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Bacteriophage That Can Distinguish Between Wild-Type *Rhizobium japonicum* and a Non-Nodulating Mutant

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A bacteriophage (phage TN1) that lyses *Rhizobium japonicum* 3I1b110 was isolated from Tennessee soil. Structurally, this phage resembles the *Escherichia coli* phage T4, having an icosahedral head (47 by 60 nm) and a contractile tail (17 by 80 nm). An interesting feature of this phage is that it lyses all of the symbiotic defective mutants derived from *R. japonicum* 3I1b110 that were tested, except one, mutant strain HS123. Mutant strain HS123 is a non-nodulating mutant that is defective in attachment to soybean roots. Since *Rhizobium* attachment to host roots is thought to be mediated by a specific cell surface interaction, it is likely that mutant strain HS123 is defective in some way in its cell surface. Mutant strain HS123 bound soybean lectin to the same extent as the wild type as measured by the binding of tritium-labeled lectin. Phage TN1 did not attach to the surface of strain HS123, nor did cells of strain HS123 inactivate phage TN1. A hot phenol-water cell extract from the wild-type inactivated phage TN1, whereas a similar cell extract from mutant HS123 did not. Capsular polysaccharide isolated from mutant or wild type did not inactivate the phage. Capsular polysaccharide and exopolysaccharide from the mutant and wild type do not differ in sugar composition. These results indicate that capsular polysaccharide may not play a role in attachment to the plant root surface and that other cell wall components may be important.

The infection of leguminous plants by *Rhizobium* spp. is species specific. The factors determining this specificity are presently unknown, although work has focused on the initial binding of *Rhizobium* spp. to the host roots as an important step at which specificity could be expressed. Lectins, proteins that recognize and bind to specific carbohydrate residues, have been implicated as being important in determining binding specificity (5, 8, 12, 15, 37).

According to this hypothesis, host-plant lectins located on the root surface recognize carbohydrate receptors on the compatible *Rhizobium* cell surface, thereby facilitating the binding between the bacteria and root. In *Rhizobium japonicum*, soybean lectin (SBL) has been shown to interact with the capsular polysaccharide (CPS) portion of the bacterial cell wall (4, 9, 26). Because of this, interest has focused on the CPS as the portion of the *R. japonicum* cell wall that is involved in root attachment.

To understand the mechanism of root attachment by *R. japonicum*, we have investigated a mutant of *R. japonicum*, strain HS123, that had been shown previously to be defective in attachment to soybean roots (30). Surprisingly, this mutant binds SBL to the same extent as the wild type. Phage TN1, isolated by its ability to lyse wild-type strain 3I1b110, does not bind to cells of strain HS123. This indicates that strain HS123 does have an alteration in its cell surface. Experiments indicate that the CPS and exopolysaccharide (EPS) of strain HS123 do not differ from those of the wild type. These data indicate that the CPS may not be involved or may not be the only cell wall component involved in root attachment by *R. japonicum* and that other cell wall components may play a role in root adherence.

MATERIALS AND METHODS

Bacterial strains. *R. japonicum* 3I1b110, colony type I-110 (22), was originally obtained from G. H. Elkan, North Carolina State University, Raleigh. All of the symbiotic defective mutants derived from strain 3I1b110 were given the prefix HS (30). A description of the isolation and phenotypes of the mutants used in this study was reported previously by Stacey et al. (30). All mutants were tested for purity by serology and compared to the wild type by two-dimensional polyacrylamide gel protein patterns. Bacterial cultures were grown in yeast extract-mannitol medium (32).

Phage isolation and purification. Bacteriophage TN1 was isolated from soybean fields on the agricultural campus of the University of Tennessee, Knoxville. Phage were isolated and purified by the method of soil enrichment described by Bishop et al. (6). Phage obtained as plate lysates were maintained over chloroform in a solution containing (per liter): 10 g of tryptone, 4.3 g of $MgCl_2 \cdot 7H_2O$, and 1 g of gelatin. Subsequently, it was found that phage TN1 can also be maintained at −70°C in the above solution plus 10% glycerol.

Negative staining for transmission electron microscopy. Phage particles were incubated with cells of *R. japonicum* for 10 min or observed directly. A drop of the mixture was placed on a 400-mesh Formvar-coated grid and allowed to settle for 1 min. Grids were stained with either 0.2% potassium phosphotungstic acid or 0.5% uranyl acetate for 5 s and then washed twice with distilled water. The grid was air dried and examined at 75 kV in a Hitachi 600 transmission electron microscope.

