyruvic acid).

Studies on the extracellular polysaccharide of *Rhizobium meliloti* have shown that it contains: glucose, galactose, pyruvic acid, and *O*-acetyl groups in the proportion 7:1:1:1 (1). The polysaccharide contains the octasaccharide repeating unit shown in Fig. 1, with all the hexose residues β-pyranosidic and with *O*-acetyl groups linked to position 6 of some glucose residues (2). By methylation analysis and fragmentation with two β-D-glycanases, it was found that the exopolysaccharides from *R. meliloti*, *Alcaligenes faecalis* var. myxogenes, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* are identical except for the acetyl or succinyl substituents (3).

Lipid intermediates are known (4) to be involved in the biosynthesis of many polysaccharides in bacteria. In *R. meliloti*, it was found that addition of UDP-Glc led to the formation of lipid-bound galactose and glucosyl β1-3 galactose (5). Studies on the characterization of the different lipid-bound saccharides in *R. meliloti* have now been continued.

EXPERIMENTAL PROCEDURES

Materials—Dr. Tokuya Harada from Osaka University, Japan generously provided the succinoglycan octasaccharide isolated from *A. faecalis* var. myxogenes strain 22 (6). This octasaccharide is similar to the repeating unit of the exopolysaccharide of *R. meliloti* but is partially esterified with succinic acid. The compound was submitted to deacylation or deacylation and depyruvylation, as will be described below, and the products obtained were referred to as pyruvylated octasaccharide and octasaccharide, respectively. Undecaprenyl-P-[^{14}C]galactose (7) was a gift from Nora Iñón from this laboratory.

Enzyme Preparation—Cells from *R. meliloti* strain R41 were grown as described previously (5) and harvested by centrifugation at the late logarithmic phase, and the pellet was resuspended in 0.01 M EDTA-Tris buffer, pH 8, frozen and thawed several times (8).

Isolation of a Galactose-negative Mutant—Cells from *R. meliloti* R41 were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Miller (9). The cells were then grown in a mineral medium with 1% galactose and 2.5 μg/ml of ampicillin and then plated out in a Bacto-Antibiotic medium 2 (Difco) with 0.005% of 2,3,5-triphenyltetrazolium chloride and 1% galactose, as described by Ohlsson *et al.* (10). In this medium, the growing colonies reduce the colorless tetrazolium dye to red, and those colonies which ferment the sugars are white because the reduction of tetrazolium is inhibited at low pH. The red colonies were picked and plated again as mentioned above. The cells were then grown in a minimal medium with galactose or glucose as carbon source. The cells that grew in the glucose medium but did not grow in the galactose medium were isolated and the enzyme was prepared as described previously.

Assay Procedure—The incubation mixture contained: 70 mM Tris-HCl buffer, pH 8.2, 12 mM $MgCl_2$, 40 mM 2-mercaptoethanol, enzyme preparation (about 7 mg of protein), and 3×10^6 cpm of UDP-[^{14}C]Glc (268 Ci/mol). Final volume: 0.5 ml. The reaction mixture was incubated for 20 min at 10 °C. Methanol (1 ml), chloroform (1.5 ml), and 7 mg of liver microsomes as carrier were added. The mixture was centrifuged, the upper phase was discarded, the lower phase was washed with chloroform, methanol, 4 mM $MgCl_2$ (3:48:47) and will be referred to as lower phase. The interphase was partitioned twice with chloroform/methanol/water (3:2:1) and then washed three times with chloroform, methanol, 4 mM $MgCl_2$ (3:48:47). The residue was extracted twice with chloroform/methanol/water (1:1:0.3). This fraction (1103 extract) plus the lower phase is referred to as the organic solvent extract.

Preparation of the Radioactive Extracellular Polysaccharide—*R. meliloti* strain R41 was grown in yeast mannitol broth (500 ml) as described previously (5). When the culture reached the late-logarithmic phase, 100 μCi of [^{14}C]glucose (278 Ci/mol) were added. After 3

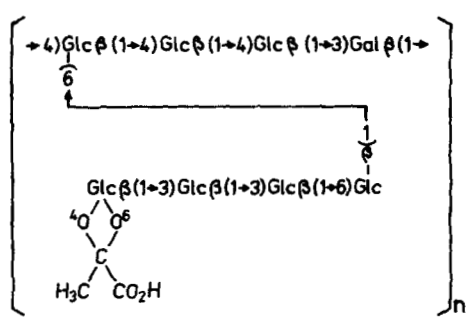

FIG. 1. **Structure of the octasaccharide repeating unit of *R. meliloti*, according to Jansson *et al.* (2).**

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‡ Fellow of the Consejo Nacional de Investigaciones Cientificas y Técnicas (R. Argentina).

§ Career Investigator of the Consejo Nacional de Investigaciones Cientificas y Técnicas (R. Argentina).

h at 28 °C, the culture was centrifuged at 3000 × *g* for 10 min. Carrier *R. meliloti* exopolysaccharide (150 mg) and 3 volumes of 1-propanol were added to the supernatant fluid, and the mixture was centrifuged at 3000 × *g* for 10 min. The precipitate was suspended in 1% KCl, 3 volumes of 1-propanol were added, and the mixture was centrifuged as above. The precipitation was repeated three times more and the final compound was used as radioactive extracellular polysaccharide of *R. meliloti*. It was excluded in a column of Bio-Gel A-50m. When treated with 1 N HCl for 4 h at 100 °C, only radioactive glucose and galactose were obtained.

Deacylation and Depyruvylation—The saccharides were deacylated by treatment with 10 mM potassium hydroxide for 5 h at 20 °C (11) and depyruvylated at pH 2 for 90 min at 100 °C (12).

