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Involvement of *Rhizobium japonicum* O Antigen in Soybean Nodulation

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Non-nodulating mutant strains of *Rhizobium japonicum* lacked a surface antigen that was present on the wild type. This surface antigen is associated with the O antigen portion of the lipopolysaccharide. Paper chromatography of hydrolyzed lipopolysaccharide and O antigen revealed three major component differences between the non-nodulating strains and the wild type.

The biochemical steps involved in establishing the N_2-fixing symbiosis between *Rhizobium* and its legume host plant are beginning to be understood. An early step appears to be a recognition between the two symbionts, resulting in binding the bacteria to the root (9, 19). This recognition is believed to occur by the cross-bridging of a root protein (lectin), which binds to the surface of the bacterium (8). The recognition seems to be quite specific—the lectin from soybeans (*Glycine max*) binds only to *R. japonicum* (2, 4, 23), and the lectin from clover (6, 8) binds only to *R. trifolii*.

The lectin recognition hypothesis necessitates that a unique receptor for lectin be present on the surface of *Rhizobium* cells. It was proposed that legume lectins interact with a polysaccharide on the surface of the bacteria (4). The lectin from clover binds to an acidic heteropolysaccharide present in the capsule of *R. trifolii* (8). Other cell-surface polysaccharides reported as the receptor site for lectin binding include a nonlipopolysaccharide (LPS) acidic heteropolysaccharide from the cell walls of *R. leguminosarum* (18) and the O antigen moiety of intact LPS (23). Preliminary findings indicate that these lectins are enzymes that degrade the LPS of their symbiont rhizobia (1).

Mutant strains of *R. japonicum* 61A76 that specifically were unable to fix N_2 for soybeans have been isolated (13). These strains were obtained by screening survivors of a mutagenesis for effective nodulation of soybeans. Two of the mutant strains, SM1 and SM2, did not nodulate soybeans. We studied these strains in more detail to determine their biochemical defects.

MATERIALS AND METHODS

Chemicals and media. Agarose type 1 (used for immunodiffusion) and protease type 6 were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade, available commercially.

The medium used for *R. japonicum* growth has been described previously (3).

LPS and O-antigen isolation. Cells were harvested in late exponential phase when the cell density had reached approximately 3×10^9 cells per ml. The cells were centrifuged in a Sharples centrifuge and freeze-dried for storage. LPS was isolated by using a procedure described previously with modifications (23). A 2.5-g amount of dry cells was suspended in 50 ml of water (65°C), and 45 ml of phenol (65°C) was added. The mixture was shaken in a water bath (65°C) for 15 min, cooled to 4°C, and centrifuged for 20 min at 8,000 × *g*. The water phase was removed, 45 ml of water (65°C) was added to the phenol phase, and the above extraction procedure was repeated. The two water phases were pooled and extracted 10 times with 25 to 35 ml of diethyl ether. The water phase (2 ml) was then dialyzed at 4°C against 2 liters of distilled water for 4 days (two daily changes) and then freeze-dried. The dried extract was dissolved in a minimal amount of water and placed onto an AG1X10 (Bio-Rad Laboratories, Richmond, Calif.) anion exchange resin in the acetate form. The water-eluted polysaccharide contains LPS and is designated fraction A. NaCl (1 M) was applied to the column, and the acidic polysaccharide was collected (designated fraction B). To monitor the elution of polysaccharide, the phenol-sulfuric acid method of sugar detection was used (16). All LPS and O-antigen concentrations are based on this sugar assay, using glucose as the standard. Fraction B amounts to less than 10% of the carbohydrate applied to the anion exchange column. The LPS in fraction A was further purified as a high-molecular-weight polysaccharide by gel filtration on Sepharose 4B equilibrated with 20 mM imidazole-HCl (pH 7.0) containing 80 mM NaCl (23).

LPS hydrolysis. The O antigen-containing portion of LPS was removed by treating LPS with 1% glacial acetic acid for 1 h at 100°C, cooling the preparation on ice for 15 min, and centrifuging the mixture for 30 min at 6,000 × *g* (15). The supernatant solution, containing the O antigen-R core was removed, and the acetic acid in this fraction was removed by rotary

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evaporation. This polysaccharide (obtained from fraction A) is designated fraction A'.

Paper chromatography. Polysaccharide samples (fractions A, A', and B) were prepared for chromatography by boiling the sample (8 mg of glucose equivalents) in 2 N HCl for 6 h. This hydrolysis was performed in tightly sealed tubes under N_2 gas. After evaporation of the acid, the samples were dissolved in water and spotted onto Whatman 3MM filter paper (57 cm in length). The paper was placed into a chromatography cabinet and equilibrated with the solvent vapors for 6 h. The solvent system used was the upper phase of a mixture of butanol, benzene, pyridine, and water (5:1:3:3, vol/vol). After 14 h of descending development, the chromatogram was dried in a fume hood for 1.5 h. Components were detected by spraying the chromatogram with 0.5 N NaOH in ethanol, after dipping the chromatogram in silver nitrate-acetone solution (21). One hour after spraying with sodium hydroxide, the chromatograms were washed with water.

