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# Role of Galactosyltransferase Activity in Phage Sensitivity and Nodulation Competitiveness of *Rhizobium meliloti*

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**A stock culture of *Rhizobium meliloti* 102F51 contains colonies of two distinct phenotypes (Handelsman et al., J. Bacteriol. 157:703–707, 1984); one colony type is agglutinated by high dilutions of the alfalfa agglutinin, is sensitive to phage F20, and is resistant to phage 16B, and the other is agglutinated only by low dilutions of the alfalfa agglutinin, is resistant to phage F20, and is sensitive to phage 16B. Cells of the latter phenotype have an inner-membrane-bound galactosyltransferase activity that transfers galactose from UDP-galactose to a water-insoluble anionic polymer. This enzymatic activity is absent in cells of the first phenotype. All of the phage 16B-resistant mutants selected from a sensitive strain were agglutinated by high dilutions of the alfalfa agglutinin, were sensitive to phage F20, and lacked galactosyltransferase activity. The galactose-containing polymer prepared in vitro was immunologically cross-reactive with the cell surface.**

Nodulation of legumes by *Rhizobium* spp. is a process of interdependent steps. It has been proposed that specific recognition of *Rhizobium* spp. at legume root surfaces is required for infection (17), and it has been suggested that bacterial cell surface polysaccharides play an important role in this recognition. A hypothesis originally developed for *Rhizobium phaseoli* (4) and *Rhizobium japonicum* (1) proposed that specific carbohydrate-binding proteins present on the surfaces of root hairs are involved in attaching the bacteria to root surfaces. A protein that specifically agglutinates *Rhizobium meliloti* cells was identified in alfalfa seeds and on the roots. A possible role in recognition and infection of alfalfa by *R. meliloti* has been proposed for this protein (13).

We previously described cell surface variants that arose from a single stock culture and differed in their abilities to recognize the alfalfa agglutinin, in their sensitivities to phages F20 and 16B, and in their competitiveness in nodulation of alfalfa (5). The strain which is highly agglutinable with the alfalfa agglutinin (HA), sensitive to phage F20 ($F20^s$), and resistant to phage ($16B^r$) nodulates alfalfa more slowly and is less competitive than the strain that is less agglutinable (LA), resistant to phage F20 ($F20^r$), and sensitive to phage 16B ($16B^s$). Some *Rhizobium* spp. cell surface mutants that interact less with plant lectins and are resistant to certain phages (3, 6, 7) are unable to nodulate their hosts ($Nod^-$ phenotypes). In contrast, the surface changes of the LA $F20^r$ $16B^s$ strains led to a more competitive phenotype (5). Characterization of the biochemical differences between cells with these two phenotypes might help in understanding the role of *R. meliloti* cell surfaces in nodulation. In this paper we suggest that some of the more competitive strains have a galactosyltransferase activity that is involved in the biosynthesis of an antigen, which may be involved in the surface changes that affect nodulation.

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## MATERIALS AND METHODS

**Media and chemicals.** Bacteria were grown on AMA medium, a yeast extract-mannitol medium (9). UDP-[U-$^{14}$C]galactose (250 to 360 Ci/mol), GDP-[U-$^{14}$C]mannose (200 Ci/mol), UDP-[U-$^{14}$C]*N*-acetyl-D-glucosamine (200 Ci/mol), and UDP-[1-$^{3}$H]galactose (10 to 25 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

**Bacteria and phages.** *R. meliloti* 102F51 was obtained from Nitragin Co., Milwaukee, Wis. All of the derivatives of strain 102F51 used and the isolation of *R. meliloti* phages F20 and 16B have been described previously (5).

**Permeabilized cells.** The bacteria were grown on AMA medium at 30°C with good aeration for 20 or 40 h, centrifuged at 10,000 × *g* for 10 min, washed once with fresh medium, and suspended in 10 mM EDTA adjusted to pH 8.0 with Tris base (ET8.0 buffer). To carry out permeabilization, cells in ET8.0 buffer were frozen and thawed eight times in a dry ice-ethanol bath. After permeabilization, the cells were collected by centrifugation at 10,000 × *g* for 10 min, and the first supernatant was saved as the source of thermostable factor (TF). The pellet was washed four times with ET8.0 buffer and then was suspended in 10 mM EDTA (pH 7.0)–10 mM β-mercaptoethanol (EB7.0 buffer) (final volume 1/1000th of the original culture volume). The cells were stored at −20°C until they were used.

**Preparation of membranes.** Cell membranes were prepared from 30-h cultures (12, 14). Inner and outer enriched membrane fractions were collected and washed by centrifugation at 100,000 × *g* for 2 h, suspended in EB7.0 buffer, and stored at −20°C until they were used.

**Galactose transferase assay.** Galactose transferase activity was assayed by using permeabilized cells or membrane fractions in 100 mM Tris hydrochloride buffer (pH 8.2) containing 40 mM $MgCl_2$, 0.16 μM UDP-[$^{14}$C]galactose, and amount of TF that gave the maximal response. The final reaction volume was 50 μl. After incubation for 30 min at 25°C, one of the methods described below was used to stop the reaction and measure the incorporation of [$^{14}$C]galactose into the product.

