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Research Note

Bradyrhizobium japonicum Mutants Deficient in Exo- and Capsular Polysaccharides Cause Delayed Infection and Nodule Initiation

Gillian Eggleston[1], Mary Christine Huber[2], Rong-ti Liang[1], Arthur L. Karr[2], and David W. Emerich[1]

[1]Department of Biochemistry, [2]Department of Plant Pathology, and [1,2]Interdisciplinary Plant Biochemistry and Physiology Program, University of Missouri, Columbia 65211

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Four strains of *Bradyrhizobium japonicum* 2143 that were unable to bind soybean lectin were isolated following Tn*5*-insertion mutagenesis. Each of the mutants had a dry colony morphology compared with the mucoid parent and were found to be deficient in the amount of exo- (EPS) and capsular (CPS) polysaccharides. Nodulation and nitrogen-fixing characteristics of four unique mutants were investigated and all four mutants exhibited delayed nodulation on soybean (*Glycine max*) cv. Williams 82 and significantly reduced acetylene reduction activities. The infection of one of the mutant strains was observed by light microscopy and was found to be delayed as early as 5 days post infection (dpi). The ultrastructure of nodules formed by these strains was examined at 31 dpi: two of the mutants appeared to be similar to nodules formed by the parent strain, but the other two mutants appeared to be defective in plant cell invasion and nodule initiation. The results presented here provide additional evidence that extracellular polysaccharides are required for efficient infection and nodulation of determinate nodules.

Additional keyword: nitrogen fixation.

The induction of nitrogen-fixing nodules on the roots of host legumes by *Rhizobium* and *Bradyrhizobium* bacteria is a complex molecular communication process between the two symbiotic partners. Bacterial factors that are involved in this process include surface polysaccharides composed of lipopolysaccharides (LPS), capsular polysaccharides (CPS), exopolysaccharides (EPS), and β-glucans. Exactly how these polysaccharides function in nodule development is still unknown, and a full understanding would allow for possible future technological manipulations. The significance of these surface polysaccharides for infection and nodule initiation depends on the type of nodules produced by the host plant, i.e., indeterminate or determinate (Ko and Gayda 1990; Parniske et al. 1993, 1994). Indeterminate nodules have continuous meristematic activity whereas determinate nodules have finite activity. EPS performs a more significant role in the formation of indeterminate nodules whereas LPS performs the more significant role in determinate nodules. However, Parniske et al. (1993, 1994) showed that although an *exo*B mutant of *B. japonicum* still produced effective determinate nodules on soybean (*Glycine max* (L.) Merr.), nodule formation was delayed by approximately 5 days and the mutants exhibited a greatly reduced competitiveness, which indicates that EPS has some function that appears to be especially important during the early stages of infection.

We previously isolated Tn*5*-generated mutants of *B. japonicum* that were unable to bind soybean lectin (Liang and Emerich 1987). Four isolates designated as strains 1251, 1252, 5141, and 5142, which gave only a faint blue color in the soybean lectin blot assay (Liang and Emerich 1987), and which did not exhibit observable fluorescence in the fluorescein isothiocyanate (FITC)–soybean lectin binding assay (Bhuvaneswari et al. 1977), were purified by single-colony isolation and confirmed to be derived from *B. japonicum* strain 2143 by their drug resistance profile (Karr and Emerich 1988) and by comparison of their genomic DNA restriction digestion pattern (Huber et al. 1984).

The amounts of EPS and CPS obtained from the various mutants and parent strain at stationary phase were determined (EPS analysis as described by Dunn and Karr 1990; CPS analysis as described by Mort and Bauer 1980). All of the mutants had markedly reduced EPS and CPS: parent strain 2143 and mutant strains 1251, 1252, and 5141 were found to have 2,267, 13.2, 61.7, and 16.4 μg of EPS/10^9 CFU, and 1,097, 27.7, 89.5, and 16.4 μg of CPS/10^9 CFU, respectively. Strain 5142 had 15.4 μg of CPS/10^9 CFU but the EPS was too low to measure reliably. It has not yet been determined whether the composition of these extracellular polysaccharides of the mutants has been altered. In general, all strains, parent and mutants, showed an increased capsule size during growth to stationary phase. An exception to this was strain 1252, which produced numerous small capsules in early log phase. Although all the mutants produced capsules in the late exponential and stationary phases of growth, the capsules were markedly smaller when compared with capsules in the parent strain 2143.