Antisera. Antiserum was prepared against wild-type whole cells that had a colony type, on a variety of media, identical to the colony type of strains SM1 and SM2. Antiserum was prepared against whole washed cells suspended in 0.85% NaCl solution. The cells were administered intravenously into New Zealand white rabbits every 3 days for a total of nine immunizations. The first, second, and third sets of three immunizations contained 1.5×10^8, 5.0×10^8, and 1.5×10^9 cells, respectively. The rabbits rested for 6 days and then were exsanguinated by cardiac puncture. The blood was clotted for 1 h at 37°C and held at 4°C for 1 h, and the clot was removed. The serum was centrifuged at $1,000 \times g$ for 10 min, and the supernatant solution was incubated at 56°C for 1 h to inactivate the complement.

Antiserum against wild-type O antigen (fraction B) was initiated in rabbits by subcutaneous injections of an emulsion made up of 0.5 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and 0.5 ml (200 μg) of O antigen in 0.85% NaCl. After 1 week, rabbits received subcutaneous injections of 200 μg of fraction B suspended in Freund incomplete adjuvant once weekly for the next 4 weeks. Beginning 5 weeks after the first immunization, 100 μg of fraction B in 0.85% NaCl was administered intravenously every other day for a total of five injections, and the rabbits were bled 3 days after the final injection.

Antisera adsorption. *R. japonicum* SM1 was grown on agar medium for 8 to 11 days and washed with 0.85% NaCl solution. Two milliliters of antiserum (prepared against wild-type whole cells or fraction B) was adsorbed four times with whole cells as described previously (8). The final adsorbed antiserum did not react with strain SM1 cells. The polysaccharide-adsorbed antiserum was prepared by five successive removals of immune precipitates after incubation of 0.5 ml of antiserum with 50 μg of fraction A or B.

Thin-layer chromatography. LPS and other polysaccharide components were separated and identified by thin-layer chromatography by using isobutyric acid–NH_4OH–water (57:4:39, vol/vol) as the solvent, followed by spraying with diphenylamine solution and heating at 85°C for 15 min (5).

Agglutination of whole cells by antisera. Cells were grown on agar plates at 30°C for 8 to 10 days and suspended to a cell density of 10^9 cells per ml in saline solution. Dilutions of the antisera were prepared so that 0.2 ml of the appropriate dilution of antisera remained in a sterile plastic tube (10 by 75 mm). Each tube received 0.2 ml of the cell suspension to be tested, and the contents of the tube were mixed. The tubes were scored for agglutination after 4 h of incubation at 37°C.

Heptose analysis. Polysaccharide samples (50 μg) were analyzed for heptose by the cysteine-H_2SO_4 method (24).

RESULTS AND DISCUSSION

Mutant strain SM1 was previously shown to be unable to nodulate soybeans, but was identical to the wild type with respect to many properties. These include lysis by seven different phages that form plaques on the wild type, growth rate on minimal and rich media, colony morphology on a variety of media, and asymbiotic reduction of acetylene (13). We wanted to determine the biochemical defect in this strain, and we reasoned that the strain might have a missing or modified cell surface component, which normally plays an integral role in the initial stages of infection.

Antiserum prepared against whole cells of the wild type was titered with whole cells of the wild type and strain SM1 after adsorption. Strain SM1 was unable to remove all of the agglutinating antibodies that were made against the wild type (Table 1). The antigen(s) missing in strain SM1 was present in strains SM3 and SM5, which form ineffective nodules (13). When the antiserum was adsorbed with strain SM5 cells, all of the agglutinating antibodies were removed. Thus, it seems that strain SM1 lacks an antigenic surface component that is present in

TABLE 1. *Agglutination of mutant strains with antiserum prepared against wild-type whole cells*

Strain	Agglutination titer[a]		
	Unadsorbed antiserum	Adsorbed with strain SM1	Adsorbed with strain SM5
Wild type	2,250	16	0
SM1	1,250[b]	0	0
SM3	2,250	16	0
SM5	2,000	8	0

[a] Reciprocal of the highest dilution giving positive cell agglutination. The antiserum was diluted 1:1 in 0.85% NaCl before use and had a previous end-point titer (using undiluted antiserum) against wild-type cells of 1:5000.

