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Biosynthesis of a Galactose- and Galacturonic Acid-Containing Polysaccharide in *Rhizobium meliloti*†

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Previous work showed that two different strains derived from a culture of *Rhizobium meliloti* 102F51 differed with respect to phage specificity, agglutinability by alfalfa seed lectin, and synthesis of a galactose-containing polysaccharide (R. A. Ugalde, J. Handelsman, and W. J. Brill, J. Bacteriol. 166:148–154, 1986). Inner membranes from the more competitive strain incorporated galactose from UDP-galactose when a thermostable factor was present. This factor has now been identified as UDP-galacturonic acid. UDP-glucuronic acid was also active as a donor; however, this activity may be due to the presence of a 4-epimerase. Galacturonic acid, together with galactose, is incorporated into the reaction product, which appears to be a polysaccharide formed by several repeating units of these two monosaccharides. Partial acid hydrolysis liberates the disaccharide with galactose at the reducing end.

Surface polysaccharides of bacteria act as immunochemical determinants (10), lectin receptors (4, 7, 11, 14), and phage receptors (1, 2) and are involved in many other interactions of the cells with their environment (6).

For *Rhizobium meliloti*, two variants have been isolated from a laboratory stock culture of strain 102F51 (4). One of the variants is highly agglutinable (HA) by an alfalfa seed agglutinin, sensitive to phage F20, and resistant to phage 16B. The inverse is true for the other variant; i.e., it shows low agglutinability (LA), resistance to F20 ($F20^r$), and sensitivity to phage 16B ($16B^s$). Furthermore, the latter is more competitive for infecting alfalfa roots. Both variants produce exopolysaccharide.

The inner membranes prepared from LA $F20^r$ $16B^s$ cells incorporated galactose from UDP-galactose into an insoluble polymer when a soluble thermostable factor was present. This reaction was not catalyzed by membranes from variant HA $F20^s$ $16B^r$ cells (16). Spontaneously arising mutants resistant to phage 16B obtained from LA $F20^r$ $16B^s$ were also HA $F20^s$ and did not synthesize the galactose-containing polymer (16). Antibodies against LA $F20^r$ $16B^s$ whole cells precipitated the galactose-containing polymer, whereas antibodies against HA $F20^s$ $16B^r$ cells were ineffective (4).

According to the facts mentioned above, agglutinability with lectin and virus sensitivity are associated with the synthesis of a cell wall polymer containing galactose. Work reported in this paper shows that, besides galactose, the polymer contains galacturonic acid and that the thermostable factor required for galactose incorporation is UDP-galacturonic acid.

MATERIALS AND METHODS

Materials. UDP-[U-^{14}C]galactose (329 Ci/mol) was prepared by a published method (3). UDP-[U-^{14}C]glucuronic acid (284 Ci/mol) and UDP-[U-^{14}C]galacturonic acid (284 Ci/mol) were a gift from M. Dankert. [^{32}P]orthophosphoric acid, carrier free, was obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were analytical grade.

Membrane preparation. Cell membranes were prepared from 30-h cultures (9) of the less agglutinable strain WL131 or WL100 as described previously (16). Total membranes instead of purified inner fractions were used.

Thermostable factor assay. The reaction mixture contained 0.2 mg of total membrane proteins, 100 mM Tris hydrochloride buffer (pH 8.2), 40 mM $MgCl_2$, and 0.85 μCi of UDP-[U-^{14}C]galactose. Total volume was 50 μl. After 30 min at 25°C, the reaction was stopped by adding 0.5 ml of cold 5% trichloroacetic acid (TCA). After filtration through a glass microfiber filter (2.4 cm; Whatman GF/A; Whatman, Inc., Clifton, N.J.), the sample was washed with 20 ml of 5% TCA and 5 ml of methanol and then dried. Radioactivity in the filter was then measured in a scintillation counter. The

