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Lipid-bound Saccharides Containing Glucose and Galactose in *Agrobacterium tumefaciens*

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The incubation of an enzyme preparation of *Agrobacterium tumefaciens* with UDP-[^{14}C]Glc led to the formation of radioactive substances soluble in chloroform/methanol/water (1 : 1 : 0·3, by vol.). These substances had properties similar to the polyprenol diphosphate saccharides. The lipid moiety appears to be an unsaturated polyprenol. Mild acid hydrolysis of the substances liberated five water-soluble products which were separated by TLC and characterized by borohydride reduction–acid hydrolysis, partial acid hydrolysis, methylation analysis, TLC and paper chromatography and paper electrophoresis. The products behaved like: galactose; the disaccharide Glc(β1-3)Gal; the tetrasaccharide Glc(β1-4)Glc(β1-4)Glc(β1-3)Gal; an octasaccharide and a pyruvylated octasaccharide. The last two substances were compared by paper electrophoresis, TLC and methylation analysis with an octasaccharide obtained by the action of a hydrolytic enzyme on the exopolysaccharide of *Agrobacterium tumefaciens*. The octasaccharide and the pyruvylated octasaccharide from the lipid behaved like those obtained by enzyme action on the *A. tumefaciens* exopolysaccharide. These results suggest that the lipid-bound saccharides are involved in the biosynthesis of the extracellular polysaccharides.

INTRODUCTION

The genera *Agrobacterium* and *Rhizobium* are related taxonomically and they infect some dicotyledonous plants. The first induces tumours, while *Rhizobium* induces nitrogen-fixing nodule formation in legumes. Both produce extracellular polysaccharides and β-linked glucans. It has been proposed that these compounds participate in the bacterium–plant cell interaction (Bauer, 1981; Abe *et al.*, 1982). The extracellular polysaccharides produced by both genera are similar; they contain glucose, galactose, with pyruvic acid and acyl substituents in the approximate proportions of 7 : 1 : 1 : 1 (Bjorndal *et al.*, 1971; Zevenhuisen, 1973).

Lipid intermediates have been found to be involved in the biosynthesis of lipopolysaccharides (Wright *et al.*, 1967), peptidoglycans (Higashi *et al.*, 1967), and other cell wall polysaccharides (Braun & Hantke, 1974; Sutherland, 1979) in bacteria and the glycosylation of protein in animals (Staneloni & Leloir, 1982). Their role in exopolysaccharide biosynthesis has been proved by Ielpi *et al.* (1981). Recently, it was found that a lipid-bound octasaccharide was formed when a particulate enzyme of *R. meliloti* was incubated with UDP-Glc (Tolmasky *et al.*, 1982). Here we report the formation of lipid-linked saccharides by enzymes from *A. tumefaciens* and provide evidence that the saccharide moieties of some of these compounds are similar to the repeating unit of the extracellular polysaccharide produced by these bacteria.

METHODS

Materials. The succinoglycan octasaccharide isolated from *Alcaligenes faecalis* var. *myxogenes* strain 22 was a gift from T. Harada, Osaka University, Japan. According to Hisamatsu *et al.* (1980*b*), the composition and structure of this octasaccharide is identical to the repeating unit of the extracellular polysaccharide of

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A. tumefaciens. The octasaccharide was deacylated or deacylated and depyruvylated as described below and the products are referred to as the pyruvylated octasaccharide and octasaccharide of *Alcaligenes faecalis*.

Bacterial strains and growth conditions. The wild-type *A. tumefaciens* LBA 4001 containing the virulent plasmid pTiAch5, and a plasmidless strain, LBA 4011, were provided by P. J. J. Hooykaas, State University of Leiden, The Netherlands (Klapwijk *et al.*, 1978). The bacteria were grown at 28 °C on a medium containing 0·5% tryptone and 0·3% yeast extract as described by Hooykaas *et al.* (1980). *Flavobacterium* sp. M64 was obtained from T. Harada. It was grown with 1% (w/v) extracellular polysaccharide of *R. meliloti* as carbon source, as described by Amemura *et al.* (1974).

Enzyme preparation. The cells were harvested by centrifugation, resuspended in 10 mM-EDTA/Tris buffer pH 8, and then frozen and thawed several times (Garcia *et al.*, 1974).

Assay procedure. The assay and methods were as described previously (Tolmasky *et al.*, 1982). The standard incubation mixture was as follows: 70 mM-Tris/HCl buffer pH 8·2, 12 mM-$MgCl_2$, 3 × 10^6 c.p.m. UDP-[^{14}C]Glc (268 Ci mol^{-1}; 9·916 TBq mol^{-1}) and enzyme preparation (about 7 mg protein) in a final volume of 0·5 ml. After 30 min at 10 °C, the reaction was stopped by the successive addition of 1 ml methanol, 1·5 ml chloroform and 7 mg liver microsomal protein as carrier. The mixture was centrifuged, and the lower and upper phase and the solid interphase were separated. The latter was washed three times with chloroform/methanol/4 mM-$MgCl_2$ (3 : 48 : 47, by vol.) and extracted twice with chloroform/methanol/water (1 : 1 : 0·3, by vol.) as described by Tolmasky *et al.* (1982). The last fraction is referred to as the organic solvent extract.