Methylation of Saccharides—Methylation of radioactive oligosaccharides was carried out by the method of Hakomori (13). The methylated samples were hydrolyzed with 1 N HCl at 100 °C for 4 h, desalted on a column of Amberlite MB-3 (acetate form), and separated by thin layer chromatography on Silica Gel 60 (0.25 mm Merck) in solvent E as described by Li *et al.* (14).

Acid Hydrolysis—Mild acid hydrolysis was carried out at pH 2 for 10 min at 100 °C as described previously (15). For partial acid hydrolysis, the samples were heated at 100 °C for 1 h in 0.5 N HCl. Total acid hydrolysis was performed in 1 N HCl at 100 °C for 4 h.

Chromatography—Paper chromatography was performed with the following solvents: solvent A, 1-butanol/pyridine/water (6:4:3); solvent B, 1-butanol/pyridine/water (4:3:4); solvent C, nitromethane/acetic acid/ethanol/water saturated with boric acid (8:1:1:1). Radioactivity on paper chromatograms was detected with a scanner and the reducing compounds by the alkaline silver nitrate method (16).

Thin layer chromatography was carried out on Silica Gel 60 (0.25 mm Merck) with the following solvents: solvent D, 1-propanol/nitromethane/water (5:2:2); solvent E, acetone/benzene/ammonium hydroxide/water (200:50:1.35:1). Radioactive compounds were localized by radioautography and the saccharides with 5% concentrated sulfuric acid in ethanol as described by Li *et al.* (14).

Electrophoresis—Paper electrophoresis was carried out with solvent F, pyridine/acetic acid/water (1:0.04:9), pH 6.5, for 90 min at 40 V/cm, and solvent G, 0.05 M sodium borate buffer, pH 9.0, for 3 h at 20 V/cm.

RESULTS

Properties of the Substances Soluble in the Lower Phase and 1103 Extract—When the enzyme preparation was incubated with UDP-[^{14}C]Glc in the presence of Mg^{2+}, products were formed which, after fractionation as described under "Experimental Procedures" were recovered in the lower phase and 1103 extract. These products had properties similar to polyprenyl diphosphate saccharides. Their mobility in paper chromatography with solvent B was about the same (R_F about 0.8). Their behavior on chromatography on DEAE-cellulose with chloroform/methanol/water (1:1:0.3) as solvent and ammonium formate as eluent (17) was also similar. Thus, the radioactive substances present in the 1103 extract were eluted with 114 mM ammonium formate. The corresponding value for dolichyl diphosphate $(Glc)_3$ $(Man)_9$ $(GlcNAc)_2$ and dolichyl-P-Glc was 100 mM and 23 mM of ammonium formate, respectively.

Treatment at pH 2 for 10 min at 100 °C of the compounds soluble in the organic solvent extract led to decomposition, yielding radioactive water-soluble products. The products of the lower phase, after mild acid hydrolysis, chromatographed like galactose and glucose β1-3 glactose, and those of the 1103 extract gave several higher saccharides which will be described later.

Like unsaturated polyprenyl diphosphate derivatives, the substances found in the lower phase and 1103 extract were decomposed by treatment with concentrated ammonia at room temperature while dolichyl-P-Glc and undecaprenyl-P-Gal were not affected (Table I).

The polyprenyl phosphates are known to be decomposed when heated at 70 °C in 50% phenol. The half-life of undecaprenyl diphosphate sugars is 4 to 10 min and that of undecaprenyl monophosphate sugars, 60 min (8). Dolichyl monophosphate glucose (which has an α-saturated isoprene residue) is decomposed only 10% in 3 h (17). As shown in Table I, the radioactive substances of the lower phase and 1103 extract were degraded 75% and 77%, respectively, after a 30-min heating in phenol. A half-life value of 8 min was obtained for both substances, thus indicating that they are polyprenyl diphosphate derivatives.

TABLE I

Properties of the substances of the lower phase and 1103 extract

Substances	Decomposition, %		
	Alkaline treatment[a]	Phenol treatment[b]	Catalytic reduction[c]
Dolichyl-P-Glc	0	0	0
Undecaprenyl-P-Gal	0	—[d]	69
Lower phase	96	75	95
1103 extract	98	77	95

[a] Samples of the radioactive compounds were dried and 100 μl of concentrated ammonia were added. After 20 min at room temperature, the samples were dried, and 300 μl of water and butanol were added. The two phases were separated and then counted in a gas flow counter.

[b] Samples of the radioactive substances were dried, 60 μl of water and 100 μl of 80% phenol were added. After heating at 68 °C for 30 min, the samples were cooled, the two phases were separated and counted in a gas flow counter.

[c] Carried out with H_2 gas and platinum catalyst for 5 min at 30 °C as described by Pont-Lezica *et al.* (18).

[d] Not determined.

Unsaturated polyprenyl phosphates are more labile than the α-saturated derivatives toward catalytic hydrogenation. As shown in Table I, dolichyl-P-Glc was not degraded after 5 min of catalytic hydrogenation while the radioactive substances of the lower phase and 1103 extract were all degraded in 5 min. These data indicate that the substances in the lower phase and 1103 extract contain unsaturated polyprenol diphosphate saccharides.

Gel Filtration of the Lipid-bound Saccharides—Gel filtration on Sephadex G-75 in the presence of 0.5% sodium deoxycholate has been used to estimate the chain length of polyprenyl phosphate derivatives (17). The results of such an experiment are shown in Fig. 2. The compounds of the lower phase and 1103 extract were compared with some known compounds. Dolichyl diphosphate oligosaccharide which has the largest polyprenol and a 14-unit oligosaccharide appeared first, followed by dolichyl-P-Glc and later by undecaprenyl-P-Gal. The *Rhizobium* compounds were eluted in an intermediate position. Considering the molecular weight of the saccharide moieties, it may be concluded that the *Rhizobium* compounds behave as if they contained a polyprenol of about 12 isoprene residues.