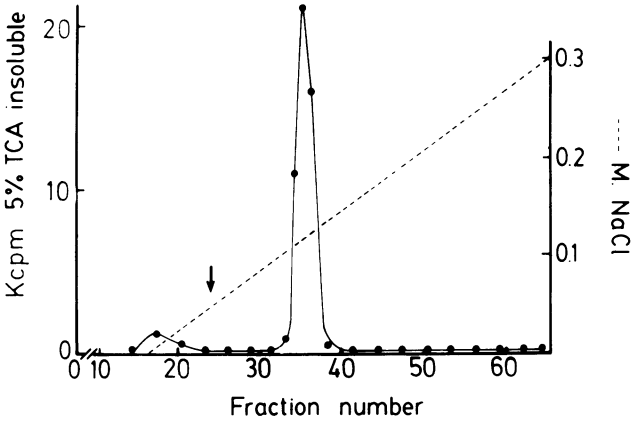

FIG. 1. DEAE chromatography of thermostable factor. The column (2 by 8 cm; model DE52; Whatman) was equilibrated with 15 mM Tris hydrochloride (pH 8.0) and eluted with a linear gradient (0 to 0.3 M) of NaCl. Fractions (3 ml) were collected and assayed for thermostable factor activity. The arrow indicates the elution position of UDP-galactose.

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† This paper is dedicated to Luis F. Leloir on the occasion of his 80th birthday, 6 September 1986.

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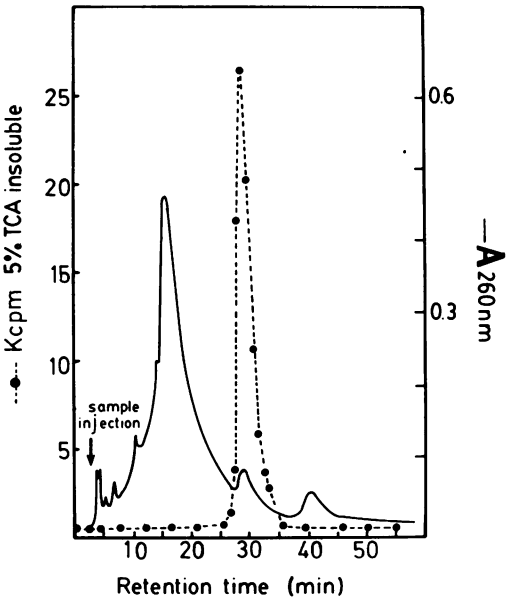

FIG. 2. High-performance liquid chromatography of thermostable factor. Fractions from the experiment described in the legend to Fig. 1 were pooled and concentrated with UM2.0 Diaflo membranes (Amicon). The high-performance liquid chromatography column (Synchropak AX300; Synchron Inc., Linden, Ind.) was eluted with 20 mM phosphate buffer (pH 6.5) at 1,000 lb/in^2 with a flow rate of 1 ml/min. Retention time was 28 min (with 25 mM phosphate [pH 6.5] plus 75 mM NaCl, retention time was 12 min; data not shown).

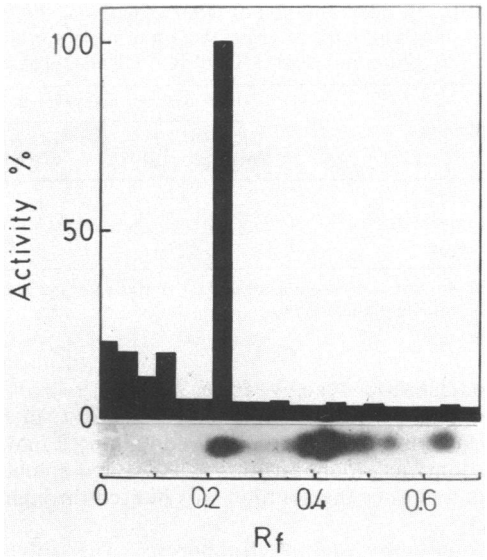

FIG. 4. TLC of thermostable factor. A partially purified preparation was run on TLC plates with solvent F. After radioautography, the substances were eluted with water and tested for biological activity.

stimulation of the incorporation of galactose into the TCA precipitate was taken as a measure of the activity of the thermostable factor.