**(i) Method A.** The reaction mixture was adjusted to a

chloroform-methanol-water ratio of 3:2:1 by adding 0.4 ml of methanol, 0.6 ml of chloroform, and 0.15 ml of 4 mM $MgCl_2$. Heat-inactivated cells or membranes were added to form an interphase. After centrifugation at 400 × *g* and room temperature for 10 min, the upper phase was discarded, and the lower phase and the insoluble interphase were separated and recovered. Three additional extractions with chloroform-methanol-water (3:2:1) were performed on the insoluble interphase. All of the lower phases were washed three times with chloroform-methanol-water (3:48:47) and dried in a glass scintillation vial, and the $^{14}C$ in this fraction was counted with a Packard Tri-carb scintillation counter. The insoluble interphase was washed three times with methanol and dissolved in Protosol (New England Nuclear Corp.), and the amount of $^{14}C$ incorporated was determined.

**(ii) Method B.** Alternatively, the reaction was stopped by adding 0.5 ml of cold 5% trichloroacetic acid (TCA), the mixture was filtered through glass microfiber filters (Whatman type GF/A; diameter, 2.4 cm), washed with 20 ml of 5% TCA and 5 ml of methanol, and dried, and the amount of $^{14}C$ retained in the filter was determined as described above for method A. When this procedure was used, 2.0 mM UMP was added to the incubation mixture (see Results).

**Isolation of the [$^{14}C$]galactose-containing polymer.** From the insoluble interphase (method A) or the 5% TCA-insoluble fraction (method B), the [$^{14}C$]galactose-containing polymer was solubilized by acid treatment (5% TCA, 3 min, 100°C) or alkaline treatment (0.1 N NaOH, 5 min, 65°C). The reaction product solubilized by either of these methods was partially purified by gel chromatography as described below.

**Total acid hydrolysis.** The $^{14}C$-labeled reaction product partially purified by gel chromatography was treated with 1.0 M HCl at 100°C for 4 h. $^{14}C$-labeled sugars were identified by thin-layer chromatography on cellulose plates (Polygram Cel 300; Macherey-Nagel Co., Durin, Federal Republic of Germany) which were developed with butanol-pyridine-water 6:4:3 (solvent A). Radioactivity was detected by autoradiography, and standards were detected by using alkaline silver nitrate.

**Periodate oxidation.** Oxidation was performed by using 0.05 M $NaIO_4$ at 5°C for 72 h. The reaction was stopped by adding an excess of ethylene glycol (10%, vol/vol). The [$^{14}C$]formic acid formed was analyzed by paper electrophoresis (0.1 M Tris hydrochloride buffer, pH 8.0; 400 V; 12 mA; 40 min). Under these conditions, the ratio of the migration of formic acid to the migration of ATP was 1.7. [U-$^{14}C$]methylgalactoside was used as the standard, and oxidation was considered complete when 16% of the radioactivity was recovered as [$^{14}C$]formic acid. The periodate oxidation product described above was reduced with 100 mg of $NaBH_4$ per ml for 48 h at room temperature in the dark, and the reduction was stopped by acidification with acetic acid, followed by desalting in a Bio-Gel P2 column (Bio-Rad Laboratories, Richmond, Calif.). The periodate oxidation product was subjected to total acid hydrolysis as described above, and the products were analyzed by thin-layer chromatography on cellulose plates by using solvent A. Radioactivity and standards were detected as described above.

**Preparation of antisera against bacterial cells.** A 300-ml culture that had been grown for 24 h was centrifuged and suspended in 5 ml of phosphate-buffered saline (100 mM potassium phosphate, pH 6.8, 150 mM NaCl). Three-month-old New Zealand White rabbits were bled from the central ear vein to obtain preimmune sera. The marginal ear vein was injected with 0.1, 0.2, 0.3, and 0.4 ml of cell suspension on subsequent days. After skipping 1 day, 0.5 ml was injected, and after 2 weeks 0.5 ml was injected. The rabbits were boosted twice at 2-week intervals with 0.5 ml and 1 week after the last boost the rabbits were bled from the central ear vein. The blood was allowed to clot for 30 min at room temperature and then overnight at 4°C and then centrifuged, and the supernatant was stored at −20°C.

TABLE 1. Incorporation of [$^{14}C$]galactose into the organic phase and insoluble fraction by permeabilized cells

| Strain | Phenotype | Age of culture (h) | Amt of galactose incorporated (pmol/min per mg of protein)[a] | | | |
|---|---|---|---|---|---|---|
| | | | Organic phase | | Insoluble fraction | |
| | | | −TF | +TF | −TF | +TF |
| WL200 | HA $F20^s$ $16B^r$ | 20 | 5.2 | 4.3 | 1.8 | 1.9 |
| | | 40 | 20.0 | 18.5 | 1.4 | 1.8 |
| WL100 | LA $F20^r$ $16B^s$ | 20 | 9.4 | 5.3 | 6.9 | 23.8 |
| | | 40 | 19.3 | 14.8 | 4.0 | 15.0 |
| WL131 | LA $F20^r$ $16B^s$ | 20 | 2.8 | 1.9 | 3.4 | 23.1 |
| | | 40 | 18.7 | 13.0 | 4.9 | 19.5 |
| WL199 | LA $F20^r$ $16B^s$ | 20 | 5.0 | 3.5 | 6.2 | 26.3 |
| | | 40 | 20.8 | 20.7 | 3.1 | 15.6 |

[a] The reactions were carried out as described in Materials and Methods at 15°C for 30 min by using washed permeabilized cells (100 μg of protein). A saturating amount of crude TF (10 μl) was added to some of the reaction mixtures.