Corresponding author: David W. Emerich, Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia 65211; E-mail: bcemeric@muccmail.missouri.edu

Present address of G. Eggleston: SRRC-USDA-ARS, 1100 Robert E. Lee Blvd., P.O. Box 19687, New Orleans, LA 70179.

Since the reduced levels of EPS and CPS of the mutants could have been the result of increased polysaccharide depolymerase activity, the levels of EPS depolymerase activity were measured in ruptured cells (Dunn and Karr 1990). However, no significant differences were observed in the depolymerase activity between the parent and the mutant strains (data not shown).

The LPS of the parent and mutant strains was determined qualitatively by polyacrylamide gel electrophoresis (Stacey et al. 1991) and no apparent differences were found (data not shown).

When these mutants were inoculated onto soybeans it was apparent that they caused a symbiotic defect based on the leaf color of the nodulated plants. Compared with the healthy green leaf color of the plant inoculated by the parent strain 2143 at 28 days post inoculation (dpi), all the plants inoculated with the mutants showed some leaf chlorosis as an indicator of nitrogen deficiency. Acetylene reduction activity of the nodules (Karr et al. 1984) confirmed that the visual appearance of the plants was the result of reduced nitrogen fixation (Table 1). All of the mutants demonstrated lower acetylene reduction activity than that of the parent strain. Strain 1251 was the most effective of the mutants in terms of maximal acetylene reduction activity and it had the least initial delay in expression of acetylene reduction activity. Strains 1252, 5141, and 5142 all displayed measurable acetylene reduction activity by 22 dpi and the activity of all four mutants increased by 28 dpi. The maximum acetylene reduction of strain 1251 (28.4 μmoles/h/g.fr.wt.) was 58% that of the parent strain (49.0 μmoles/h/g.fr.wt.) and was delayed by approximately 6 days. The maximum acetylene reduction activity of the other three mutants was only about 10 to 15% of the parent strain and also was delayed by about 6 days. The acetylene reduction activity of all strains declined by 31 dpi. Acetylene reduction activity determined on a dry weight basis followed the same general pattern as the fresh weight.

The mass of the nodules was lower for the mutants compared with the parent at most plant ages (Table 1). Nodules formed by inoculation with mutant 1251 were larger in mass than the wild type at the early stages of nodule development but were of comparable size later. All the other mutants were considerably smaller throughout development until day 31, when they appeared to reach maximal size.

Soybean seedlings inoculated with the mutants had delayed nodule appearance compared with the wild type (Table 1). Plants inoculated with parent strain 2143 had nodules clustered at the crown of the tap root, whereas the plants infected by strains 1251 and 5141 had nodules both on the tap root and along the lateral roots. Strains 1252 and 5142 were largely infected on peripheral roots, an indication of even further delay of nodulation (Law et al. 1982). Unlike inoculation with strains 2143 and 1251, which resulted in infection loci in a well-defined region in the growing root (Calvert et al. 1984), the varied location of nodules formed by strains 1251 and 5142 made it difficult to find infection loci for microscopic examination.

The relative development of infection loci, after 5 and 8 dpi, by the parent strain 2143 and the mutant 1251 was examined (Fig. 1). At 5 dpi, the infection loci initiated by strain 2143 were predominately at stage 5 with a few in stage 4 (Fig. 1A) as defined by Calvert et al. (1984). Cortical cells in the outermost layers had divided anticlinically and cell division activity was often evident in the inner cortex as well. The infection loci initiated by mutant 1251 were more or less equally distributed between stages 2 and 3 at day 5 (Fig. 1C). The extent of the delay of infection by mutant 1251 compared with strain 2143 at day 5 dpi was apparent (Fig. 1A versus 1C). The difference between the two strains remained apparent at 8 dpi; many of the seedlings inoculated with mutant 1251 showed infection development at stages 5/6 (Fig. 1D). In comparison, there were many more well-developed infection sites on the roots inoculated with strain 2143; most were at stage 8 with

Table 1. Nodulation and nitrogen fixation (acetylene reduction) characteristics of selected non-lectin binding mutants of *Bradyrhizobium japonicum* strains unable to bind lectin[a]