Octasaccharide of A. tumefaciens. Radioactive extracellular polysaccharide of *A. tumefaciens* LBA 4011 was prepared as previously described (Tolmasky *et al.*, 1982). The exopolysaccharide depolymerizing enzyme was obtained from the culture fluid of *Flavobacterium* sp. M64 as described by Amemura *et al.* (1974) up to the $(NH_4)_2SO_4$ precipitation step. The enzyme preparation (about 40 mg) was incubated with the radioactive extracellular polysaccharide (30000 c.p.m.) in 20 mM-sodium acetate buffer pH 5·5 at 37 °C for 2 h. The degradation products were separated from the undegraded exopolysaccharide with a gel filtration column (Bio-Gel P-4, 2 × 30 cm) and the octasaccharide was isolated by TLC with solvent D (see below).

Acid hydrolysis. Acid hydrolysis was carried out at 100 °C under three conditions: mild, 0·01 M-HCl for 10 min; partial, 0·5 M-HCl for 60 min and total, 1 M-HCl for 240 min.

Deacylation and depyruvylation of the saccharides. The samples were deacylated by treatment with 10 mM-KOH for 5 h at 20 °C and depyruvylated with 0·01 M-HCl for 90 min at 100 °C (Hisamatsu *et al.*, 1980*a*).

Analytical methods. The saccharides were methylated as described by Hakomori (1964), hydrolysed with 1 M-HCl for 4 h at 100 °C and processed as described by Li *et al.* (1978). The phenol treatment and sodium borohydride reduction were performed as described by Garcia *et al.* (1974).

Chromatography. Descending paper chromatography was performed on SS2043a paper with the following solvents: solvent A, butanol/pyridine/water (6 : 4 : 3, by vol.); solvent B, butanol/pyridine/water (4 : 3 : 4, by vol.); solvent C, nitromethane/acetic acid/ethanol/water saturated with boric acid (8 : 1 : 1 : 1, by vol.). Radioactivity was detected with a scanner and the sugars by the alkaline silver nitrate method (Trevelyan *et al.*, 1950). TLC was carried out on silica gel-60 plates 0·25 mm thick and developed three times (15 cm) with solvent D [1-propanol/nitromethane/water (5 : 2 : 2, by vol.)]. Methylated sugar derivatives were chromatographed for 2 h with solvent E [acetone/benzene/ammonium hydroxide/water (200 : 50 : 1·35 : 1, by vol.)]. Radioactive compounds were detected by autoradiography and the sugars with 5% (v/v) sulphuric acid in ethanol as described by Li *et al.* (1978).

Electrophoresis. Paper electrophoresis was performed with solvent F [pyridine/acetic acid/water (1 : 0·04 : 9, by vol.)] pH 6·5 for 90 min at 40 V cm^{-1}; solvent G (50 mM-sodium borate buffer pH 9) for 3 h at 20 V cm^{-1} and solvent H (0·1 M-ammonium molybdate adjusted to pH 5 with sulphuric acid) for 2 h at 20 V cm^{-1}.

RESULTS

Studies on the lipid-bound saccharides

Radioactive compounds soluble in organic solvent were formed when the enzyme preparation of *A. tumefaciens* strain LBA 4011 was incubated with UDP-[^{14}C]Glc and Mg^{2+}. These substances had properties similar to polyprenyl diphosphate saccharides. Thus they gave a similar R_F (0·6) on paper chromatography with solvent B, and were decomposed on treatment with 13 M-ammonia for 20 min at 25 °C or at pH 2 for 10 min at 100 °C. The rate of decomposition of polyprenyl phosphate saccharides on heating with aqueous phenol has been used to characterize these compounds (Garcia *et al.*, 1974). A half-life value of 2 min was obtained when the radioactive compounds were heated at 70 °C in 50% (v/v) phenol. This half-life value was similar to that reported by Garcia *et al.* (1974) for undecanoprenyl diphosphate sugars (4 to 10 min) and different from undecaprenyl monophosphate-sugar (60 min) and dolichyl monophosphate glucose (10% hydrolysed in 3 h) (Pont Lezica *et al.*, 1975).