**Immunological precipitation of the galactose-containing polymer.** The reaction between antibodies raised against bacterial cells and the galactose-containing polymer prepared in vitro was measured by using the *Staphylococcus aureus* cell precipitation method, with some modifications (8). Antiserum diluted in phosphate-buffered saline was incubated with the polymer for 15 min at room temperature and for 15 min at 4°C. *S. aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.) were added in *S. aureus* cell buffer (8) modified to contain 50 mM Tris hydrochloride, 140 mM NaCl, 5 mM $NaN_3$, and 0.5% Nonidet P-40 (pH 7.6) and incubated on ice for 30 min. The cells were removed by centrifugation and washed twice with *S. aureus* buffer. The final pellet was suspended in 0.1 ml of *S. aureus* cell buffer, and the radioactivity in the pellet was determined by counting in 5 ml of Bray solution (2).

## RESULTS

**Incorporation of [$^{14}C$]galactose by different strains of *R. meliloti*.** Permeabilized cells of 15 strains with the LA $F20^r$ $16B^s$ phenotype incorporated 8 to 13 times more galactose into an insoluble fraction than 10 strains with the HA $F20^s$ $16B^r$ phenotype. Washing the permeabilized cells released a low-molecular-weight TF into the solution. Addition of the TF to the galactose incorporation reaction mixture significantly increased the incorporation of galactose into the insoluble fraction by washed permeabilized LA $F20^r$ $16B^s$ cells but had no effect on galactose incorporation in HA $F20^s$ $16B^r$ cells (Table 1).

The TF was present in both HA and LA cells and in other *Rhizobium* species. The TF had a molecular weight of approximately 600, as determined by ultrafiltration and gel chromatography on Sephadex G-15 performed by using glucose, maltose, rafinose, and stachiose as standards. It was heat stable at pH 8.0 (100°C, 10 min) and heat labile at pH 2.0 (100°C, 10 min). The identity of the TF is being studied and will be the subject of a forthcoming paper. The TF did not stimulate the incorporation of galactose into the

TABLE 2. Effect of 5'-UMP and 5'-UDP on the incorporation of [$^{14}$C]galactose into the organic and insoluble fractions

| Prepn | Amt of galactose incorporated (pmol/min per mg of protein)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Without UMP | | With 2 mM UMP | | With 2mM UDP | |
| | −TF | +TF | −TF | +TF | −TF | +TF |
| Organic phase | 19.2 | 14.7 | 0.5 | 0.2 | 11.5 | 9.0 |
| Insoluble fraction | 4.0 | 15.0 | 3.2 | 12.5 | 0.8 | 3.0 |

[a] Reactions were carried out with washed permeabilized cells from a 40-h culture of strain WL100 as described in Table 1, footnote *a*.

organic phase by washed permeabilized cells of either phenotype (Table 1).

Incorporation of [$^{14}$C]galactose into the organic phase under conditions similar to those used in this study has been examined in other strains of *R. meliloti* (16). The products that accumulated were polyprenyl diphosphate galactose and polyprenyl diphosphate galactose-β-1,3-glucose, which are precursors in the biosynthesis of lipid-linked saccharides identical to the repeating unit of the exopolysaccharide (15, 16). The $^{14}$C-labeled products recovered from the organic phase became water soluble after mild acid treatment (pH 2, 100°C, 10 min), yielding a monosaccharide and a disaccharide. The monosaccharide migrated as galactose, and the disaccharide migrated as cellobiose on paper chromatograms (Whatman no. 1 filter paper) when solvent A was used. The disaccharide was eluted from the chromatograms with water and was subjected to total acid hydrolysis (4 M HCl, 100°C, 4 h), yielding glucose and galactose at a ratio close to 1, as identified by thin-layer chromatography with solvent A (see Materials and Methods). Similar results have been reported for another strain of *R. meliloti* (16). These compounds were identified as lipid intermediates in the synthesis of the exopolysaccharide (15). However, incorporation of galactose into the insoluble fraction differed from incorporation into the lipid intermediate in a number of ways. First, incorporation of galactose into the lipid intermediates depended on the growth phase of the culture. Maximum incorporation of galactose into the lipid intermediates was obtained at stationary phase when the cells were actively producing exopolysaccharides (40 h after inoculation under our culture conditions). In late logarithmic phase (20 h), the incorporation was 52 to 85% lower (Table 1). Similar results have been reported for another strain of *R. meliloti* (16). However, the culture growth phase had little effect on the incorporation of galactose into the insoluble fraction (Table 1). These results suggest that the lipid intermediates are not precursors in the biosynthesis of the product that is recovered in the insoluble fraction. Furthermore, as shown in Table 2, 2 mM 5'-UMP inhibited 94 to 98% of the incorporation of [$^{14}$C]galactose from UDP-[$^{14}$C]galactose into the organic phase. However, 5'-UMP inhibited only 10 to 25% of the incorporation of galactose into the insoluble fraction. 5'-UMP inhibited incorporation of galactose into the lipid intermediate because it was one of the products of the reversible reaction that transferred galactose 1-phosphate from UDP-galactose to the polyprenyl monophosphate acceptor (16). The lack of inhibition by 5'-UMP of incorporation of galactose into the insoluble fraction suggests that the lipid intermediates are not precursors of this product and indicates that, in this reaction, galactose and not galactose 1-phosphate is transferred to the product. The strong inhibition of incorporation of galactose into the insoluble fraction by 5'-UDP (Table 2) supports this conclusion.