[b] The difference in end-point titer between strain SM1 and the wild type is more than one tube dilution. Intermediate dilutions tested include 1,500, 1,750, and 2,000.

nodulating strains. The SM1-adsorbed antiserum (Table 1) was readsorbed with strain SM1 and was still as reactive with the wild type. The difference between strain SM1 and the wild type, therefore, is not due to a quantitative difference in the amount of common antigenic material produced by the strains.

To identify the site on *R. japonicum* that was responsible for the antigenic difference between the wild type and strain SM1, various cell-surface components were purified and analyzed. Antigenic differences between infective and noninfective strains of *R. trifolii* (8) were due to differences in an acidic capsular polysaccharide antigen. Therefore, an initial goal was to purify a capsular polysaccharide from the wild type and strain SM1. Although we could detect small amounts of acidic polysaccharide (by light and electron microscopy) on the surface of cells after staining with alcian blue (14) and ruthenium red (17), we could not isolate polysaccharide material from the strains by 1% phenol extraction and precipitation with quaternary ammonium salts (8, 16). Most strains of *Rhizobium* are gummy because they have a polysaccharide capsule (10); however, the wild-type strain used in these experiments does not form gummy colonies.

Other surface polysaccharides were then examined. LPS is thought to be involved in binding *Rhizobium* to the lectin from the corresponding host plant (23). During purification of LPS (fraction A) from the wild type and mutant strain SM1, we detected another polysaccharide fraction that bound to the anion exchange column (fraction B). This fraction could be eluted with 1 M NaCl. Fraction B seems to be related antigenically to LPS. Passive hemagglutination of rabbit erythrocytes coated with fraction B, by antiserum prepared against wild-type whole cells, was eliminated by preadsorption of the antiserum with fraction A. Fraction A removed antibodies capable of binding to fraction B. Antiserum was prepared against fraction B and tested for reaction with whole wild-type cells after adsorption with fraction A. This adsorbed antiserum no longer reacted with wild-type whole cells. These experiments show that fraction B shares at least one common antigenic determinant with fraction A.

A study on *Serratia marcescens* endotoxin (22) showed that the O-specific side chain of LPS can be generated from whole cells or LPS when the hot phenol-water method is used for polysaccharide extraction. To determine whether fraction B was possibly generated from fraction A during the extraction procedure, we repeated this extraction procedure with fraction A as the starting material. The resulting polysaccharide fractions were separated by anion-exchange chromatography. A polysaccharide (fraction B′) analogous to fraction B was produced by this phenol treatment of fraction A. Both fraction B and B′ migrated identically in thin-layer chromatograms. Thus, fraction B could have been generated from fraction A by the extraction procedure.

We wondered whether fraction B contained the O antigen portion of the LPS. LPS was treated by a mild acid hydrolysis, a method commonly used to split the O antigen portion from the LPS (15, 20). The resulting O antigen portion, fraction A′, migrated identically to the fraction B on thin-layer chromatograms. Fraction A had a significantly different R_f on the same chromatogram. Both fractions A′ and B reacted with antiserum prepared against fraction B, forming reactions of identity in an Ouchterlony plate.

The linkage between the O antigen and R core is sensitive to cleavage by phenol (22). To test the possibility that fraction B is the specific O-antigen portion (unlinked to the R core) of LPS, heptose was assayed as a marker for the R core. Fractions A and A′ contained heptose, whereas fraction B did not. Therefore, fraction B is probably the O-specific portion of LPS. Alternatively, fraction B could be a lipid-free acidic polysaccharide present on the cell surface in addition to the O-antigen polysaccharide, which is linked to lipid LPS. Some strains of *Escherichia coli* contain a lipid-free acidic polysaccharide, which has the same chemical composition and structure as the O antigen obtained from their LPS (11, 12). The acidic heteropolysaccharide in the capsule of *R. trifolii* also is a lipid-free O antigen (F. B. Dazzo and W. J. Brill, unpublished data).

Fractions analogous to A, B, B′, and A′ also were prepared from strain SM1, and differences between these fractions and those obtained from the wild type were examined. Antiserum prepared against the wild-type whole cells was adsorbed with strain SM1 fractions A and B. The adsorbed antiserum was still reactive with wild-type fraction B (Fig. 1); however, this antiserum did not react with strain SM1 fraction B. This shows that the wild-type O antigen contained an antigenic determinant that was absent in fractions A or B from strain SM1. This antigenic difference between the polysaccharide fractions of the wild type and strain SM1 does not appear to be in the amounts of antigen produced. Further adsorption of the antiserum with fractions A or B, from strain SM1, yielded the same result as that depicted in Fig. 1. When fraction B from the wild type was treated with sodium periodate, and then placed in the immunodiffusion well, no precipitin reaction was observed. When fraction

B was treated with protease, the band was still present. These results indicate that the antigen is a polysaccharide.