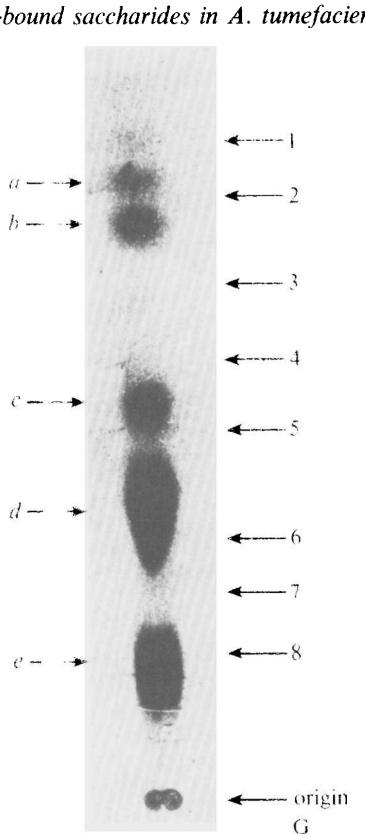

Fig. 1. TLC of the saccharides released from lipid. The organic solvent extract (7800 c.p.m.) obtained as described in Methods was treated with 0·01 M-HCl for 10 min at 100 °C. The water-soluble radioactive products were chromatographed on a thin-layer plate (solvent D, run three times). The numbers indicate the degree of polymerization of the maltooligosaccharides (G). The compounds were detected as described in Methods.

The compounds also behaved like polyprenyl diphosphate saccharides when chromatographed on DEAE-cellulose with chloroform/methanol/water (1 : 1 : 0·3, by vol.) and with an ammonium formate gradient (Behrens *et al.*, 1971). Thus, the compounds were eluted with 112 mM-ammonium formate, while dolichyl monophosphate glucose and dolichyl diphosphate ($Glc_3Man_9GlcNAc_2$) eluted with 23 mM and 100 mM-ammonium formate, respectively.

Separation of the saccharide moiety of lipid-bound saccharides

The *A. tumefaciens* strain LBA 4011 enzyme preparation was incubated with UDP-[^{14}C]Glc in the presence of magnesium ions and the lipid-bound saccharides were extracted with chloroform/methanol/water (1 : 1 : 0·3, by vol.) as described. Mild acid hydrolysis of the extract and chromatography of the water soluble products revealed the presence of five different radioactive compounds (Fig. 1). Compound *a*, the fastest moving substance, had the same mobility as galactose on paper chromatography with solvent A and C. Compound *b*, the second fast migrating substance, chromatographed like a disaccharide. After elution from the thin-layer plate (Fig. 1), it was hydrolysed with acid and the products were chromatographed on paper. As shown in Fig. 2(*a*) two equal peaks appeared, one moving like galactose and the other like glucose. In order to find out which of the hexoses occupied the reducing end, compound *b* was reduced with sodium borohydride, hydrolysed with acid and chromatographed on paper with solvent C. As shown in Fig. 2(*b*) two substances appeared, one moving as glucose and the other as galactitol, thus showing that galactose occupied the reducing end.

Compound *b* was compared with the disaccharide glucosyl(β1-3)galactose that had been

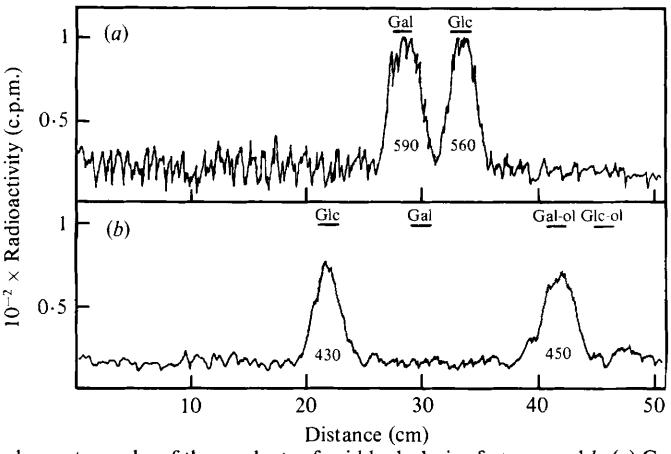

Fig. 2. Paper chromatography of the products of acid hydrolysis of compound *b*. (*a*) Compound *b* was eluted from the thin-layer plate (Fig. 1), treated with 1 M-HCl for 4 h at 100 °C, dried and then chromatographed on paper with solvent A. (*b*) Compound *b* reduced with sodium borohydride was subjected to total acid hydrolysis and chromatographed with solvent C. The numbers under the peaks are c.p.m. in that region.