Separation by Gel Filtration of the Water-soluble Moiety of the Lipid-bound Saccharides—As shown in Fig. 3 when the mixture of octasaccharide and pyruvylated octasaccharide from *A. faecalis* was run in a Bio-Gel column, the pyruvylated octasaccharide (fractions 190 to 205) was separated from the octasaccharide (fractions 228 to 240). The resolution obtained was markedly better than that expected for oligosaccharides differing in 1 hexose unit. The pyruvylated octasaccharide eluted as a larger compound; this difference can be attributed to pyruvic acid. Similar results have been described for other charged substances (20). The radioactive oligosaccharides liberated by mild acid hydrolysis from the organic solvent extract gave four peaks when chromatographed on a gel filtration column (Fig. 3). Peak I (fractions 180 to 203) chromatographed in a position similar to the pyruvylated octasaccharide, and peak II (fractions 216 to 245) like the octasaccharide of *A.*

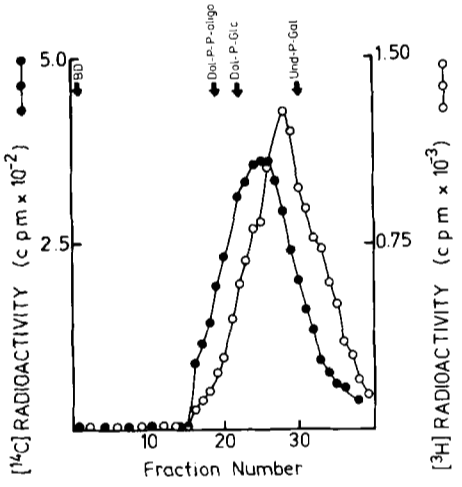

FIG. 2. **Gel filtration in the presence of deoxycholate.** The lower phase (○); and 1103 extract (●), prepared as described under "Experimental Procedures," were chromatographed on a column (76 × 1.5 cm) of Sephadex G-75 in 50 mM Tris-HCl buffer, pH 8.2, containing 0.5% of sodium deoxycholate. The column was maintained at 37 °C with a jacket. Fractions of 1.3 ml were collected and samples of 0.5 ml were counted in a scintillation counter. The markers are: *Dol-P-Glc*, dolichyl monophosphate glucose; *Dol-P-P-oligo*, dolichyl diphosphate $(Glc)_3$ $(Man)_9$ $(GlcNAc)_2$; *Und-P-Gal*, undecaprenyl monophosphate galactose; and *BD*, blue dextran.

faecalis. Two more peaks appeared: peak III (fractions 372 to 398) and peak IV (fractions 405 to 438). These arose from substances found mainly in the lower phase and they were characterized as glucose β1-3 galactose and galactose by various criteria as described previously (5).

The compounds appearing in the eluate depended on the pretreatment of the organic solvent extract. These seemed to be very labile and suffered alterations if dried with a nitrogen current before being subjected to mild acid hydrolysis. The radioactive compounds appearing in the Bio-Gel P-4 column (fractions 160 to 174 and 332 to 350) corresponded to degradation products (phosphate esters) and were negatively charged since they were bound to a quaternary aminoethyl-Sephadex column. When the compounds of these fractions were treated with alkaline phosphatase, the radioactivity moved to the positions of peaks II and III, respectively, and ran through the quaternary aminoethyl-Sephadex column.

Thin Layer Chromatography of the Substances Separated by Gel Filtration—Thin layer chromatography of peak I gave two radioactive substances: I_a and I_b (Fig. 4*I*). Compound I_a chromatographed in a position similar to that of a maltooligosaccharide of 4 glucose residues, and I_b in the position of the pyruvylated octasaccharide of *A. faecalis*. Peak II also gave two peaks, II_c and II_d, the first chromatographed a little behind the pyruvylated octasaccharide and II_d in the same position of the octasaccharide (Fig. 4, *II*). In order to facilitate the understanding, it is anticipated that the previously mentioned compounds correspond to the octasaccharide shown in Fig. 1 with different substitutions as follows: I_a, acetylated pyruvylated octasaccharide; I_b, pyruvylated octasaccharide; II_c, acetylated octasaccharide; and II_d, octasaccharide.

Paper Electrophoresis of the Saccharide Moiety Isolated from the 1103 Extract—When the substances separated by thin layer chromatography (Fig. 4) were eluted and subjected to paper electrophoresis, some compounds behaved as neutral and others as negatively charged substances. The neutral substances corresponded to peak II (II_d and II_c) (Fig. 5, *A* and *E*), while the negatively charged compounds (I_a and I_b) were those of peak I (Fig. 5*B* and *F*). The negatively charged compounds I_a and I_b ran the same as the pyruvylated octasaccharide standard (Fig. 5, *B* and *F*). They were not substituted by succinic acid or phosphate since after deacylation or alkaline phosphatase treatment both ran the same as before treatment (Fig. 5, *C* and *G*). When the substances of peaks I_a and I_b were depyruvylated and then submitted to paper electrophoresis they became neutral (Fig. 5, *D* and *H*). These results suggested that the negative charge of peaks I_a and I_b was due to a pyruvic acid residue.

Change in the Chromatographic Mobility of Compounds I_a, II_d and $I_b + II_c$ by Deacylation and Depyruvylation—The organic solvent extract was submitted to mild acid hydrolysis and the products obtained were separated by thin layer chromatography. This procedure yeilds a mixture of substances I_b and II_c because these compounds migrate very close in thin layer chromatography (Fig. 4, I_b and II_c).

Compound II_d migrated the same as the octasaccharide of *A. faecalis* (Fig. 6, ●, and II_d). Its mobility did not change if it was depyruvylated or deacylated (not shown) and it was neutral as judged by electrophoresis (Fig. 5). Therefore, II_d seems to be identical with the unsubstituted octasaccharide of Fig. 1.