When 5'-UMP was included in the incubation mixture, the galactosyltransferase activity which we studied could be assayed by determining the incorporation of galactose into the 5% TCA-insoluble fraction. This procedure could not be used when 5'-UMP was not added since the lipid diphosphate sugars formed were recovered in the 5% TCA-insoluble fraction and, as a result, the effect of the TF was masked and the difference between LA F20$^r$ 16B$^s$ and HA F20$^s$ 16B$^r$ strains could not be attached. Using the TCA precipitation method, we studied the presence of galactosyltransferase activity in many strains derived from the original culture. The enzyme activity was found only in LA F20$^r$ 16B$^s$ cells (Table 3).

No incorporation of radioactivity into the 5% TCA-insoluble fraction was obtained when the permeabilized cells were incubated with GDP-[$^{14}$C]mannose or UDP-[$^{14}$C]*N*-acetyl-D-glucosamine with or without added TF. When UDP-[$^{14}$C]glucose was used as the substrate, however, significant amounts of radioactivity were recovered in the 5% TCA-insoluble fraction. To determine the effect of the TF on glucose incorporation, the incorporation of [$^3$H]galactose and [$^{14}$C]glucose from UDP-[$^3$H]galactose and UDP-[$^{14}$C]glucose into the insoluble fraction was assayed simultaneously with the permeabilized LA F20$^r$ 16B$^s$ cells. The addition of the TF increased the incorporation of [$^3$H]galactose from 1.6 to 19.8 pmol/min per mg of protein; the values obtained with [$^{14}$C]glucose were 1.6 and 1.8 pmol/min per mg of protein without and with added TF, respectively. These results indicated that the effect of the TF is specific for the incorporation of galactose into the product recovered in the insoluble fraction.

TABLE 3. Presence of galactosyltransferase activity in permeabilized cells and inner membranes prepared from different strains of *R. meliloti*

| Strain | Phenotype | Galactosyltransferase activated by TF | | |
|---|---|---|---|---|
| | | Permeabilized cells[a] | Inner membranes[b] | |
| | | | −TF | +TF |
| WL200 | HA F20$^s$ 16B$^r$ | 1.1 | 5.7 | 5.7 |
| WL201 | HA F20$^s$ 16B$^r$ | 0.9 | ND[c] | ND |
| WL203 | HA F20$^s$ 16B$^r$ | 1.2 | ND | ND |
| WL205 | HA F20$^s$ 16B$^r$ | 1.0 | ND | ND |
| WL218 | HA F20$^s$ 16B$^r$ | 1.3 | ND | ND |
| WL251 | HA F20$^s$ 16B$^r$ | 1.1 | 7.6 | 7.6 |
| WL252 | HA F20$^s$ 16B$^r$ | 0.8 | 7.6 | 9.5 |
| WL299 | HA F20$^s$ 16B$^r$ | 1.0 | 13.3 | 13.3 |
| WL131-1 | HA F20$^s$ 16B$^r$ | 1.4 | 5.7 | 5.7 |
| WL131-2 | HA F20$^s$ 16B$^r$ | 0.9 | 5.7 | 5.7 |
| WL100 | LA F20$^r$ 16B$^s$ | 10.0 | 5.7 | 83.6 |
| WL109 | LA F20$^r$ 16B$^s$ | 13.5 | ND | ND |
| WL112 | LA F20$^r$ 16B$^s$ | 12.0 | ND | ND |
| WL199 | LA F20$^r$ 16B$^s$ | 14.4 | 9.5 | 121.6 |
| WL131 | LA F20$^r$ 16B$^s$ | 10.6 | 7.6 | 95.0 |
| WL113 | LA F20$^r$ 16B$^s$ | 13.0 | 7.6 | 106.4 |
| WL101 | LA F20$^r$ 16B$^r$ | 0.64 | ND | ND |
| WL105 | LA F20$^r$ 16B$^r$ | 0.9 | ND | ND |
| WL111 | LA F20$^r$ 16B$^r$ | 1.0 | ND | ND |
| WL121 | LA F20$^r$ 16B$^r$ | 1.2 | ND | ND |

[a] The values indicate the stimulation of the incorporation of galactose into the insoluble fraction upon the addition of a saturating amount of TF.