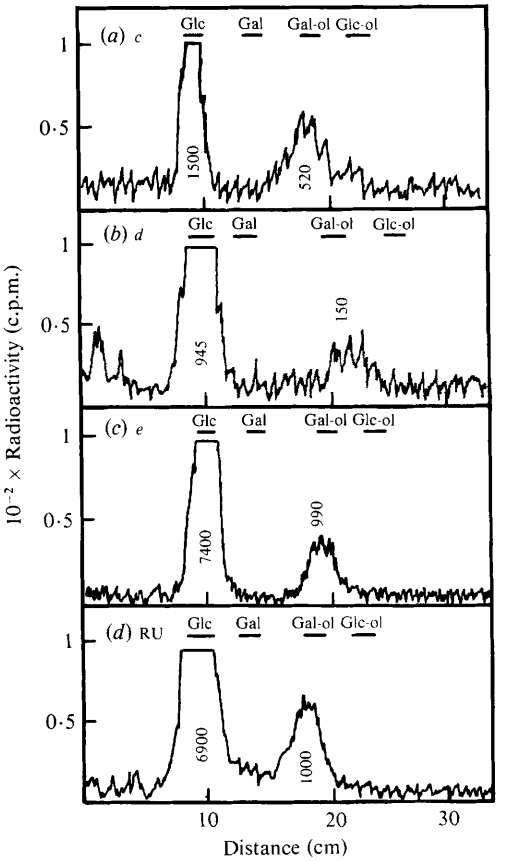

Fig. 3. Products of acid hydrolysis after reduction. The compounds *c*, *d* and *e* were eluted from the thin-layer plate (Fig. 1) and the octasaccharide repeating unit (RU) of *Agrobacterium tumefaciens* was isolated as described in Methods. The compounds were reduced with sodium borohydride, treated with 1 M-HCl for 4 h at 100 °C, dried and chromatographed with solvent C. The numbers indicate the radioactivity in each peak (c.p.m.).

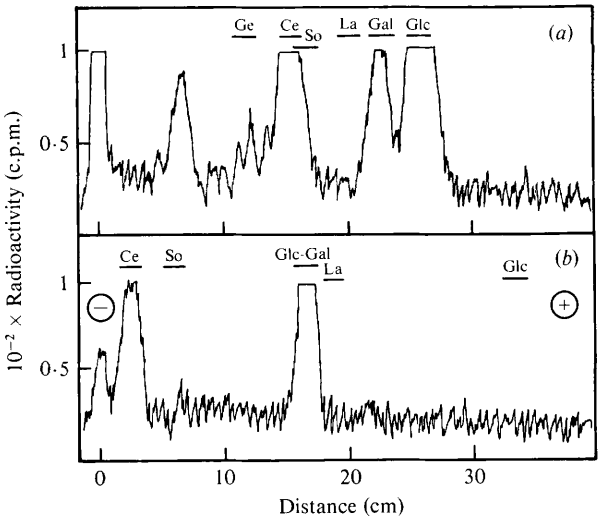

Fig. 4. Products of partial acid hydrolysis of compound *c*. (*a*) Compound *c* was isolated by TLC, treated with 0·5 M-HCl for 1 h at 100 °C, dried, and the products chromatographed with solvent A. (*b*) The peak that migrated like cellobiose in A was eluted and submitted to paper electrophoresis with solvent G for 3 h at 20 V cm^{-1}. The standards were: Ge, gentiobiose [Glc(β1-6)Glc]; Ce, cellobiose [Glc(β1-4)Glc]; So, sophorose [Glc(β1-2)Glc]; La, laminaribiose [Glc(β1-3)Glc]; Glc-Gal, [Glc(β1-3)Gal]; Gal and Glc.

found lipid-bound in *R. meliloti* (Tolmasky *et al.*, 1980). Both disaccharides chromatographed in the same position with solvent A, which separates these compounds from others such as gentiobiose [Glc(β1-6)Glc], sophorose [Glc(β1-2)Glc] and laminaribiose [Glc(β1-3)Glc)]. In other experiments, compound *b* and glucosyl(β1-3)galactose were reduced with sodium borohydride and submitted to electrophoresis with solvent H. Both substances had the same mobility but were different from maltitol and cellobitol. By methylation analysis, compound *b* gave 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose. These results indicate that compound *b* is glucosyl(β1-3)galactose.

Compound *c* chromatographed as a maltooligosaccharide of four hexose residues. When the substance separated by TLC was eluted and subjected to paper electrophoresis with solvent F, it behaved as if it were neutral. When run in a Bio-Gel P-2 column (110 × 1·2 cm) with 0·1 M-pyridine acetate, pH 5, as solvent, it chromatographed like the tetrasaccharide stachyose.