A similar experiment was performed with antiserum prepared specifically against the O antigen (fraction B) from the wild type (Table 2). The serum was adsorbed with whole cells, fraction A, and fraction B from the wild type, strain SM1, and strain SM5. Only strain SM1 cells and the corresponding polysaccharide fractions from this strain failed to adsorb out agglutinating antibodies against wild-type whole cells. These results provide further evidence that there are antigenic differences specifically in the O antigen between the wild type and the non-nodulating strain, SM1.

We hydrolyzed the fractions and looked for differences in the components of the polysaccharides by paper chromatography. Fractions A, A', and B are quite similar to each other (Fig. 2). Fraction A contains the greatest number of components. This agrees with the previous conclusion that fractions A' and B are composed of a portion of fraction A.

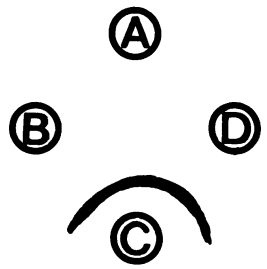

FIG. 1. *Immunodiffusion analysis of wild type and non-nodulating mutant strain SM1 antigens with antiserum to wild-type whole cells. Polysaccharide antigens were mixed with sodium deoxycholate (final concentration, 1%). Antigen wells contained fraction B from (A) SM1; (C) wild type. Wells (B) and (D) contained antiserum adsorbed with SM1 fractions B and A, respectively. Conditions have been described previously (7).*

TABLE 2. *Agglutination of wild-type cells by antiserum prepared against wild-type O antigen*[a]

Antiserum adsorbed with	End-point titer of wild-type cells
Wild-type cells	0
SM1 cells	16
SM5 cells	0
Wild-type LPS	0
SM1 LPS	8
SM5 LPS	0
Wild-type O antigen	0
SM1 O antigen	16
SM5 O antigen	0
Unadsorbed	2,500

[a] SM1-adsorbed antiserum was no longer reactive with SM1 whole cells.

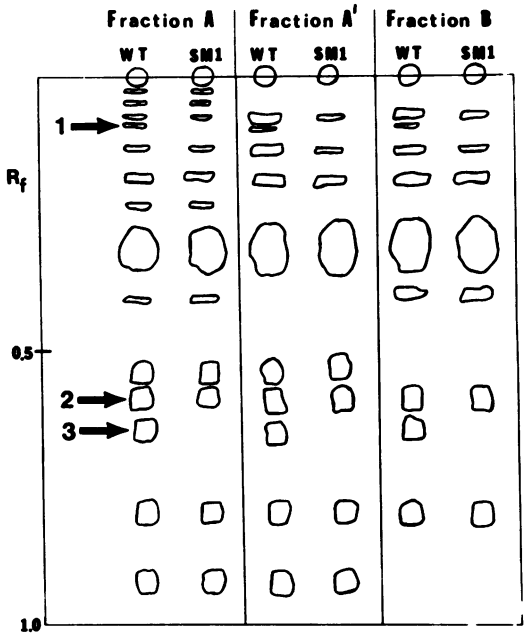

FIG. 2. *Paper chromatography of polysaccharide fractions. Each polysaccharide fraction from the wild type and strain SM1 was hydrolyzed, and paper chromatography was performed as described in the text. The migrated components were detected by spraying with sodium hydroxide after dipping the chromatogram in silver nitrate solution. Spots no. 1 and 2 were evident 15 min after spraying the chromatograms with sodium hydroxide, whereas spot no. 3 took a longer period of time (to 24 h) to develop. Spot no. 2 was considerably darker in all three fractions of the wild type than in the fractions from mutant strain SM1. WT, Wild type.*

Three major component differences (identified by arrows in Fig. 2) were observed between the wild type and strain SM1 in all three polysaccharide fractions. Two spots (labeled 1 and 3) were present in the fractions from the wild type, but were absent in the fractions from the mutant strain. Spot 2 was considerably darker in the fractions from the wild type than the corresponding fractions from strain SM1. All of the other spots were of the same intensity and had the same R_f value in both strains.

These data indicate that certain silver nitrate-staining components of the O antigen are required to establish nodulation of soybean roots. The missing components might be sugars or other groups, perhaps attached to the sugars. Presently, we are attempting to determine the chemical nature of these alterations in the O

antigen. All of the experiments described in this paper also were performed with the non-nodulating mutant strain SM2 (13), yielding the same results as those observed with strain SM1. It is possible that strains SM1 and SM2 are siblings since they were obtained from a single mutagenized culture. Detailed studies on the interactions between the O antigen and plant lectin should yield greater insight into the initial step of the infection process.

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