Strain	Days post inoculation	Nodules per plant	Average mg/nodule	Specific acetylene reduction activity (ηmole/h/g.fr.wt.)[b]	Specific acetylene reduction activity (ηmole/h/g.d.wt.)
2143	16	10	5.6	29.0	165.3
1251	16	13	27.0	12.7	77.4
1252	16	1	7.0	<0.1	0.0
5141	16	0	0.0	<0.1	0.0
5142	16	5	3.3	<0.1	15.6
2143	22	23	12.2	49.0	228.6
1251	22	8	11.0	22.7	132.4
1252	22	23	2.0	2.0	10.7
5141	22	29	3.3	4.2	22.8
5142	22	6	6.8	6.2	32.9
2143	28	21	13.4	12.5	52.1
1251	28	27	18.3	28.4	149.8
1252	28	40	1.7	6.4	49.5
5141	28	31	1.0	5.8	31.3
5142	28	42	0.9	6.7	36.2
2143	31	31	7.4	1.8	8.9
1251	31	36	9.3	<0.1	1.1
1252	31	19	10.0	<0.1	0.0
5141	31	21	13.1	<0.1	0.1
5142	31	28	4.7	<0.1	0.0

[a] Represents three separate experiments.
[b] Average of four separate determinations; SD < 17% for all measurements.

the remainder at stage 7 (Fig. 1B). The vascular differentiation between the nodule meristem and root stele was apparent in the roots inoculated with strain 2143 but was not obvious in the roots inoculated with mutant 1251.

Strain 5141 was slightly more delayed in the development of infection loci than strain 1251, but strains 1252 and 5142 were even further delayed. The location of the nodules formed by mutants, other than strain 1251, on the peripheral roots made location of infection sites very difficult and, therefore, few were found. Because of the small sample size observed, it is not known how typical they were of the general population and, thus, we do not present them.

Nodules induced by *B. japonicum* strain 2143 and the mutants were examined at 31 dpi with transmission electron microscopy. This age was selected because sufficient nodule mass was available so that nodules could be easily harvested. Some of the morphological features of the nodules can be observed in Figure 2A–F.

At 31 dpi, nodule cells infected by mutant strains 1251 (Fig. 2B) and 5141 (Fig. 2D) were similar to those infected with the parent strain 2143. Several bacteroids were enclosed within each of the symbiosome membranes, and many bacteroids had electron-translucent deposits of poly-β-hydroxybutyrate. Some features of bacteroids and infected cells in each of these combinations were similar to those observed in nodules undergoing senescence. Many of the symbiosome membranes were convoluted rather than ellipsoid in shape. Some bacteroids were electron dense, irregular in shape, or were localized in large vesicles rather than in symbiosomes.

In contrast to nodules induced by mutants 1251 and 5141, those induced by mutants 1252 (Fig. 2C) and 5142 (Fig. 2E and F) were similar to nodules formed during incompatible combinations of soybean with *B. japonicum*. As observed with light microscopy, fewer cells in the central nodule tissue were infected in each of these combinations.

The infected cells of nodules formed by inoculation with mutant 1252 had abundant empty vesicles (Fig. 2C). Only a few, spherically shaped bacteroids were observed, and these were present singly within structures resembling symbiosome membranes. As is typical of infected cells formed during