[b] Amount of galactose incorporated into the TCA-insoluble fraction (in picomoles per minute per milligram of protein).

[c] ND, Not determined.

**Localization and properties of the galactosyltransferase activity.** The results obtained with washed permeabilized cells suggested that the galactosyltransferase activity described above is not soluble. This activity was recovered in the total membrane fraction. Further fractionation of the total membranes into inner and outer membranes showed that this activity was enriched in the inner membrane fraction (Table 4). As with permeabilized cells, the activity was highest in inner membranes prepared from LA F20$^r$ 16B$^s$ cells. When the TF was not added to the inner membranes, the incorporation of galactose into the TCA-insoluble fraction was lower than the incorporation when permeabilized cells were used. This was due to the difficulty in washing the TF out of the permeabilized cells. It was possible, after extensive washing, to obtain preparations of permeabilized cells with lower basal activity (data not shown). These results suggest that the galactosyltransferase activity that distinguishes LA F20$^r$ 16B$^s$ strains from HA F20$^s$ 16B$^r$ strains has an absolute requirement for the TF (Tables 3 and 4).

Several properties of the galactosyltransferase were studied by using inner membranes as the enzyme source. The reaction had an optimum temperature of 25°C, with a sharp decline at 30°C (Fig. 1). The same temperature response was observed with permeabilized cells. Experiments in which the reaction was carried out with UDP-[$^{14}$C]galactose at 25°C for 20 min and then chased at 25 or 30°C with an excess of unlabeled UDP-galactose demonstrated that the lower accumulation at 30°C was not due to a loss of radioactive counts from the TCA-insoluble fraction.

At 25°C the reaction was linear up to 30 min and had an optimum pH of 8.2, and the effect of the TF was saturable (Fig. 1). The reaction followed Michaelis-Menten kinetics with an apparent $K_m$ for UDP-galactose of 1.6 μM. The reaction had an absolute requirement for $Mg^{2+}$, which could not be replaced by $Ca^{2+}$ or $Mn^{2+}$.

The following results obtained were comparable when inner membranes or permeabilized cells were used: (i) the TF was required, and its effect was specific for the incorporation of galactose; (ii) the optimum temperature for galactose incorporation was 25°C, with a sharp decrease in incorporation at 30°C; (iii) the reaction had a narrow pH range, with a pH maximum of 8.2; (iv) the reaction was not inhibited by UMP at concentrations that inhibited 97 to 98% of the incorporation of galactose into the lipid intermediate; and (v) the products recovered from the TCA-insoluble fraction were identical by several criteria (see below).

We assayed the galactosyltransferase activity in the inner membranes prepared from different HA F20$^s$ 16B$^r$ and LA F20$^r$ 16B$^s$ strains. This enzyme activity was present only in strains with the latter phenotype. Strains WL199 and WL299 are examples of strains that have a distinctive colony morphology when they are grown on agar plates (glossy) which distinguishes them from all other strains (which have a matte type of colonies). As shown in Table 3, galactosyltransferase activity was present only in LA F20$^r$ 16B$^s$ cells and was independent of the colony morphology.

Spontaneous 16B$^r$ mutants were obtained from different LA F20$^r$ 16B$^s$ strains. As previously described (5), 90 to 95% of the 16B$^r$ colonies were also HA and F20$^s$. Inner membranes prepared from these mutants were tested for galactosyltransferase activity. All of the HA F20$^s$ 16B$^r$ strains selected from LA F20$^r$ 16B$^s$ strains by using phage resistance had lost galactosyltransferase activity (Table 3, strains WL251, WL252, WL131-1, and WL131-2). These results further demonstrated that galactosyltransferase activity is associated with cell surface differences between two phenotypes.

Attempts to obtain LA F20$^r$ 16B$^s$ cells from HA F20$^s$ 16B$^r$ cells by selecting phage F20-resistant mutants were unsuccessful. All of the F20-resistant mutants yielded a third phenotype (LA F20$^r$ 16B$^r$). Galactosyl-transferase activity was not present in any of these isolates (Table 3, strains WL101, WL105, WL111, and WL121). Thus, galactosyltransferase activity is present only in cells that are sensitive to phage 16B, and the transition from 16B$^s$ to 16B$^r$ correlates with the loss of the enzymatic activity.