Substance I_a ran on thin layer chromatography like a maltooligosaccharide of four glucose residues. When deacylated, it chromatographed like a maltooligosaccharide of 6 glucose residues (Fig. 6, I_a (*Da*)). After deacylation and depyruvylation almost all the radioactivity of substance I_a migrated like the octasaccharide of *A. faecalis*. These results indicate that the substance I_a corresponds to the acetylated pyruvylated

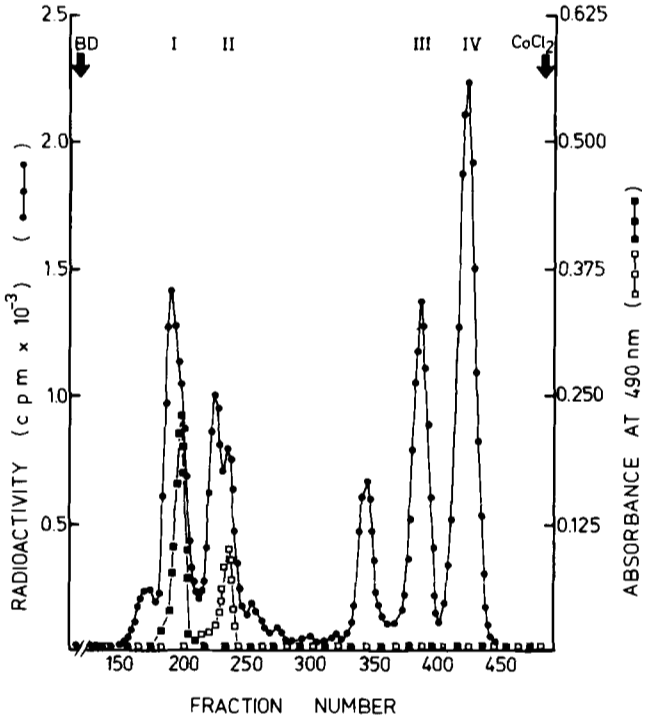

FIG. 3. **Gel filtration of the oligosaccharides obtained by mild acid hydrolysis.** The organic solvent extract (300,000 cpm) obtained as described under "Experimental Procedures," was subjected to mild acid hydrolysis and poured into a column (200 × 2 cm) of Bio-Gel P-4 (200 to 400 mesh) with 5 mg of pyruvylated octasaccharide and 5 mg of octasaccharide of *A. faecalis*. The solvent was 0.1 M pyridine acetate of pH 5, the flow rate was 10 ml/h, and the temperature was maintained at 55 °C. Fractions of 1 ml were collected and aliquots of 0.4 ml were monitored for ^{14}C radioactivity (●). The unlabeled saccharides were determined in aliquots of 0.2 ml with the phenolsulfuric acid method (19). ■, pyruvylated octasaccharide; □, octasaccharide; *BD*, blue dextran.

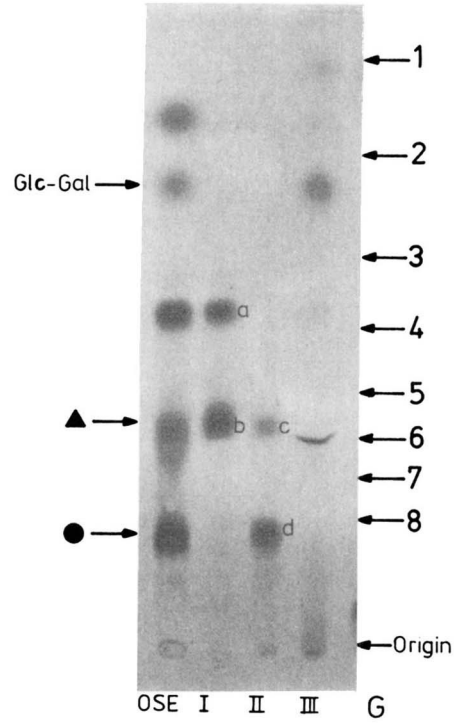

FIG. 4. **Thin layer chromatography of the substances isolated by gel filtration chromatography.** A sample of the radioactive peaks (*I*, *II*, and *III*) isolated by gel filtration chromatography (Fig. 3) and the saccharides obtained by mild acid hydrolysis from the organic solvent extracts (*OSE*) were chromatographed three times on thin layer plates with solvent D. The following standards were used: glucosyl β1-3 galactose (*Glc-Gal*); pyruvylated octasaccharide (▲), and octasaccharide (●) of *A. faecalis* and maltooligosaccharides (*G*).

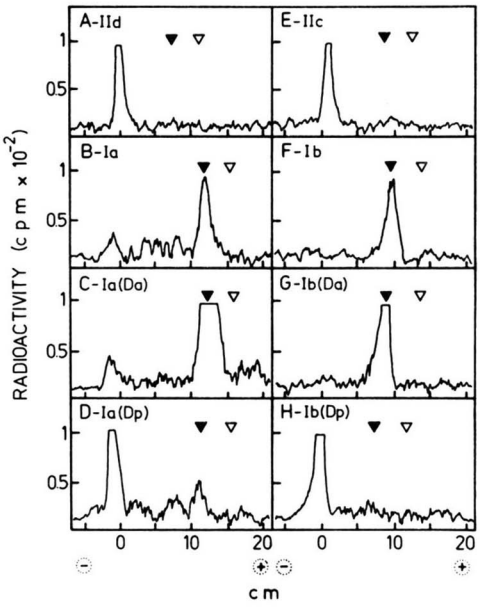

FIG. 5. **Paper electrophoresis of the compounds of Fig. 4.** Samples of the radioactive compounds isolated as shown in Fig. 4 and 0.4 mg of succinylated, pyruvylated octasaccharide of *A. faecalis* were submitted to paper electrophoresis with solvent F as described under "Experimental Procedures." *A*, *B*, *E*, and *F* correspond to *peaks* II_d, I_a, II_c, and I_b, respectively. Aliquots of I_a and I_b were deacylated and depyruvylated as described under "Experimental Procedures." *C* and *G* are deacylated (*Da*) I_a and I_b, respectively. *D* and *H* correspond to depyruvylated (*Dp*) I_a and I_b, respectively. ▲, pyruvylated octasaccharide; and ▽, succinylated, pyruvylated octasaccharide.

octasaccharide (Fig. 6, I_a (*Da*) (*Dp*)).