^{32}P labeling. Cells were grown for 30 h at 30°C in 300 ml of a modified minimal medium (12). The K_2HPO_4 concentration was lowered to 0.13 mM, and the pH was adjusted to 7.2 with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. Carrier-free orthophosphoric acid (2 mCi; New England Nuclear) was added at the beginning of the culture. Cells were harvested by centrifugation (10,000 × *g*, 20 min), washed once with 150 ml of minimal medium with 1.3 mM K_2HPO_4, and suspended in 3 ml of 10 mM Tris-EDTA buffer (pH 8.0). After the cells were frozen and thawed eight times in an acetone–dry-ice bath, the ^{32}P-labeled thermostable factor was recovered from supernatant fluid (centrifugation at 10,000 × *g* for 20 min).

Acid hydrolysis and $NaBH_4$ reduction. Total acid hydrolysis was carried out in sealed ampoules with 1 N HCl at 100°C for 5.5 h, and partial acid hydrolysis was carried out with 0.5 N HCl at 100°C for 30 min. Reduction with $NaBH_4$ was performed as described elsewhere (15).

Phosphodiesterase treatment. TCA-insoluble polysaccharide was incubated with 0.1 U of phosphodiesterase

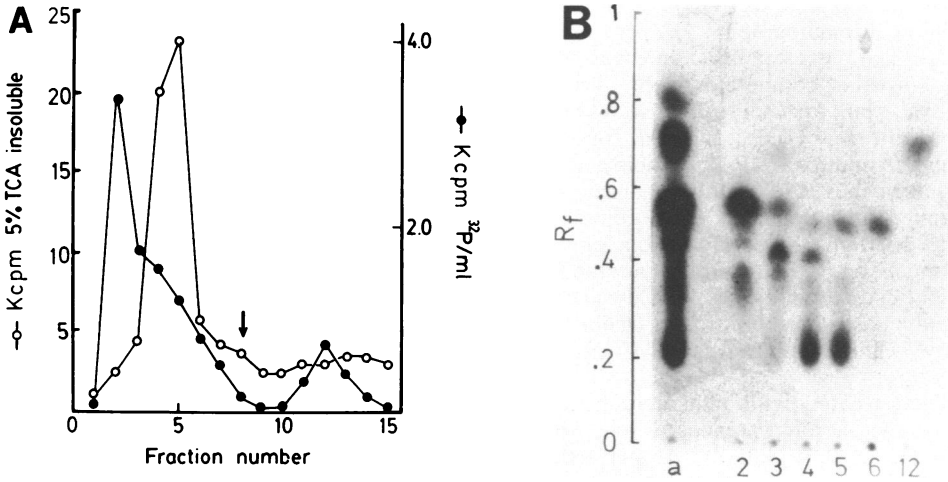

FIG. 3. Chromatography of ^{32}P-labeled thermostable factor on a phenylboronate agarose column. (A) A preparation purified by DEAE chromatography and concentrated by Diaflo filtration was chromatographed in a phenylboronate agarose column of 1.3-ml bed volume and was eluted with 50 mM HEPES buffer (pH 8) with 20 mM $MgCl_2$. Fractions (0.8 ml) were collected, and thermostable factor activity was assayed as described in Materials and Methods. ●, Radioactivity of ^{32}P; ○, biological activity. (B) Radioautography of the TLC run of fractions 2 to 6 and fraction 12. Lane a, Crude extract. Arrow, Elution of column with 100 mM Tris hydrochloride buffer (pH 8.0).