Hot phenol-water extraction of *R. japonicum* cells. Cells were grown in 16-liter carboys in yeast extract-mannitol medium at 25°C and harvested during late log-early stationary phase. The cells were harvested, washed, and dried according to the procedure of Galanos et al. (14). Bacterial cells (1 to 2 g [dry weight]) were extracted with hot phenol and water according to the method of Westphal and Jann (33). The aqueous cell extract was dialyzed exhaustively against distilled water and then lyophilized. In some cases, this crude cell extract was further purified by passage through a Dowex AG1X1 (acetate form; Bio-Rad Laboratories, Richmond, Calif.) anion-exchange column, followed by treatment with DNase and RNase by the procedure of

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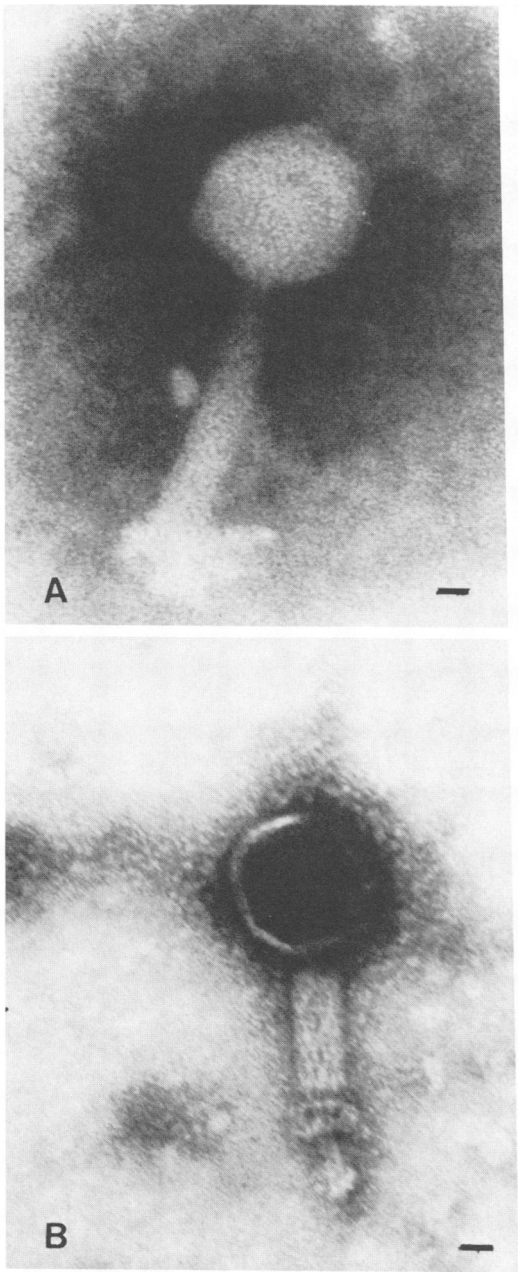

FIG. 1. Electron microscope visualization of the structure of bacteriophage TN1. (A) Particle of phage TN1 with uncontracted tail sheath. The polyhedral nature of the phage head is obvious. Stained with 0.2% potassium phosphotungstic acid. (B) Phage TN1 with contracted tail sheath. Stained with 0.5% uranyl acetate. Bar, 2.5 nm.

Carlson et al. (10). After this treatment, the cell extract was again extensively dialyzed against distilled water and lyophilized.

Isolation and compositional analysis of *R. japonicum* CPS and EPS. Bacteria were grown in 6- to 12-liter batches in a defined medium that favors CPS and EPS synthesis (26). The cells were harvested by centrifugation, and the CPS adhering to cells was removed by treatment in a Waring blender for 5 min in phosphate-buffered saline buffer (6.8 g of KH_2PO_4, 8.7 g of K_2HPO_4, 8.7 g of NaCl per liter of water). The CPS was dialyzed extensively against distilled water and lyophilized. The EPS remaining in the culture fluid was isolated by concentrating the culture fluid by rotary evaporation, followed by extensive dialysis against distilled water and lyophilization. The lyophilized CPS and EPS were then dissolved in distilled water and purified by passage over a column of DEAE-Biogel (Bio-Rad Laboratories) and elution with a gradient of 0 to 1 M NaCl (26, 27). The purity of the polysaccharide obtained was monitored by measuring the ratio of neutral sugar (using the phenol-sulfuric acid method [13]) to that of uronic acid determined colorimetrically (7). This ratio was constant throughout the peak of polysaccharide obtained.