When the mixture of substances I_b + II_c was deacylated and chromatographed on thin layer, a small part of the radioactive substances migrated like a maltooligosaccharide of 8 glucose residues, but most of it ran like an oligosaccharide of 6 glucose residues (Fig. 6, *line* I_b + II_c (*Da*)). After deacylation and depyruvylation, substances I_b + II_c ran on thin layer chromatography like the octasaccharide standard (Fig. 6, I_b + II_c (*Da*) (*Dp*)). These experiments were repeated with the substances separated by gel filtration. Each substance was then deacylated and/or depyruvylated and the products were analyzed by thin layer chromatography. As shown in Table II, it was confirmed that I_b and II_c corresponded to the pyruvylated and acetylated octasaccharide, respectively.

Reduction with Borohydride and Acid Hydrolysis—Substances I_a, I_b + II_c, and II_d were obtained by mild acid hydrolysis of the organic solvent extract and separated by thin layer chromatography. In order to determine their sugar composition and which hexose occupied the reducing end, the radioactive compounds, after reduction with sodium borohydride, were hydrolyzed with acid. For substances I_a, I_b + II_c, and II_d only two products which chromatographed like galactitol and glucose were detected (Fig. 7). The ratio of radioactivities of glucose to galactitol was 7.2, 6.8, and 6.6 for sub-

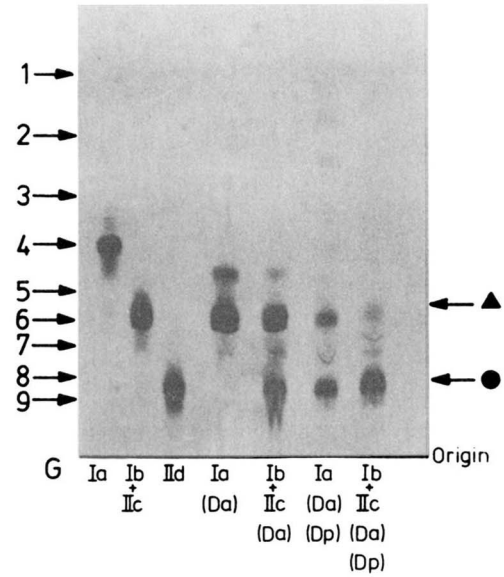

FIG. 6. **Thin layer chromatography of peaks I_a, I_b + II_c, and II_d.** Saccharides obtained by thin layer chromatography were rechromatographed directly (I_a, I_b + II_c, and II_d) after deacylation (*Da*) or depyruvylation (*Dp*) as described in text. The following standards were used: pyruvylated octasaccharide (▲) and octasaccharide (●) of *A. faecalis* and maltooligosaccharides (*G*). Thin layer chromatography was developed three times with solvent D.

TABLE II

Thin layer chromatography of substances I_b and II_c

Substances I_b and II_c were isolated by gel filtration and thin layer chromatography. The samples were then deacylated or depyruvylated and analyzed by thin layer chromatography as described in Fig. 6.

Substance	Mobility relative to glucose		
	Untreated	Deacylated	Depyruvylated
I_b	0.43	0.43	0.18
II_c	0.36	0.17	0.17
Py-octasaccharide[a]	0.43	0.43	0.19

[a] Pyruvylated octasaccharide from *A. faecalis*.

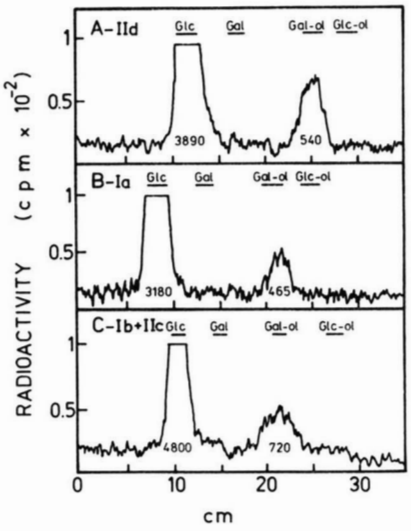

FIG. 7. **The products of acid hydrolysis after reduction.** Substances II_d, I_a, and $I_b + II_c$ isolated by thin layer chromatography were reduced with sodium borohydride (8) and then treated with 1 N HCl for 4 h at 100 °C, dried, and chromatographed with solvent C for 72 h, as shown in *frames A*, *B*, and *C*, respectively. The radioactive peaks were located with a scanner and the corresponding pieces of paper were counted in a scintillation counter. The numbers indicate the radioactivity in each peak.