TABLE 1. Incorporation of radioactivity into the 5% TCA-insoluble product after incubation of membranes with UDP-[U-^{14}C]glucuronic acid or UDP-[U-^{14}C]galacturonic acid[a]

Addition	Radioactivity incorporated (kcpm)	
	UDP-[U-^{14}C]-glucuronic acid	UDP-[U-^{14}C]-galacturonic acid
None	1.7	1.5
Thermostable factor[b]	0.7	0.6
UDP-galactose[c]	4.8	15.0

[a] The reaction was carried out as described in Materials and Methods.
[b] 10 μl of partially purified factor.
[c] 20 nmol.

(phosphodiesterase type V from *Bothrops atrox*; Sigma Chemical Co., St. Louis, Mo.) for 12 h at 30°C in 100 mM Tris hydrochloride buffer (pH 8.2) containing 2 mM $CaCl_2$. The reaction was stopped with 5% TCA, and solubilization was estimated after the solution was filtered through a glass fiber filter.

Chromatography and electrophoresis. The solvents for paper chromatography were butanol-pyridine-water (6:4:3; solvent A), isopropanol-glacial acetic acid-water (29:4:9; solvent B), and nitromethane-ethanol-acetic acid-water saturated with boric acid (8:1:1:1; solvent C). The solvents for electrophoresis were 0.1 M sodium molybdate (pH 5; solvent D) and 1.2 M pyridine acetate (pH 6.5; solvent E). The runs lasted 3 h at 16.6 V/cm. The paper used for chromatography or electrophoresis was Schleicher and Schuell 2043 a. Thin-layer chromatography (TLC) was carried out with Polygram Cell 300 plates (20 by 20 cm; Macherey-Nagel Co., Duren, Federal Republic of Germany). The solvent was isobutyric acid–water–35% ammonia–0.1 M disodium EDTA (100:56.6:5.8:1.6; solvent F).

RESULTS

Chromatography of the thermostable factor. The thermostable factor obtained from the supernatant (20,000 × *g*, 20 min) of permeabilized cells as previously described (16) was retained on an anion-exchange column and eluted as a symmetrical peak with 0.12 M NaCl (Fig. 1). It needed a stronger eluate than did UDP-galactose, which was eluted at 0.05 M NaCl. The thermostable factors could be purified by high-performance liquid chromatography. The peak of activity coincided with a peak at A_{260}. Retention time was 28 min, and retention times for other compounds were 13 min for UMP, 12 min for AMP, 3 min for UDP-galactose, 14 min for GDP-mannose, 56 min for UDP, 28 min for UDP-galacturonic acid, and 28 min for UDP-glucuronic acid (Fig. 2). Therefore, the factor behaved like the UDP-uronic acids. When chromatographed in a Sephadex G-15 column, the factor was eluted in a position corresponding to a molecular weight of 660 by comparison to the neutral sugar standards. This molecular weight estimation agrees with the fact that the thermostable factor passed freely through Diaflo UM10 but was retained by Diaflo UM2.0 (Amicon Corp., Lexington, Mass.).

Acid stability. When treated with mild acid (pH 2 at 100°C), the thermostable factor was inactivated, with a half-life of 1.8 min (K = 0.38 min^{-1}). At pH 8.0, the compound was stable.

Presence of phosphate. To detect the presence of phosphate in the compound, a ^{32}P-labeled preparation (see Materials and Methods) was purified by DEAE chromatography, and the active fractions were spotted on TLC plates. Radioactive compounds were localized by radioautography. A ^{32}P-containing compound with an R_f of 0.23 was present only in the active fractions. Pooled active fractions were then chromatographed on phenylboronate agarose. The active compound was retarded in the column, thus indicating that it may have some *cis*-hydroxyl groups (Fig. 3). It was confirmed by a run on TLC that the compound runs with an R_f of 0.23 (Fig. 3). In another experiment, the product partially purified by DEAE chromatography was run on TLC plates as previously described. Radioactive compounds were localized by radioautography, eluted, and tested for biological activity. The results (Fig. 4) show that biological activity clearly corresponded with the radioactive compound with an R_f of 0.23.