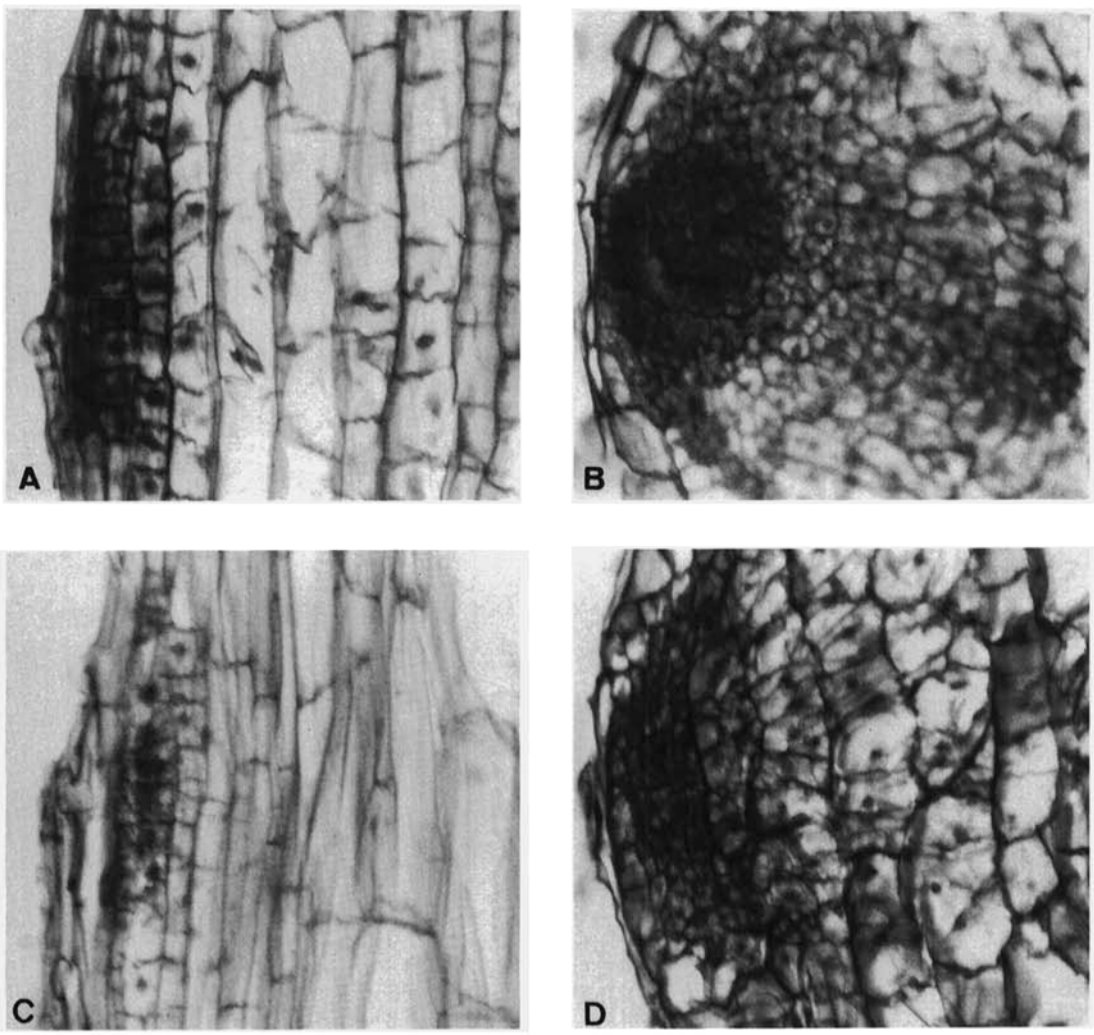

Fig. 1. Infection loci formed from inoculation of soybean seedlings with either *Bradyrhizobium japonicum* strain 2143 or strain 1251. **A,** Infection loci at 5 days after inoculation with strain 2143. **B,** Infection loci at 8 days after inoculation with strain 2143. **C,** Infection loci at 5 days after inoculation with strain 1251. **D,** Infection loci at 8 days after inoculation with strain 1251.