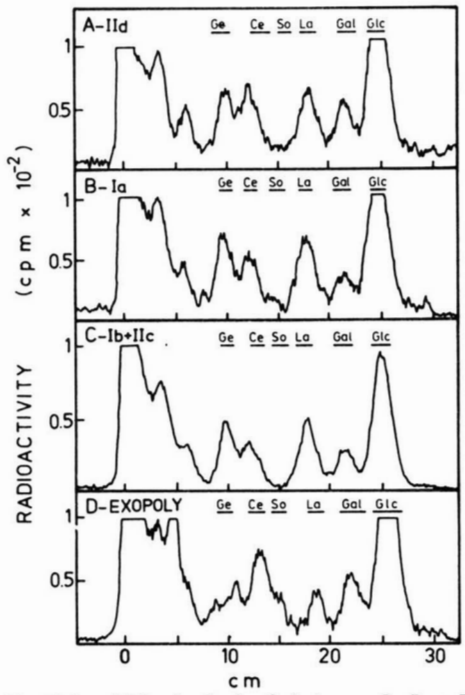

FIG. 8. **Partial acid hydrolysis.** Substances I_a, $I_b + II_c$, and II_d isolated by thin layer chromatography and the radioactive exopolysaccharide of *R. meliloti* were treated with 0.5 N HCl for 1 h at 100 °C, dried, and chromatographed with solvent A for 36 h. Standards were run at the same time: *Ge*, gentiobiose (Glc β1-6 Glc); *Ce*, cellobiose (Glc β1-4 Glc); *So*, sophorose (Glc β1-2 Glc); *La*, laminaribiose (Glc β1-3 Glc); *Gal* and *Glc*.

stances II_d, I_a, and $I_b + II_c$, respectively. These results indicate that the radioactive substances are oligosaccharides containing 7 glucose residues and 1 galactose residue at the reducing end.

Partial Acid Hydrolysis—In order to obtain more information on the structure of substances II_d, I_a, and $I_b + II_c$, these were compared with the radioactive exopolysaccharide of *R. meliloti*. The products obtained after treatment with 0.5 N HCl at 100 °C were chromatographed with solvent A. As shown in Fig. 8, substances II_d, I_a, and $I_b + II_c$ and the exopolysaccharide gave a similar chromatographic pattern. Part of the radioactivity remained at the origin, while the degradation products moved like glucose, galactose, laminaribiose (Glc β1-3 Glc), cellobiose (Glc β1-4 Glc), and gentiobiose (Glc β1-6 Glc). In order to check the identity of the latter two disaccharides, they were eluted from the paper and submitted to paper electrophoresis with solvent G. The results obtained confirmed the formation by partial hydrolysis of cellobiose, gentiobiose, and in addition, glucosyl β1-3 galactose (not shown).

Methylation Analysis—The different saccharide moieties of the lipid derivatives separated by thin layer chromatography were compared with the exopolysaccharide of *R. meliloti* and with the *A. faecalis* octasaccharide by methylation analysis. The results are shown in Fig. 9. All the compounds gave 2,3,6; 2,4,6; 2,3,4-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose, as expected for a saccharide that has 4-, 3-, and 6-substituted glucose and 3-substituted galactose. The first lane, from left to right, shows the methylation products of the *Alcaligenes* pyruvylated octasaccharide. It yielded 2,3-di-*O*-methylglucose which arises from the terminal glucose residue when it is pyruvylated (substituted in 4 and 6). As shown in the second lane, methylation of the depyruvylated *Alcaligenes* octasaccharide gave no 2,3-di-*O*-methylglucose and instead yielded 2,3,4,6-tetra-*O*-methylglucose from the unsubstituted terminal glucose. The same results have been obtained by Hisamatsu *et al.* (3) who estimated the methylated sugars by gas-liquid chromatography. The same changes produced by depyruvylation were observed when substance I_a

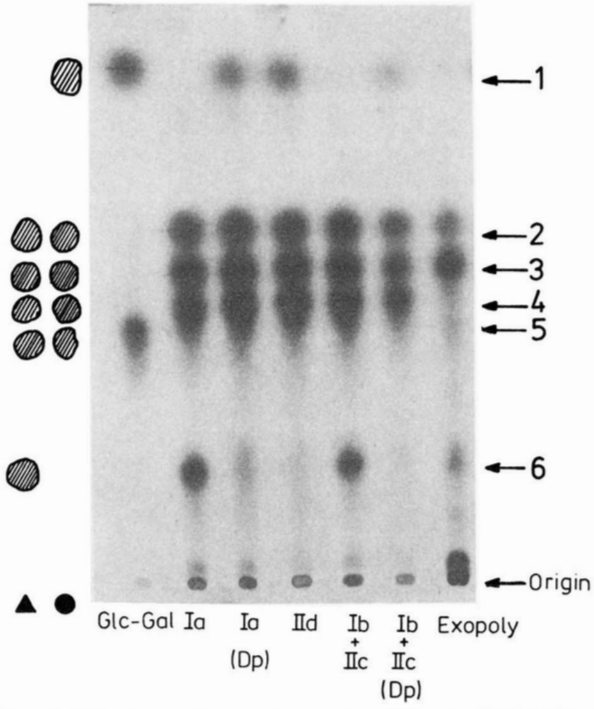

FIG. 9. **Thin layer chromatography of the methylated products.** Substances II_d, I_a, $I_b + II_c$ depyruvylated (*Dp*) or not; the exopolysaccharide of *R. meliloti* and samples of *A. faecalis* octasaccharide, pyruvylated (▲) and depyruvylated (●) were methylated and chromatographed as described under "Experimental Procedures." Radioactive substances were localized by radioautography and the nonradioactive ones with 5% concentrated sulfuric acid in ethanol (14). The standards were (*1*) 2,3,4,6-tetra-*O*-methylglucose; (*2*) 2,3,6-tri-*O*-methylglucose; (*3*) 2,4,6-tri-*O*-methylglucose; (*4*) 2,3,4-tri-*O*-methylglucose; (*5*) 2,4,6-tri-*O*-methylgalactose; and (*6*) 2,3-di-*O*-methylglucose.

(fourth lane) was depyruvylated (I_a (*Dp*)). It has been mentioned that substance I_a is believed to be the acetylated pyruvylated octasaccharide and since acetyl residues are lost in the methylation reaction, the results shown in Fig. 9 are consistent with this belief.