After compound *c* was reduced with sodium borohydride, hydrolysed with acid and chromatographed on paper with solvent C, two radioactive substances were detected (Fig. 3*a*). One migrated like glucose and the other like galactitol. The ratio of radioactivity of the first to the second substance was 3, as if compound *c* were a tetrasaccharide containing three glucose molecules and one galactose molecule. In another experiment, compound *c* was subjected to partial acid hydrolysis (0·5 M-HCl for 1 h at 100 °C) and the products were chromatographed with solvent A. The degradation products behaved like glucose, galactose, and cellobiose (Fig. 4*a*). The products that migrated like cellobiose were eluted and submitted to paper electrophoresis with solvent G. Two substances were detected, one migrating like cellobiose, and, the other like glucosyl(β1-3)galactose (Fig. 4*b*). Compound *c* was methylated, hydrolysed with acid and subjected to TLC with solvent E. The main products (Fig. 9, lane *c*) were 2,3,6-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose as expected for a saccharide containing 4-substituted glucose and 3-substituted galactose. The presence of 2,3,4,6-tetra-*O*-methylglucose and a dimethyl derivative was also detected. The first corresponds to the terminal glucose and the second could be the result of an insufficient methylation, or less probably, the product of a branching point. From gel filtration, borohydride reduction–acid hydrolysis, partial hydrolysis and methylation results, it was concluded that compound *c* is the following tetrasaccharide: Glc(β1-4)Glc(β1-4)Glc(β1-3)Gal.

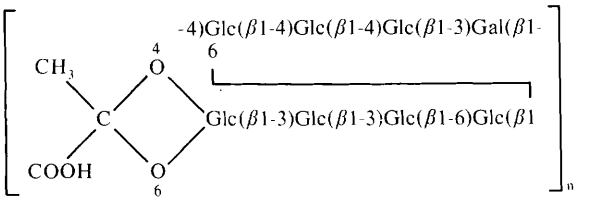

Fig. 5. Proposed structure of the octasaccharide repeating unit of *R. meliloti*, *A. tumefaciens* and *Alcaligenes faecalis* var. *myxogenes*, according to Jansson *et al.* (1977) and Hisamatsu *et al.* (1980*a*). Acyl substituents not shown.

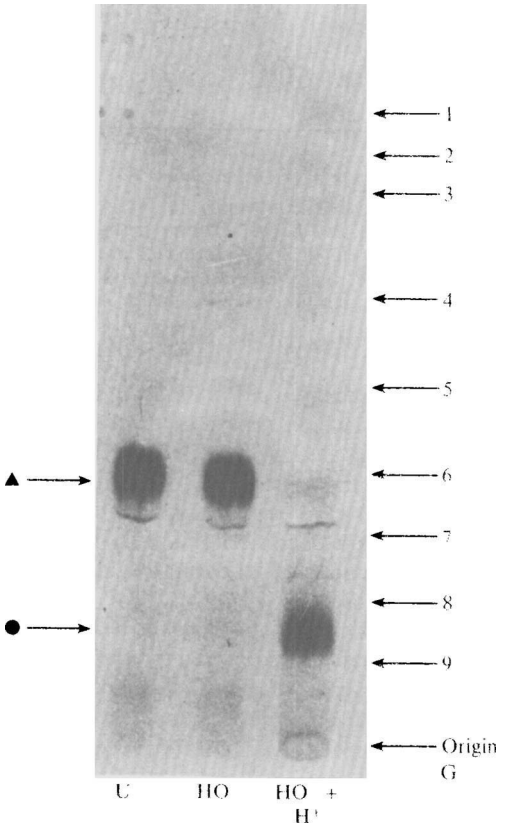

Fig. 6. TLC of the repeating unit of *A. tumefaciens*. The octasaccharide repeating unit of *A. tumefaciens* was obtained by treatment of the radioactive exopolysaccharide with the depolymerase of *Flavobacterium* sp. M64 as described in Methods. This substance, untreated (U), treated with 10 mM-KOH for 5 h at 20 °C (HO^-) or with 10 mM-HCl for 90 min at 100 °C (H^+) was chromatographed on thin-layer plates (solvent D, run three times). The standards were: pyruvylated octasaccharide (▲) and octasaccharide (●) of *Alcaligenes faecalis* and maltooligosaccharides (G). The substances were detected as described in Methods. See Fig. 1 legend for definition of numbers.

Octasaccharide repeating unit of A. tumefaciens exopolysaccharide

The structure of the exopolysaccharide of *R. meliloti* has been determined by Jansson *et al.* (1977) (Fig. 5) and it has been reported that those produced by *R. meliloti*, *A. tumefaciens*, and *Alcaligenes faecalis* var. *myxogenes* have a similar structure (Zevenhuisen, 1973; Hisamatsu *et al.*, 1980*b*). The former two occurred partially esterified with acetic acid and the latter with succinic acid. The octasaccharide repeating units of these polysaccharides have been isolated after hydrolysis of the exopolysaccharide with an extracellular specific glucanase from