The content of neutral sugars in trifluoroacetic acid-hydrolyzed (2 N trifluoroacetic acid for 2 h at 120°C) CPS and EPS was determined as their alditol acetate derivatives by gas-liquid chromatography. A Sigma 3B gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a 3% SP-2330 (Supelco, Bellafonte, Pa.) column was used. The sugars were derivatized by the method of Albersheim et al. (1). In each case, 2 to 3 mg (dry weight) of polysaccharide was analyzed.

Phage inactivation. Inactivation of phage TN1 by whole cells, CPS, or hot phenol–water-extracted material was done by a modification of procedures found in Wilson et al. (36) and Hancock and Reeves (16). Cells (3×10^8) were mixed at a multiplicity of infection of 0.1 to 0.2 with phage TN1 and incubated at room temperature for 15 min. Cells were then pelleted, and the titer of the phage in the supernatant fluid was determined. Lyophilized CPS or phenol–water-extracted material at a final concentration of 5 μg/ml was mixed in a volume of 0.2 ml with 2×10^8 PFU of phage TN1. This mixture was incubated for 15 min at room temperature and then diluted to determine the titer of active phage.

Lectin binding assay. Purified soybean seed lectin was either prepared by the method of Allen and Neuberger (2) or purchased from E-Y Laboratories, Inc., San Mateo, Calif. Radiolabeled SBL (^{3}H-labeled SBL) was prepared by periodate oxidation and borotritiide reduction of the carbohydrate side chains by the procedure of Lotan et al. (23). Lectin was labeled to a specific activity of 10,000 cpm/μg of protein. The binding of ^{3}H-labeled SBL to cells of *R. japonicum* was measured as described by Bhuvaneswari et al. (5) in the presence or absence of the hapten *N*-acetyl-D-galactosamine (5 mM). Results are presented as hapten-inhibitable lectin binding. Assays were performed in 1 ml containing ca. 4×10^8 *R. japonicum* cells and 50 to 300 μg of SBL.

RESULTS AND DISCUSSION

Lectin binding. One of the symbiotic defective mutants previously isolated from the wild-type strain 3I1b110, mutant strain HS123, was shown to be defective in initial binding to soybean roots (30). Since this step is thought to involve mutual, specific cell-cell surface interaction, it is likely that this mutant is defective in some component of its cell surface. Therefore, by finding the defective component of the cell surface of this strain one should be able to identify which surface structure is important to root attachment. SBL has been suggested to mediate the binding of *R. japonicum* cells to soybean roots by attaching to the CPS portion of the cell wall (4, 9, 26). To test whether strain HS123 was defective in lectin binding we used the sensitive method of measuring ^{3}H-labeled lectin binding to cells. Under optimum conditions (determined by the stage of