The sixth lane shows that II_d, believed to be the unsubstituted octasaccharide, yields the same products as the depyruvylated octasaccharide. Sample I_b + II_c, which was a mixture of pyruvylated octasaccharide and acetylated octasaccharide, gave 2,3-di-*O*-methylglucose but no 2,3,4,6-tetra-*O*-methylglucose, presumably because the acetylated octasaccharide (II_c) was present in only small amounts. The same mixture after depyruvylation (I_b + II_c (Dp)) gave no 2,3-di-*O*-methylglucose and a clear spot of 2,3,4,6-tetra-*O*-methylglucose.

The lane on the right corresponds to the *R. meliloti* exopolysaccharide. It gave the same pattern as the other pyruvylated saccharides. It seems clear that the lipid-bound saccharide, the *A. faecalis* octasaccharide, and the *R. meliloti* exopolysaccharide have the same primary structure.

The Biosynthetic Relationship between the Different Lipid-linked Saccharides—In order to study the sequence of labeling of the different saccharides some experiments were carried out with UDP-[^{14}C]Gal instead of UDP-[^{14}C]Glc. The idea was that since galactose is the residue directly linked to the lipid phosphate, it should be introduced first and that it would be possible to introduce the glucose residues in a second step with nonradioactive UDP-Glc. However, incubation of either UDP-[^{14}C]Glc or UDP-[^{14}C]Gal with the enzyme preparation yielded the same spectrum of saccharides. It was then thought, that an UDP-Gal 4-epimerase-less mutant should give more clear-cut results because octasaccharides could be formed only when the two sugar nucleotides were present. After treatment with a mutagen and selection of galactose-negative colonies, a strain was selected and used for an experiment in which enzyme prepared from the mutant was incubated separately with UDP-[^{14}C]Glc or UDP-[^{14}C]Gal. The saccharides obtained by mild acid hydrolysis were then chromatographed on thin layer plates. The results are shown in Fig. 10 and it may be observed that, while UDP-[^{14}C]Gal yielded mainly lipid-bound galactose and glucosyl β1-3 galactose, the sample incubated with UDP-[^{14}C]Glc gave, in addition, the various types of octasaccharides.

In another experiment, the enzyme prepared with the mutant was incubated with labeled UDP-Gal, the lipid phosphate saccharides were then extracted, hydrolyzed, and the products were separated by gel filtration. As shown in Fig. 11, most of the radioactive compounds chromatographed like *peak III* (glucosyl β1-3 galactose) and *peak IV* (galactose) (Fig. 11*A*). After the accumulation of the radioactive lipid-bound galactose and lipid-bound glucosyl β1-3 galactose, an excess of unlabeled UDP-Glc was added to the mixture and the incubation was continued at 12 °C for additional periods of 3, 15, and 90 min (Fig. 11, *frames B*, *C*, and *D*). After 3 min of incubation in presence of UDP-Glc, most of the radioactivity was found in *peak II* and a small amount in *peaks I* and *III* (Fig. 11*B*). Thus, the results show that by addition of UDP-Glc, the lipid-bound galactose and lipid-bound glucosyl β1-3 galactose were transformed in lipid-bound octasaccharide (*peak II*). Besides, Fig. 11*B* shows five small radioactive peaks between *peaks III* and *II*, these peaks may correspond to the lipid-bound compounds with 3, 4, 5, 6, and 7 hexose residues. After incubation for 15 and 90 min at 12 °C in the

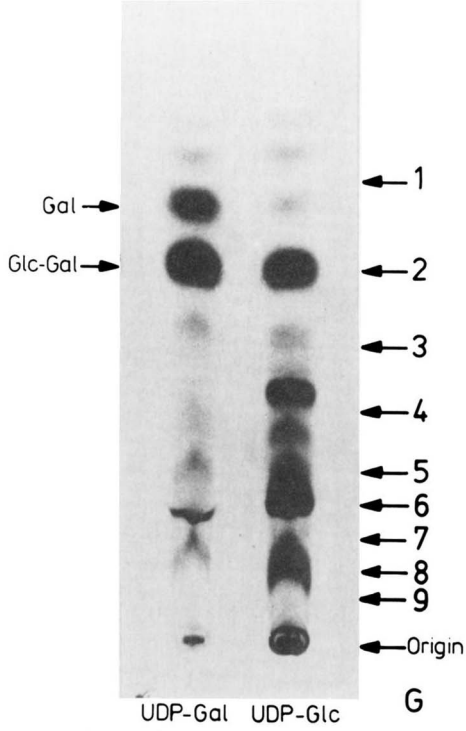

FIG. 10. **Thin layer chromatography of the substances obtained with the enzyme preparation of the galactose-negative mutant.** The incubation mixture was the same as described under "Experimental Procedures" with about 70 μg of enzyme preparation of the galactose-negative mutant and 5 × 10^5 cpm of UDP-[^{14}C]Glc (268 Ci/mol) (*right side*) or UDP-[^{14}C]Gal (196 Ci/mol) (*left side*) in a total volume of 50 μl. After 20 min at 10 °C, the organic solvent extract was obtained as described under "Experimental Procedures" and submitted to mild acid hydrolysis. The products were chromatographed three times on thin layer plates with solvent D. Standards were run at the same time: glucosyl β1-3 galactose (*Glc-Gal*) and maltooligosaccharides (*G*).