Activity of UDP-uronic acids. The thermostable factor seems to have a molecular weight of about 600 and a more negative charge than UDP-galactose. It contains phosphate, absorbs at 260 nm, and is destroyed by heating at 100°C for 10 min at pH 2. These properties are those of a UDP-uronic acid. The chromatographic (TLC and high-performance liquid chromatography) behaviors of UDP-glucuronic acid and UDP-galacturonic acid were the same as that of the thermostable factor. Both compounds were active in the biological test. When the assay was carried out as described above, with no supplement, 4 × 10^3 cpm of ^{14}C-galactose was incorporated into the 5% TCA-insoluble fraction. Supplementation with 10 μl of partially purified thermostable factor gave 32 × 10^3 cpm, whereas 22 × 10^3 or 20 × 10^3 cpm were incorporated when 20 nmol of UDP-galacturonic or UDP-glucuronic, respectively, was added. The addition of 10 μl of partially purified thermostable factor plus 20 nmol of UDP-galacturonic acid incorporated 25 × 10^3 cpm. Other sugar-nucleotides such as GDP-mannose, UDP-glucosamine, UDP-*N*-acetylglucosamine, and UDP-glucose were inactive (data not shown). To ascertain which of the two UDP-uronic acids was the most active substrate, another experiment was carried out (Table 1). The two UDP-uronic acids were incubated under identical conditions with UDP-galactose and membranes from strain WL131. The incorporation was higher with UDP-galacturonic acid. However, the fact that both compounds were active suggested the presence of an epimerase. In agreement with this possibility, when labeled UDP-glucuronic acid was preincubated with

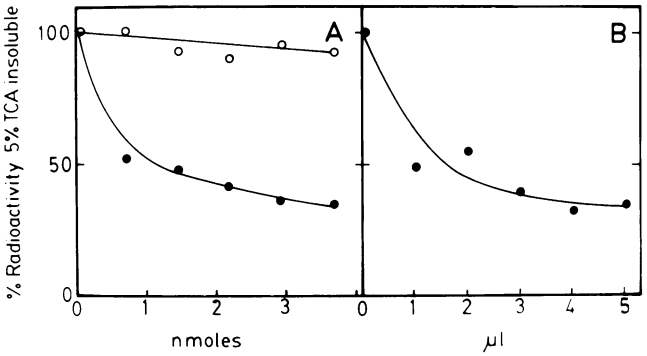

FIG. 5. Competition of the incorporation of radioactivity into the 5% TCA-insoluble fraction with UDP-glucuronic acid, UDP-galacturonic acid, or thermostable factor. Membranes (0.2 mg of protein) were incubated with UDP-[U-^{14}C]glucuronic acid (0.85 μCi) for 30 min. After that, 0.02 μmol of UDP-galactose and the indicated amounts of UDP-glucuronic acid (○) or UDP-galacturonic acid (●) were added as competitors (A). Competition was also carried out with partially purified thermostable factor (B).