We performed an experiment to determine whether the lack of incorporation of galactose by HA F20$^r$ 16B$^s$ strains was due to the absence of the enzymatic activity or was due to (i) a lack of an appropriate endogenous acceptor, (ii) the

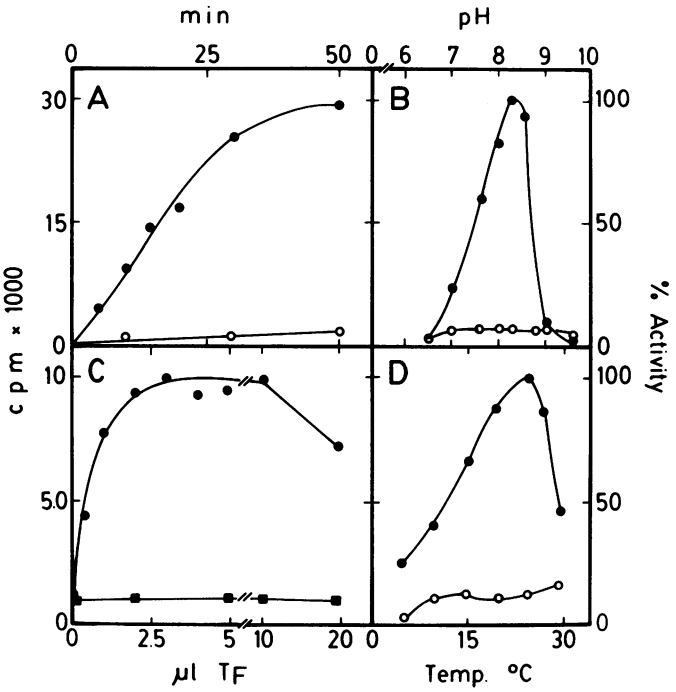


FIG. 1. Effects of time of incubation (A), pH (B), amount of TF (C), and temperature (D) on the activity of the galactosyltransferase in inner membranes of *R. meliloti* WL100. Symbols: ●, with TF; ○, without TF; ■, effect of the addition of TF on the incorporation of galactose into the 5% TCA-insoluble product by inner membranes of strain WL/100.

TABLE 4. Galactosyltransferase activities in inner and outer membranes

| Strain | TF addition | Amt of galactose incorporated (pmol/min per mg of protein)[a] | |
|---|---|---|---|
| | | Inner membranes | Outer membranes |
| WL200 | − | 3.8 | 5.7 |
| | + | 3.8 | 5.7 |
| WL100 | − | 3.8 | 1.9 |
| | + | 87.4 | 24.7 |
| WL131 | − | 3.8 | 3.8 |
| | + | 87.4 | 30.4 |

[a] Incorporation of galactose into the 5% TCA-insoluble fraction. The inner and outer membrane fractions were prepared as described in Materials and Methods. Where indicated, the reaction mixture was supplemented with crude TF (10 μl).

TABLE 5. Galactosyltransferase activity in mixtures of inner membranes from *R. meliloti* strains WL100 (LA F20$^r$16B$^s$) and WL251 (HA F20$^s$ 168$^r$)

| Inner membranes mixture | Amt of galactose incorporated (pmol/min per mg of protein)[a] |
|---|---|
| WL100 | 114.0 |
| WL100 (heated)[b] | 1.1 |
| WL251 | 3.8 |
| WL251 (heated) | 1.1 |
| WL100 + WL251 (heated) | 76.0 |
| WL251 + WL100 (heated) | 3.8 |
| WL251 + WL100 | 96.9 |

[a] Amount of galactose incorporated into TCA-insoluble product.
[b] Heated for 5 min at 60°C.

presence of inhibitors, or (iii) inactivation of the TF. As shown in Table 5, when the reactions were performed with heat-inactivated inner membranes from a strain having one phenotype mixed with active membranes from a strain having the other phenotype, the enzymatic activity was detected only when active inner membranes from LA F20$^r$ 16B$^s$ cells were used. Heat-inactivated inner membranes from LA F20$^r$ 16B$^s$ cells did not generate any galactosyltransferase activity in the inner membranes from HA F20$^s$ 16B$^r$ cells. The addition of active or heat-inactivated inner membranes from HA F20$^s$ 16B$^r$ cells did not inhibit the galactosyltransferase activity of the LA F20$^r$ 16B$^s$ strains. These results suggested that the differences in the incorporation of galactose was due to the lack of enzymatic activity in the membranes of the HA F20$^s$ 16B$^r$ cells and not to the presence of an inhibitor(s) or inactivation of the TF.

**Galactosylated product.** The galactosylated product of the reaction described above could be solubilized from the TCA-insoluble fraction by the following two methods: (i) mild acid hydrolysis with 5% TCA at 100°C for 3 min or (ii) alkaline treatment with 0.1 N NaOH at 65°C for 3 min. Total acid hydrolysis (1 N HCl, 100°C, 4 h) of either of the soluble products released [$^{14}$C]galactose as the only radioactive sugar, as identified by paper chromatography and thin-layer chromatography (see Materials and Methods).