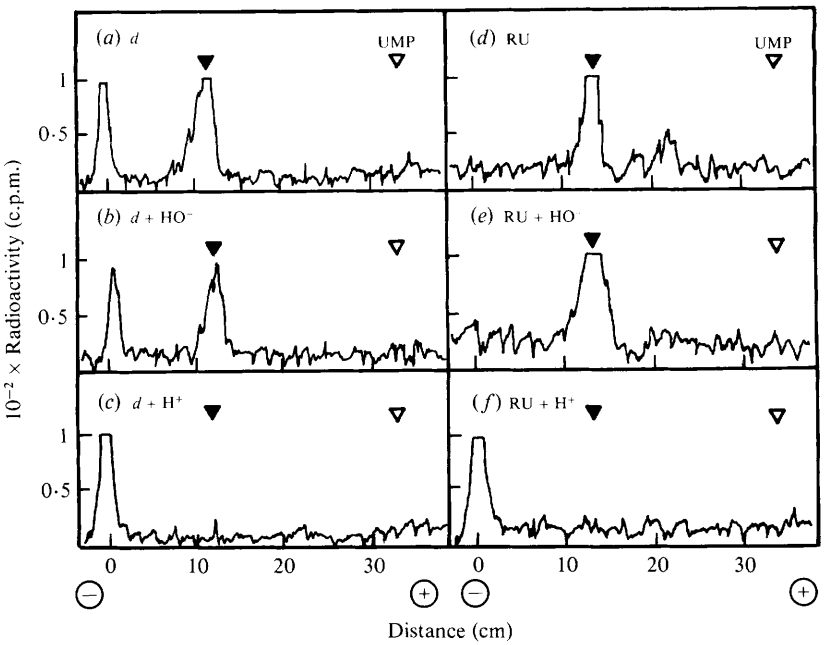

Fig. 7. Paper electrophoresis of compound *d* and the octasaccharide repeating unit of *A. tumefaciens*. (*a*), (*b*) and (*c*) Compound *d* eluted from the thin-layer plate (Fig. 1). (*d*), (*e*) and (*f*) Octasaccharide repeating unit (RU) of *A. tumefaciens* obtained as described in Fig. 6. The compounds were submitted to paper electrophoresis with solvent F for 90 min at 40 V cm^{-1} after the following treatments: (*a*) and (*d*) untreated; (*b*) and (*e*) deacylated (HO^-) with 10 mM-KOH for 5 h at 20 °C; (*c*) and (*f*) depyruvylated (H^+) with 10 mM-HCl for 90 min at 100 °C; ▼, pyruvylated octasaccharide of *Alcaligenes faecalis*; ▽, UMP.

Flavobacterium sp. M64 (Hisamatsu *et al.*, 1980*b*). By using this procedure, the repeating unit of *A. tumefaciens* was prepared (see Methods), and compared with the octasaccharide of *A. faecalis* by TLC. The radioactive substance, either untreated or after deacylation, had a mobility similar to the pyruvylated octasaccharide of *A. faecalis* (Fig. 6). After depyruvylation, the compound migrated like the octasaccharide of *A. faecalis* (Fig. 6). Therefore, the oligosaccharide of *A. tumefaciens* obtained with the depolymerizing enzyme was a pyruvylated octasaccharide. The presence of pyruvic acid was confirmed when a sample of the exopolysaccharide of *A. tumefaciens* was treated with 0·01 M-HCl for 90 min at 100 °C, and the products were submitted to paper electrophoresis as described by Parodi *et al.* (1970). A product having the mobility of pyruvic acid could be detected.

Compounds *d* and *e* were compared with the octasaccharide obtained from the exopolysaccharide of *A. tumefaciens* by different procedures.

(1) Paper electrophoresis: compound *d*, which migrated in TLC like a maltooligosaccharide of six glucose residues (see Fig. 1), was eluted and submitted to paper electrophoresis using solvent F. Two products were detected, one neutral (d_I) and the other (d_{II}) that migrated to the positive pole like the pyruvylated octasaccharide of *A. faecalis* (Fig. 7*a*). The mobility of the latter two compounds was similar to that of the octasaccharide of *A. tumefaciens* (Fig. 7*d*). They were not substituted by succinic acid since after deacylation treatment their electrophoretic mobility did not change (Fig. 7*b–e*). After the depyruvylation treatment, compound d_{II} and the repeating unit of *A. tumefaciens* became neutral (Fig. 7*c–f*). Therefore, the negative charge of these compounds appears to be due to a pyruvic acid residue.