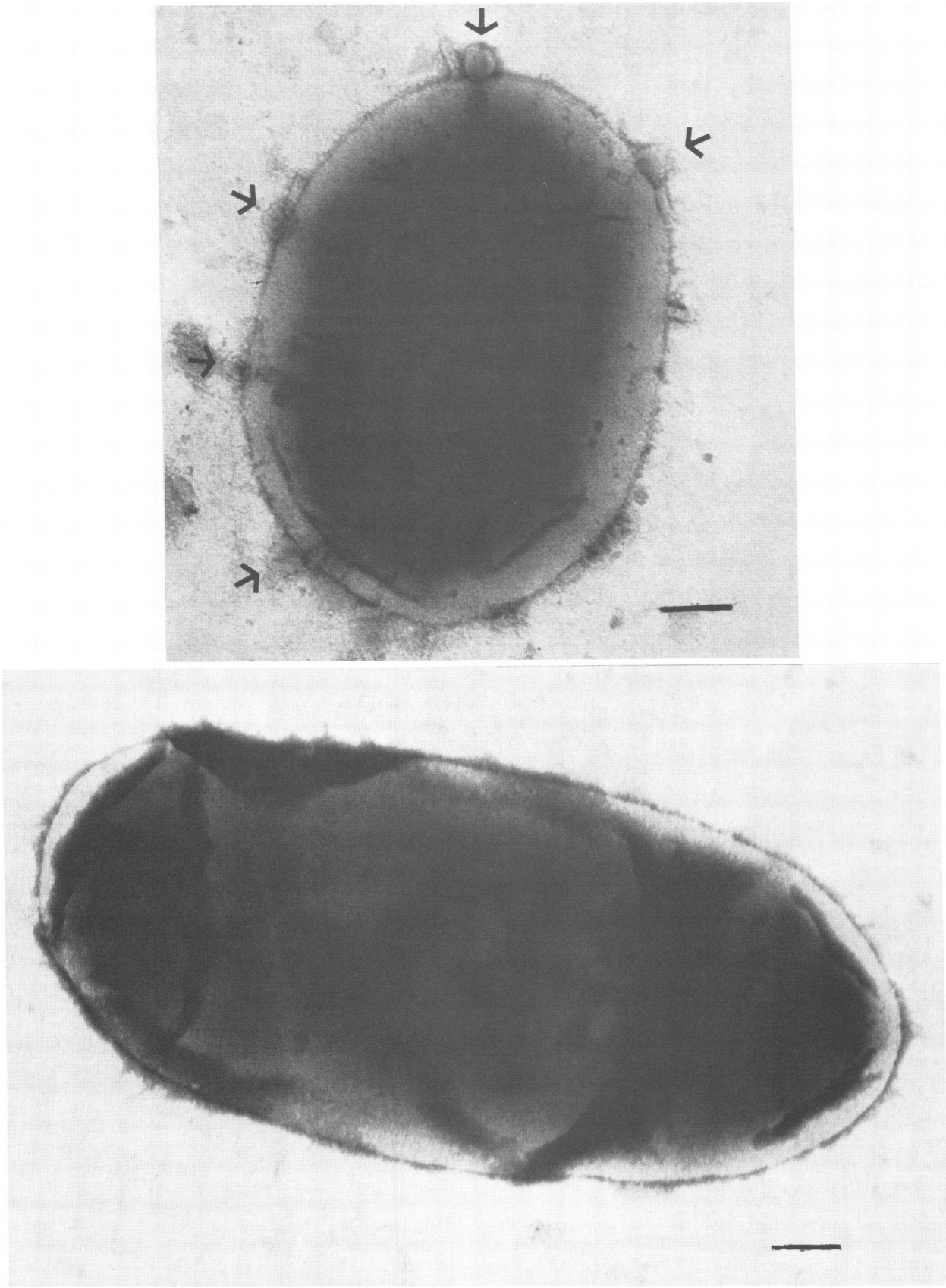

FIG. 2. Electron microscope visualization of the interaction of bacteriophage TN1 with a cell of *R. japonicum* strain 311b110 (top) and mutant strain HS123 (bottom). Stained with 0.2% potassium phosphotungstic acid. Bar. 100 nm.

TABLE 1. TN1 phage inactivation with CPS or a hot phenol-water cell extract

Phage preincubation with:	% Inactivation
No addition	0
3I1b110 phenol-water cell extract	96
HS123 phenol-water cell extract	5
3I1b110 CPS	3
HS123 CPS	2

growth [5]), both the wild-type strain and mutant strain HS123 bound identical amounts of lectin (i.e., $4 \pm 0.4 \times 10^5$ ^{3}H-labeled lectin molecules bound per cell). Therefore, although mutant strain HS123 is defective in binding to the soybean root surface, it is not defective in binding SBL.

Bacteriophage TN1. As a tool for dissecting the cell surface defect in mutant strain HS123, we sought bacteriophage that would lyse the wild-type strain but not mutant strain HS123. One such phage was isolated and designated phage TN1. The activity of this phage was tested by plaque assay against several nodulation-defective mutants of *R. japonicum*. These mutants include slow-to-nodulate strains (HS104, HS106, HS109, and HS111), ineffective mutants (HS124 and HS146), and non-nodulating mutants (HS114, HS132, HS143, and HS148) and the non-nodulating, non-root-binding mutant strain HS123 (for a more detailed description of the phenotypes represented in these mutants see reference 30). This phage lysed strain 3I1b110 and all the other symbiotic defective mutants tested, but it did not lyse mutant strain HS123. Under the transmission electron microscope, phage TN1 resembles the *E. coli* phage T4, having an icosahedral head (47 by 60 nm) and a contractile tail (17 by 80 nm) (Fig. 1).

Rhizobium mutants resistant to bacteriophage have been isolated previously (3, 21, 30). Many of these mutants were also defective in their symbiotic properties (3, 21, 30). However, it should be stressed that mutant strain HS123 was not selected as phage resistant but was obtained by screening of mutagenized clones for a non-nodulating phenotype (30).