Gel chromatography of the radioactivity solubilized by the two methods showed that [$^{14}$C]galactose was incorporated into a polymer. The alkaline treatment solubilized a product (designated the alkaline product) that eluted in the void volume on a Sephadex G-75 column. The radioactivity solubilized by the acid treatment (designated the acid product) eluted from the same column at positions that suggested

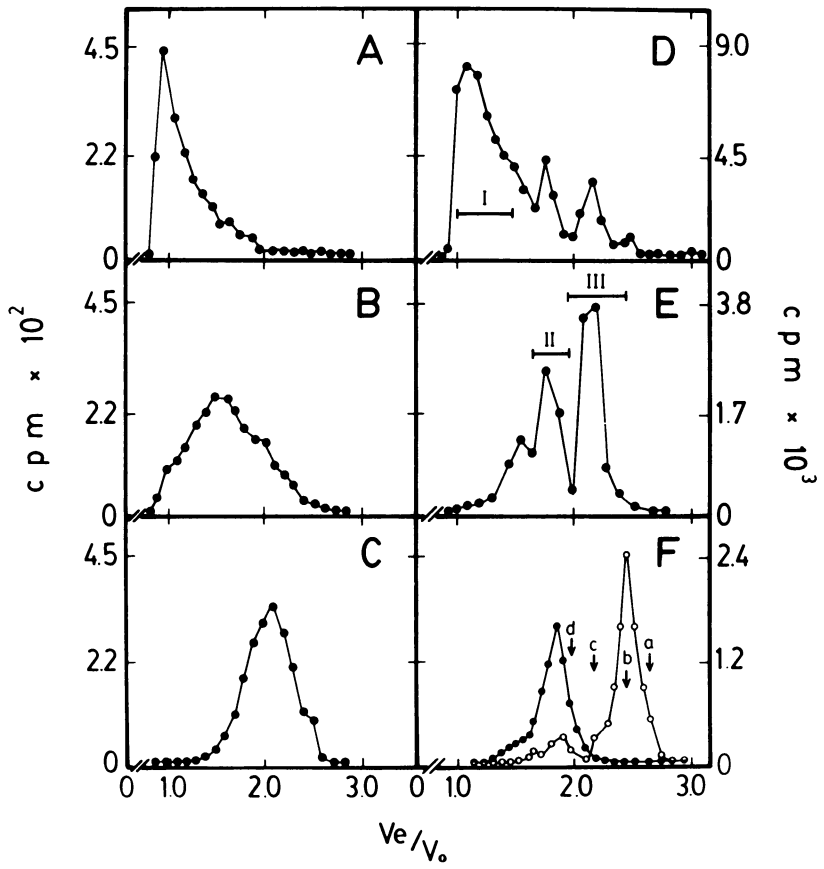


FIG. 2. Partial acid degradation of the $^{14}$C-labeled galactosylated product. (A through C) elution profiles in Sephadex G-50 columns of the products obtained after 3 min (A), 10 min (B), and 20 min (C) of heating at 100°C in 5% TCA. (D) Gel chromatography in Sephadex G-25 columns of the products recovered from the experiment shown in panel C. (E) Gel chromatography in a Sephadex G-25 column of product I pooled and recovered from the experiment shown in panel D after further treatment (20 min) with 5% TCA at 100°C. (F) Gel chromatography in Bio-gel P2 of products II (●) and III (○) recovered from the experiment shown in panel E. The standards used were galactose (a), maltose (b), rafinose (c), and stachiose (d).

a mixture of lower-molecular-weight products. No free [$^{14}$C]galactose was recovered by either method.

When the alkaline product that was recovered from the Sephadex G-75 column was treated with 5% TCA under the same conditions that were used to recover the acid product from the insoluble fraction, it became smaller and heterogeneous according to the elution profile from a Sephadex G-75 column. This indicated that the acid products were generated by partial acid degradation from the alkaline product.

To determine whether the products that were recovered after the acid treatment were heterogeneous due to partial degradation, we determined a time course of solubilization. From either permeabilized cells or inner membranes, 95% of the radioactivity was solubilized after 3 min of heating at 100°C in 5% TCA. The half-time for solubilization was the same (0.8 min) whether the product was prepared with permeabilized cells or inner membranes. An analysis of the products recovered at different times by gel chromatography in Sephadex G-50 columns showed that the longer the treatment was, the smaller and more heterogeneous the products were (Fig. 2A through C). When the products recovered after 20 min of acid treatment (Fig. 2C) were further acid treated and the resulting products were analyzed by gel chromatography in Sephadex G-25 columns, we found that three compounds containing [$^{14}$C]galactose accumulated (Fig. 2D and E). These compounds eluted from a Bio-Gel P2 column as oligomers containing two and four hexose units (Fig. 2F). Paper electrophoresis of the products recovered from the Bio-Gel P2 column, as well as of the products from the 3-min acid treatment (Fig. 2A), indicated that all of these products were negatively charged (data not shown). The product was a complex structure rather than a homopolymer of galactose since partial acid degradation did not release free [$^{14}$C]galactose but did release charged fragments.

It seemed possible that in vitro galactose was terminally added to a charged acceptor present in the membrane. This possibility was ruled out because periodate oxidation of the product labeled with [$^{14}$C]galactose did not result in the formation of detectable amounts of [$^{14}$C]formic acid. Oxidation with periodate followed by reduction with $NaBH_4$ and total acid hydrolysis of the same $^{14}$C-labeled product showed that [$^{14}$C]galactose was not destroyed during the treatment and that no detectable amounts of [$^{14}$C]glycerol, [$^{14}$C]threitol, and [$^{14}$C]erythreitol were recovered. These results indicated that the galactose residues are substituted in position 3 or in positions 2 and 4. Further analysis will be required to chemically characterize this polymer; however, our results suggest that the galactose is not terminal.