(2) TLC: compounds d_I and d_{II} were separated by paper electrophoresis, eluted and subjected to TLC. The neutral d_I and the negatively charged compound d_{II} before or after alkaline treatment, chromatographed like the pyruvylated octasaccharide standard of *Alcaligenes*

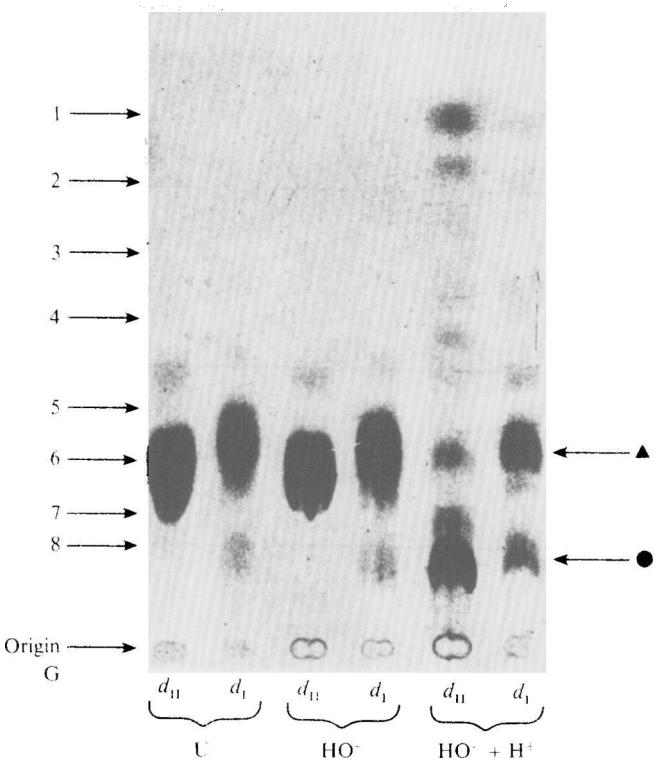

Fig. 8. TLC of compound d_I and d_{II}. Compound d was eluted from the thin-layer plate (Fig. 1) and submitted to paper electrophoresis as described in Fig. 7. The neutral (d_I) and the negatively charged compound (d_{II}) were eluted and chromatographed three times on thin-layer plates with solvent D. The compounds were untreated (U), deacylated (HO^-) or deacylated and depyruvylated ($HO^- + H^+$) as described in Fig. 7. The standards were: pyruvylated octasaccharide (▲) and octasaccharide (●) of *Alcaligenes faecalis* and maltooligosaccharides (G). The compounds were detected as described in Methods. See Fig. 1 legend for definition of numbers.

faecalis and like the maltooligosaccharide of six glucose residues (Fig. 8). After the deacylation and depyruvylation treatments, compound d_I migrated as before while most of compound d_{II} ran like an octasaccharide (Fig. 8). These results indicate that the neutral compound d_I was an unsubstituted hexasaccharide, while the negatively charged compound d_{II} behaved as the pyruvylated octasaccharide of *Alcaligenes faecalis*.

(3) Reduction with borohydride and acid hydrolysis: compound *e* was eluted from the thin layer (Fig. 1) and submitted to deacylation and depyruvylation treatment. The treated and untreated substances were neutral as judged by paper electrophoresis with solvent F and ran like an unsubstituted octasaccharide of *Alcaligenes faecalis* on TLC with solvent D.

Samples of compounds *d* and *e* and the *A. tumefaciens* octasaccharide were reduced with sodium borohydride, hydrolysed and the products chromatographed with solvent C. This experiment was carried out in order to determine the sugar composition of the compounds and the identity of the residue at the reducing end. The three compounds gave only two products, one that migrated like glucose, and the other like galactitol (Fig. 3). The ratio glucose to galactitol was 6·3, 7·5 and 6·9 for compounds *d*, *e* and for *A. tumefaciens* octasaccharide, respectively. The results indicate that *e* and the octasaccharide have seven glucose molecules and one galactose molecule, with the latter at the reducing end. The low ratio for compound *d* is understandable since it turned out to be a mixture of d_I and d_{II}, which contain six and eight hexose residues, respectively.

(4) Methylation analyses: compounds d_{II} and *e* and the *A. tumefaciens* octasaccharide were

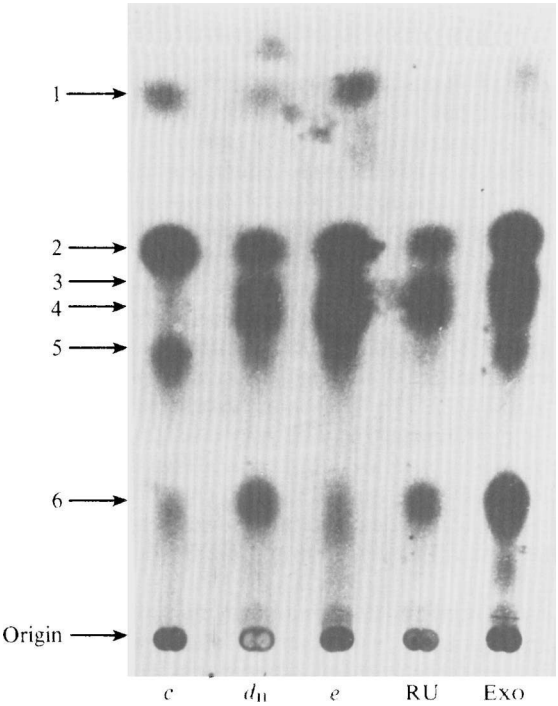

Fig. 9. TLC of the methylation products. Compounds *c*, d_{II}, *e*, the octasaccharide repeating unit (RU) and exopolysaccharide (Exo) of *A. tumefaciens* were methylated, hydrolysed and chromatographed as described in Methods. The standards were: (1) 2,3,4,6-tetra-*O*-methylglucose; (2) 2,3,6-tri-*O*-methylglucose; (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. The compounds were detected as described in Methods.