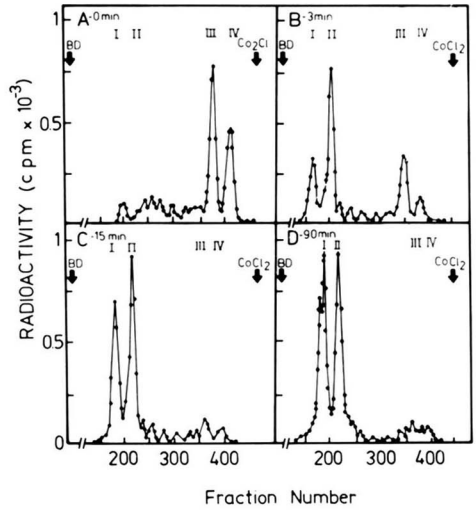

FIG. 11. **Gel filtration of the labeled saccharides after incubation for different times.** The incubation mixture contained: 70 mM Tris-HCl buffer, pH 8.2, 12 mM $MgCl_2$, 40 mM 2-mercaptoethanol, about 0.7 mg of enzyme preparation of the galactose-negative mutant and 2.5 × 10^6 cpm of UDP-[^{14}C]Gal (268 Ci/mol) in a total volume of 0.5 ml. After 20 min of incubation at 10 °C, 5 μmol of UDP-Glc was added and the incubation continued for 0, 3, 15, and 90 min (*frames A*, *B*, *C*, and *D*, respectively). The reaction was stopped by the addition of 1 ml of methanol and 1.5 ml of chloroform. The organic solvent extract was obtained as described under "Experimental Procedures" and submitted to mild acid hydrolysis. The products were poured into Bio-Gel P-4 columns and processed as described in Fig. 3. Fractions of 1 ml were collected and aliquots of 0.6 ml were monitored for ^{14}C radioactivity. The position corresponding to peaks I, II, III, and IV of Fig. 3 is indicated. *BD*, blue dextran.

presence of UDP-Glc, nearly all the radioactivity was found in *peaks I* and *II* (Fig. 11, *C* and *D*).

In some experiments, after the incubation of the enzyme prepared with the mutant with UDP-[^{14}C]Gal, the particulate fraction was washed with water until the supernatant fluid was essentially free of radioactivity. Then, the particulate fraction was suspended with the incubation mixture in the presence of an excess of unlabeled UDP-Glc and incubated at 12 °C for different times. The samples were processed as indicated above, and the results were similar to those of Fig. 11.

DISCUSSION

The study of lipid-bound oligosaccharides is of interest because these compounds are intermediates in the biosynthesis of polysaccharides (4) and the latter are believed to have a role in the infection process which leads to symbiotic nitrogen fixation (21).

Several compounds become labeled when UDP-[^{14}C]Glc is incubated with *R. meliloti* enzymes. Some of these compounds have the properties of polyprenyl diphosphate sugars; their solubility in organic solvents is similar, they are decomposed by treatment with ammonia or by acid at pH 2 for 10 min at 100 °C. The *R. meliloti* compounds are eluted from DEAE-cellulose under the same conditions as dolichyl diphosphate saccharides, and are decomposed by hot phenol or catalytic hydrogenation like unsaturated polyprenyl derivatives. The behavior of the compounds on gel filtration in the presence of deoxycholate is consistent with the presence of a polyprenyl of about 12 isoprene residues.

The study of the oligosaccharides liberated by mild acid treatment was facilitated by having available a similar saccharide obtained from the culture fluid of *A. faecalis* var. myxogenes strain 22 (6). The radioactive saccharide moieties of the *R. meliloti* compounds could be separated by gel filtration chromatography into four main peaks. Two of the substances of these peaks (*III* and *IV*) have been previously characterized as glucosyl β1-3 galactose and galactose (5). The substances that appeared in the first two peaks (*I* and *II*) were eluted in the same position as the pyruvylated octasaccharide and unsubstituted octasaccharide, respectively, and behaved as negatively charged and neutral compounds on paper electrophoresis. After depyruvylation, the substances of *peak I* became neutral. Thin layer chromatography led to the separation of *peaks I* and *II* into two substances each designated as I_a, I_b, II_c, and II_d. By thin layer chromatography and electrophoresis after deacylation or depyruvylation, it was concluded that the compounds were: I_a, pyruvylated acetylated octasaccharide; I_b, pyruvylated octasaccharide; II_c, acetylated octasaccharide, and II_d, unsubstituted octasaccharide (see Figs. 5 and 6). Therefore, the pyruvylated compounds appeared before the nonpyruvylated on gel filtration and the acetylated substances had a higher mobility than the nonacetylated in thin layer chromatography.

All the compounds were found to have galactose at the terminal reducing end, they gave by partial acid hydrolysis: laminaribiose, gentiobiose, cellobiose, and glucosyl β1-3 galactose (Fig. 8). These data are consistent with the presence in the saccharide of β(1-3)-, β(1-6)-, β(1-4)-linked glucosyl residues, and a β(1-3)-linked galactosyl residue, these are the products expected to arise from the octasaccharide shown in Fig. 1.

The same pattern of methylated sugars was obtained from the exopolysaccharide of *R. meliloti* and from the octasaccharide of *A. faecalis* var. myxogenes strain 22 as well as from substances I_a and I_b (Fig. 9). The latter three compounds, after depyruvylation, gave the same methylated sugars as the substances II_c and II_d (Fig. 9). These results indicate that the saccharide bound to polyprenyl diphosphate is the same as the repeating unit of the exopolysaccharide.

Studies on the labeling of the saccharide moieties with an enzyme obtained from a galactose negative mutant of *R. meliloti* at different times have shown that radioactivity appears first in the galactose and glucosyl β1-3 galactose and later, in a second step, the other 6 glucose residues become radioactive practically without accumulation of intermediates. At the initial stages of incubation, the lipid diphosphate octasaccharide was found mainly without substitutions, but later on, it became acetylated and/or pyruvylated. This agrees with the results of Ielpi *et al.* (22) who detected a transfer of pyruvic acid from phosphoenolpyruvate to a lipid-bound saccharide with enzymes of *Xanthomonas campestris*.

The participation of isoprenyl phosphate derivatives in the biosynthesis of polysaccharides which have repeating structural unit has been demonstrated (23, 24). The formation of an octasaccharide linked to lipid which is identical with the repeating unit of the exopolysaccharide of *R. meliloti* points to a biosynthetic relationship between both compounds, but experiments intended to prove this have been negative.

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