When the alkaline product that was recovered from the void volume of the Sephadex G-75 column (eluted with 0.1 M pyridine acetate buffer, pH 5.0) was dried, it was no longer possible to solubilize it in water. When this dried alkaline product was treated for 3 min at 100°C with 5% TCA, 100% of the [$^{14}$C]galactose became soluble as a product that was identical to the product recovered by acid treatment from the insoluble fraction, as shown in Fig. 2A. The dried alkaline product could be solubilized in 1% sodium dodecyl sulfate or in triethylamine–0.1 M EDTA buffer (pH 7.2). In these two solvents the product eluted in the void volume of a Sephadex G-200 column, suggesting that it had a very high molecular weight, although it is possible that the apparent high molecular weight was due to the formation of micelles. The results which we obtained suggest that galactoses are transferred to an endogenous nonlabeled acceptor which is present in the membranes and confers to the product insolubility in water and 5% TCA, and the high-apparent-molecular-weight [$^{14}$C]galactose-labeled portion of the product can be removed from the endogenous acceptor by acid treatment (3 min, 100°C), resulting in the recovery of a galactose-containing anionic polymer which is freely soluble in water. Lipopolysaccharide (LPS) would have similar properties, but we were unable to recover any radioactivity in phenol-water extracts or with phenol-chloroform-petroleum-ether (1:2.5:0.4). This suggests that this product is not related to LPS.

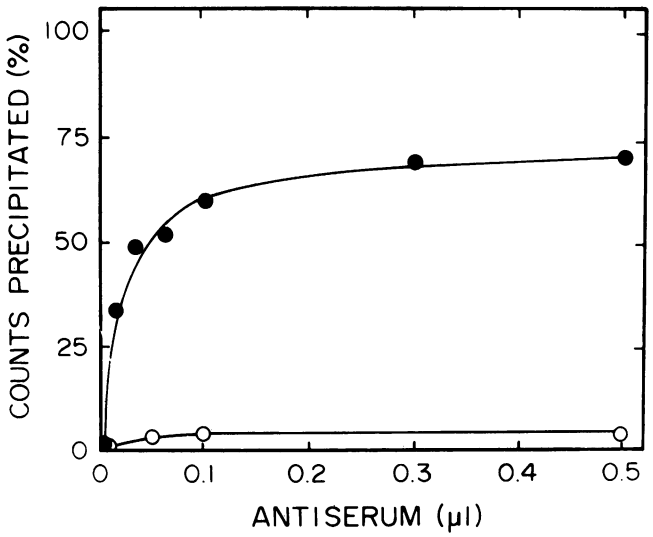


FIG. 3. Immunoprecipitation of the galactose-containing polymer by antibodies raised against strain WL200 cells (○) or strain WL100 cells (●). Both antisera precipitated the cells against which they were raised at the same final titer.

The alkaline product that was partially purified by Sephadex G-200 chromatography was precipitated by antibodies raised against LA F20$^r$ 16B$^s$ whole cells. Antibodies raised against HA F20$^s$ 16B$^r$ cells did not precipitate the product (Fig. 3). When the alkaline product was acid treated, the immunoreactivity was lost. These results showed that the product of the galactosyltransferase synthesized in vitro was present on the surfaces of LA F20$^r$ 16B$^s$ cells and was absent from LA F20$^s$ 16B$^r$ strains. The fact that the acid treatment destroyed the immunoreactivity suggested that the acid product was degraded in such a way that the original structure and the antigenicity present in the alkaline product were modified.

## DISCUSSION

We identified one step in the in vitro synthesis of an *R. meliloti* antigen whose presence is correlated with the ability of phage 16B to infect cells. The galactose-containing antigen is probably the receptor for phage 16B and is probably also the cell surface entity that modifies the interaction of the cells with the alfalfa agglutinin and with phage F20. The agglutinin receptor is probably the phage F20 receptor since every F20$^r$ cell is agglutinated by low dilutions of the alfalfa agglutinin. According to this interpretation, there are the following two mechanisms for obtaining LA strains: (i) when galactosyltransferase activity is present, as in LA F20$^r$ 16B$^s$ strains, it modifies or masks the F20 receptor, generating the receptor for phage 16B on the cell surface; or (ii) when galactosyltransferase is absent, the F20 receptor itself can be modified, as it may be in the LA F20$^r$ 16B$^r$ cells selected by resistance to phage F20 from HA F20$^s$ 16B$^r$ strains. Prelim-

inary evidence suggests that some of the LA strains selected for resistance to phage F20 have altered LPSs which may be the receptors for the alfalfa agglutinin and phage F20 (Handelsman, unpublished data).

Examples of modification of phage receptors by glycosyltransferases are well documented in *Salmonella* spp. (10, 11), in which the addition of galactose to an incomplete LPS core structure allows the cells to complete the O antigen of the LPS, resulting in the organism becoming sensitive to phages that use the O antigen as a receptor and resistant to phages that use the incomplete LPS as a receptor.

The greater competitiveness of LA strains (5) may be due to the action of the galactosyltransferase, which may modify the bacterial cell surface so as to increase its interaction with the root surface.

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