methylated, hydrolysed and the products subjected to TLC with solvent E. They gave the 2,3,6-, 2,4,6-, 2,3,4-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose (Fig. 9). These are the methylation products expected to be formed from the polysaccharide shown in Fig. 5. Compound d_{II}, the octasaccharide repeating unit and the exopolysaccharide of *A. tumefaciens* also gave 2,3-di-*O*-methylglucose that corresponds to the terminal glucose residue substituted in positions 4 and 6 by pyruvic acid (see Figs 9 and 5). A similar pattern of methylated sugars has been obtained with the exopolysaccharides of *R. meliloti*, *A. radiobacter*, *A. rhizogenes*, *A. tumefaciens* and *Alcaligenes faecalis* by Hisamatsu *et al.* (1980*b*); in this case the methylated sugars were separated by GLC.

Compound *e* did not yield the dimethylglucose derivative and gave instead 2,3,4,6-tetra-*O*-methylglucose that corresponds to the unsubstituted terminal glucose residue. This result is in agreement with the fact that compound *e* behaved as an uncharged substance when it was submitted to paper electrophoresis.

DISCUSSION

Polysaccharides are believed to be involved in the interaction between *Rhizobiaceae* and plant cells. Therefore, the study of the biosynthetic intermediates, the polyprenol-bound saccharides, is of interest.

The incubation of an enzyme preparation of *A. tumefaciens* with radioactive UDP-Glc leads to the labelling of several compounds soluble in organic solvents. They behaved like polyprenyl diphosphate saccharides on paper and DEAE-cellulose chromatography, on treatment with ammonia or hot phenol, and were hydrolysed with 0·01 M-HCl for 10 min at 100 °C.

When the substances were decomposed by mild acid hydrolysis and the water-soluble products were separated by TLC, five radioactive substances were obtained. The fastest moving substance (compound *a*) was identified as galactose. Compound *b* was the disaccharide glucosyl(β1-3)galactose, which had been obtained before with an enzyme from *R. meliloti* (Tolmasky *et al.*, 1980). Compound *c* was characterized as the following tetrasaccharide: Glc(β1-4)Glc(β1-4)Glc(β1-3)Gal. It has the same sequence as a part of the repeating unit of the exopolysaccharide (Fig. 5). The characterization of compounds *d* and *e* was more difficult. Compound *d* could be separated by paper electrophoresis into two components, one neutral (d_I) and the other negatively charged (d_{II}). The neutral compound d_I could not be fully characterized and seemed to be an oligosaccharide containing six hexose residues. Compound d_{II} was characterized as a pyruvylated octasaccharide. It became neutral after the depyruvylation treatment. It behaved like the *A. tumefaciens* octasaccharide after methylation and hydrolysis and gave a glucose:galactitol ratio of 7 after reduction and hydrolysis. Therefore its structure corresponds to that shown in Fig. 5. The lipid-bound saccharides appear to be related, as if the saccharide moiety began to build up starting with galactose (compound *a*) and then grew by the successive addition of glucose to give an octasaccharide which is the repeating unit of *A. tumefaciens*. This relationship has been studied with an enzyme preparation of *R. meliloti*. The growth of the lipid-bound octasaccharide could be followed when the enzyme preparation was incubated for a short time with UDP-[^{14}C]Gal and UDP-Glc was added for a chase period (Tolmasky *et al.*, 1982).

The results reported here were obtained with an enzyme preparation of *A. tumefaciens* LBA 4011, plasmidless strain. In order to study whether the functions of the Ti plasmid are related to the formation of the lipid-bound saccharides, experiments were carried out with an enzyme preparation obtained from the wild-type strain LBA 4001, which contained the virulent plasmid pTiAch5. The products formed with the latter enzymes were identical to the lipid-bound saccharides described in this paper. Besides, these substances are similar to the lipid-bound saccharides described in *R. meliloti* (Tolmasky *et al.*, 1982). The similarity in the structure of the repeating unit of the *A. tumefaciens* exopolysaccharide and the lipid-bound octasaccharide described in this paper indicates a precursor–product relationship. However, experiments designed to prove this point have been unsuccessful up to now. The lipopolysaccharide of *A. tumefaciens* appears to have a more complex sugar composition (Salkinoja-Salonen & Boeck, 1978) and its biosynthesis may involve additional sugar nucleotides besides UDP-glucose.

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