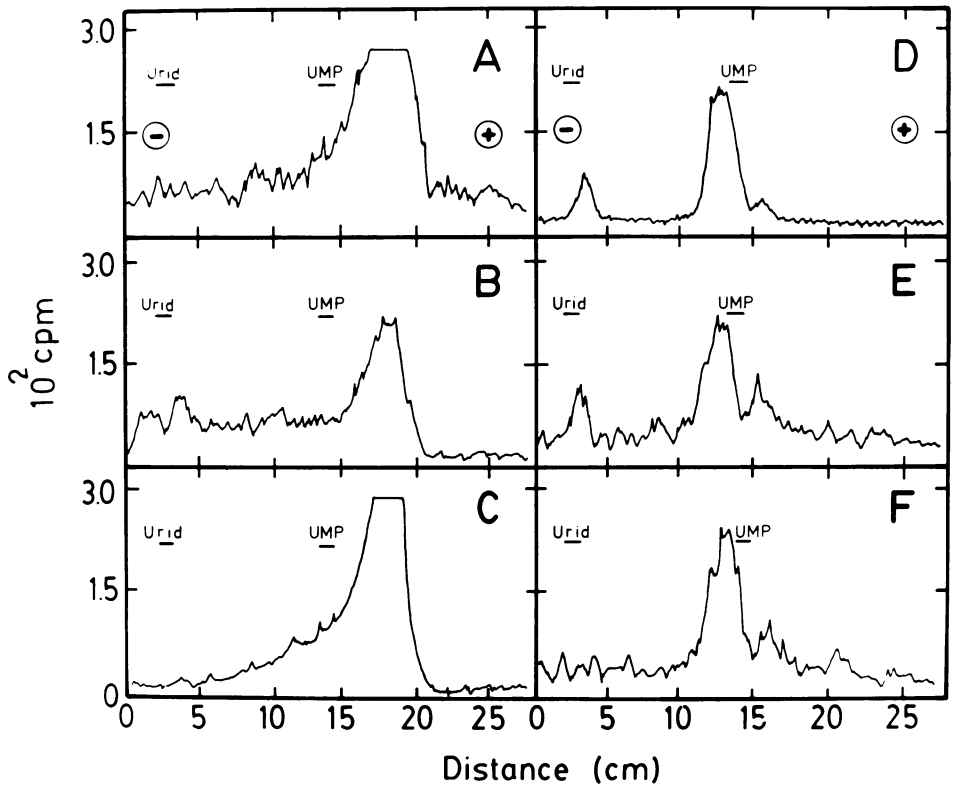

FIG. 6. High-voltage electrophoresis of the reaction products and their partial acid hydrolysis. Electrophoresis was carried out with solvent E as described in Materials and Methods. The polysaccharide was obtained from the 5% TCA-insoluble fraction by heating in 5% TCA at 100°C for 3 min, and it was desalted in Bio-Gel P2 columns. The products were prepared as follows: (A) UDP-[U-^{14}C]galactose and thermostable factor; (B) UDP-[U-^{14}C]galactose and UDP-galacturonic acid; (C) UDP-[U-^{14}C]galacturonic acid and UDP-galactose. (D, E, and F) Electrophoresis of the products tested for panels A, B, and C after partial acid hydrolysis.

the membranes before the reaction mixture was completed by adding unlabeled UDP-galactose, the transfer of radioactivity into the product was 4.8 × 10^3 cpm when the preincubation time was 15 s, and it increased to 9.1 × 10^3 and 17.4 × 10^3 cpm after 15 and 30 min, respectively.

In another experiment, membranes were preincubated with UDP-[^{14}C]glucuronic acid. After 30 min, UDP-galactose and increasing amounts of nonlabeled UDP-galacturonic acid or UDP-glucuronic acid were added. The former competed with the incorporation of radioactivity into the 5% TCA-insoluble product, while no effect was obtained with UDP-glucuronic acid (Fig. 5). The addition of increasing amounts of partially purified thermostable factor had the same effect as UDP-galacturonic acid (Fig. 5). The identity of the uronic acid transferred was ascertained by total acid hydrolysis of the reaction product followed by paper electrophoresis in solvent D. Only labeled galacturonic acid was recovered, irrespective of whether the reaction was carried out with the UDP derivative of labeled glucuronic or galacturonic acid.

Reaction product. As mentioned before, the sugar moieties of UDP-galactose and UDP-galacturonic acid were incorporated into the 5% TCA-insoluble residue. Maximal incorporation required the presence of the two UDP derivatives. The product formed from labeled-UDP-galactose and thermostable factor became soluble after being heated in 5% TCA. The solubilization was the same for the products with the label in the galactose (68%), the galacturonic acid (65%), or the thermostable factor (66%).