Cell surface defect of mutant strain HS123. The inability of phage TN1 to lyse mutant strain HS123 could be due to an inability to bind to the surface of cells of strain HS123. Examination by transmission electron microscopy of cells of both the wild type and mutant strain HS123 mixed with phage TN1 indicated that, indeed, phage TN1 does not attach to strain HS123 (Fig. 2). This is further shown when cells of strain HS123 and the wild-type strain 3I1b110 are compared for their ability to irreversibly inactivate phage TN1. Phage TN1 was 100% inactivated when mixed with strain 3I1b110 cells. When preincubated with cells of mutant strain HS123, however, the phage remained active at high levels, being inactivated only 17%. When preincubated with no addition, 0% phage inactivation was noted. These data provide proof that cells of strain HS123 do, indeed, differ in their surface from cells of the wild type.

The type of experiment represented above can be used to identify the cell wall component acting as the phage TN1 receptor. The results listed in Table 1 show that a hot phenol-water cell extract isolated from the wild-type strain 3I1b110 irreversibly inactivated phage TN1, whereas that isolated from the mutant strain HS123 did not inactivate the phage. CPS (or EPS) isolated from the wild type or mutant did not inactivate phage TN1.

We used published procedures to purify the CPS and EPS from the wild type and mutant and determine their sugar composition (Table 2). The CPS and EPS are composed of mannose, 4-*O*-methylgalactose (galactose), glucose, and uronic acid in a ratio of 1:1:2:1. These data agree with the known composition and structure of the CPS and EPS of *R. japonicum* 3I1b110 (26–28). The lectin binding data and the data in Table 2 would appear to indicate that the wild type and mutant strain HS123 do not differ in CPS or EPS structure.

Our attempts to purify and chemically characterize the material extracted with hot phenol-water have been, thus far, unsuccessful. This extraction method is routinely used to isolate lipopolysaccharide (LPS) from gram-negative cells (33). We can detect low levels of ketodeoxyoctonate, a characteristic sugar of LPS (24), in the material extracted. However, our analysis thus far would indicate that the material isolated does not possess a typical LPS structure (for example, as that of *Salmonella typhimurium* [24]). Our analyis of this portion of the cell wall of *R. japonicum* is ongoing.

A possible role for the LPS in root adherence has been suggested for various *Rhizobium*-legume interactions. For instance, Kato et al. (19, 20) reported that LPS and CPS could inhibit the binding of *Rhizobium leguminosarum* to pea roots. Kamberger (18) found that non-nodulating mutants of *R. leguminosarum* were defective in their LPS structure. Work with the *Rhizobium trifolii*-clover symbiosis has also implicated the LPS as a possible determinant in root adherence (11, 17, 29). Transient lectin binding and root adherence in batch cultures of *R. trifolii* correlated with the appearance of D-quinovosamine (2-amino-2,6-dideoxyglucose) in the LPS (17). The loss of the plasmid encoding nodulation functions in *R. trifolii* results in a non-nodulating phenotype and the loss of D-quinovosamine from the LPS (29). When the plasmid is regained, the cells are then capable of nodulation and D-quinovosamine can again be detected in the LPS (29). *Agrobacterium*, a genus of bacteria closedly related to *Rhizobium* spp. (31), causes tumors upon infection of several species of dicotyledonous plants. *Agrobacterium tumefaciens* LPS is thought to be important for the attachment to plant cells (25, 34, 35).

The cell surface of the wild type and mutant strain HS123

TABLE 2. Sugar analysis of the CPS and EPS of wild type and mutant strain HS123

Sugar	CPS				EPS			
	3I1b110		HS123		3I1b110		HS123	
	Amt (mg)	Molar ratio	Amt (mg)	Molar ratio	Amt (mg)	Molar ratio	Amt (mg)	Molar ratio
Mannose	0.32	1.0	0.42	1.0	0.54	1.0	0.43	1.0
4-*O*-Methylgalactose (galactose)	0.32	1.0	0.40	1.0	0.54	1.0	0.41	1.0
Glucose	0.65	2.0	0.85	2.1	1.09	2.0	0.86	2.0
Uronic acid	0.37	1.1	0.45	1.0	0.51	0.9	0.48	1.1

does not appear to differ with regard to CPS or EPS as witnessed by sugar composition and ability to bind SBL. The fact that cells of strain HS123 do bind lectin but are incapable of binding to plant roots is not consistent with the postulated role of SBL mediating attachment of *R. japonicum* cells to soybean roots. Therefore, if SBL is involved in nodulation, it is unlikely that it is the sole mediator of root adherence. The data presented would seem to indicate that other cell wall components, perhaps LPS, are important in mediating attachment between the rhizobial and plant root surfaces.

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