The three products prepared as described in Materials and Methods were chromatographed on Bio-Gel P2, and the substances in the void volume were then subjected to paper electrophoresis in solvent E. The results are shown in Fig. 6. The products were heterogeneous and highly anionic, the most abundant species having a R_{UMP} of 1.32. After partial acid hydrolysis (0.5 N HCl, 100°C, 30 min) followed by electrophoresis in solvent E, the products yielded a main peak with a R_{UMP} of 0.90 (Fig. 6D, E, and F). This compound, when chromatographed on paper with solvent B, gave a peak with $R_{glucose}$ of 0.44, that is, in a position between maltose and maltotriose. The same compound chromatographed on Bio-Gel P2 eluted in the position expected for a disaccharide (Fig. 7). Total acid hydrolysis yielded either labeled galactose or galacturonic acid, depending on the precursor used (Fig. 8). When the two precursors were radioactive, these appeared in the product with a ratio of 1.

Reduction of the disaccharide with borohydride followed by acid hydrolysis gave galactitol (Fig. 8), showing that galactose is at the reducing end.

These results suggest that the product contains a repetitive structure in which galactose and galacturonic acid are alternatively combined.

Other properties of the reaction product. The polysaccharide obtained by mild acid treatment (0.01 N HCl, 100°C, 10 min) of the 5% TCA-insoluble fraction was chromatographed on Bio-Gel P4 columns. Although the product was heterogeneous, the most abundant species had molecular weights of about 3,200 and 3,800. These weights would correspond to 8 to 10 repeating units. When the acid treatment was

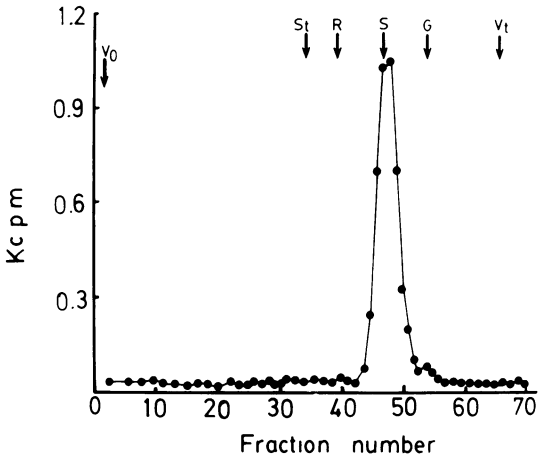

FIG. 7. Bio-Gel P2 chromatography of the disaccharide. The product obtained after partial acid hydrolysis as described for Fig. 8D (R_{UMP} = 0.9) was eluted and chromatographed on a Bio-Gel P_2 column (0.7 by 114 cm) with pyridine-acetate buffer (0.1 M, pH 5). Blue dextran and $CoCl_2$ were used as markers of the exclusion and total volume. Glucose (G), raffinose (R), stachyose (St), and sucrose (S) were used as standards. The products obtained from the electrophoresis whose results are shown in Fig. 8E and F eluted from the column at the same position (not shown).

stronger (0.1 N HCl, 100°C, 10 min), a small proportion of the product became smaller, corresponding to 4 and 5 repeating units (data not shown). Under both conditions, more than 90% of the radioactivity incorporated into the polysaccharide was solubilized.

Preliminary results showed that more than 90% of the polysaccharide was solubilized after treatment with phosphodiesterase (data not shown). This result suggested that the polysaccharide is linked to a high-molecular-weight TCA-insoluble acceptor through a phosphodiester.

Acid treatment (0.5 N HCl, 100°C) for different times led to a progressive decrease in the size of the product with the final accumulation of the disaccharide (after 30 min). The pattern was the same whether the label was in the galactose or in the uronic acid.

DISCUSSION

The transfer of galactose from UDP-galactose to an anionic polymer by membranes prepared from LA F20^r 16B^s variants of *R. meliloti* has been further studied. The thermostable factor which activated the reaction has now been identified as UDP-galacturonic acid.

The reaction product is a polysaccharide formed by a repeating unit containing galactose and galacturonic acid. The polysaccharide is somewhat heterogeneous; the most abundant species contains about 10 repeating units according to its elution volume from Bio-Gel P4 (data not shown). The disaccharide chains are joined to a high-molecular-weight residue which is precipitable by TCA. Mild-acid treatment liberates the chains, and they become soluble in TCA. The nature of the linkage between the chains and the insoluble residue is not known, but the acid lability and the result of phosphodiesterase treatment suggest that the link is a phosphodiester. Further acid treatment leads to hydrolysis of the bond between the C-1 of the galactose and galacturonic acid, leading finally to the liberation of a disaccharide. This hydrolysis occurs as expected, since it is known that the glycosidic linkage of glucuronides is relatively stable. The product containing the galactose-galacturonic acid repeating unit can be included within the teichuronic acid family (8, 17). Several members of this family with varied monosaccharides and uronic acid repeating units have been detected in gram-positive microorganisms. In capsular polysaccharides of *Pneumococcus* sp. type III, a similar structure with glucuronic acid-glucose as the repeating unit was found (5, 13). So far, these types of polysaccharides have not been described for *Rhizobium* species.

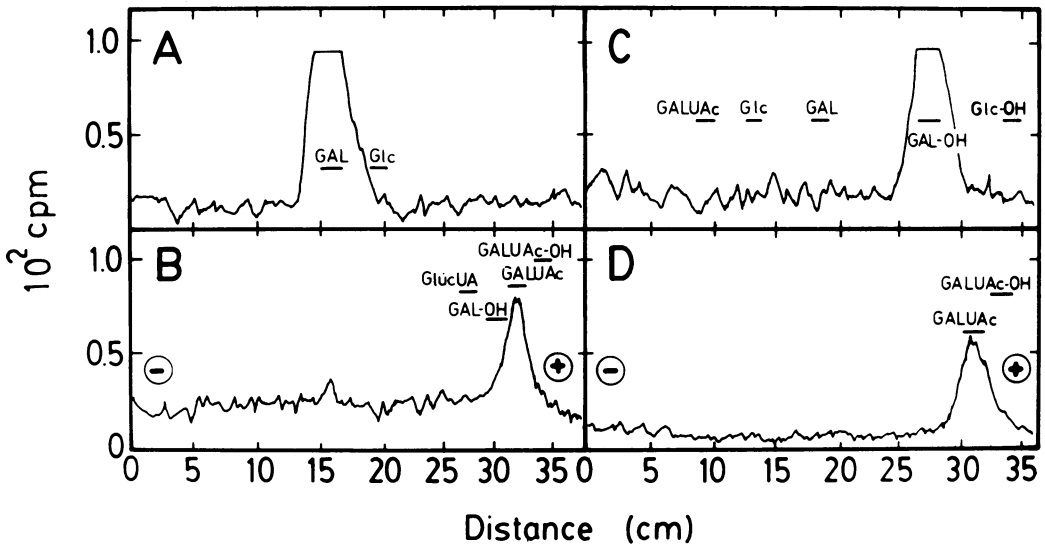

FIG. 8. Total acid hydrolysis of the disaccharide. Results are from (A) paper chromatography with solvent A of the total hydrolysis product of the substance described in the legend to Fig. 6E; (B) electrophoresis with solvent D of substance described in the legend to Fig. 6F after total hydrolysis; (C) paper chromatography with solvent C of substance described in the legend to Fig. 6E after reduction with $NaBH_4$ and total hydrolysis; (D) electrophoresis with solvent D of substance described in the legend to Fig. 6F after reduction and hydrolysis. GAL, Galactose; Glc, glucose; GALUAc, galacturonic acid; GlucUA, glucuronic acid; GAL-OH, galactitol; Glc-OH, glucitol; GALUAc-OH, galactitol uronic acid.

It is interesting that the presence of the galacturonic acid-galactose polysaccharide correlates with several physiological properties. The polysaccharide presumably interferes with the interaction of the bacteria with alfalfa agglutinin and is probably the receptor of the 16B phage. Furthermore, it seems to have a role in competitiveness between rhizobia for nodulation sites, since it is present in the more competitive strains of *R. meliloti*. This study seems to be the first report of a defined chemical change found to be associated with competitiveness.

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