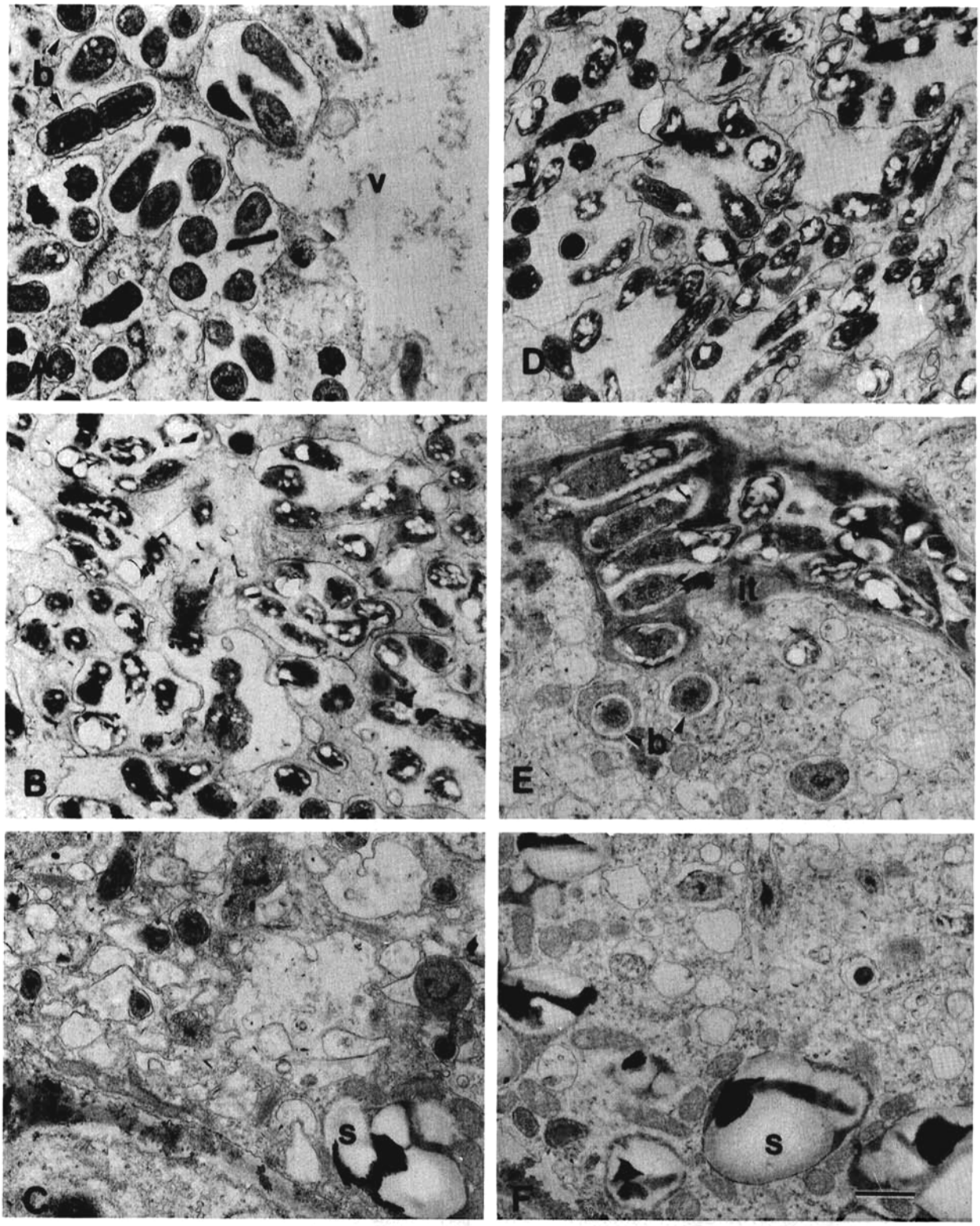

Fig. 2. Transmission electron micrographs of soybean nodule thin sections induced by the parent *Bradyrhizobium japonicum* strain 2143 and the mutants. Nodules were harvested 31 days postinfection. **A,** *B. japonicum* 2143; **B,** mutant strain 1251; **C,** mutant strain 1252; **D,** mutant strain 5141; **E,** mutant strain 5142; **F,** mutant strain 5142. it = infection thread; b = bacteroid; s = starch grain; v = vacuole. Nodules, 31 dpi, were prepared for fixation in 0.1 M sodium cacodylate buffer, fixed overnight in a solution of 2% (v/v) paraformaldehyde, 2% (v/v) glutaraldehyde, and 0.1 M sodium cacodylate at 4°C, washed in 0.1 M sodium cacodylate, and then washed in 1% sucrose (v/v), both at 4°C. Nodules were post-fixed in a solution of 1% (w/v) OsO_4, 1% (w/v) sucrose, and 0.1 M sodium cacodylate at 4°C for 2 h. Secondary fixative was removed by washing samples in glass distilled water at room temperature. Specimens were then placed in 1% uranyl acetate for 1 h at 4°C. After washing in glass distilled water, samples were dehydrated in an ethanol series (20, 40, 60, 80, 96, 100, 100, 100% v/v) and then infiltrated 3 times with propylene oxide for 30 min each. Specimens were embedded in Epon-Araldite resin (Mollenhauer 1964; Hayat 1986). Thin sections were prepared from the central area of the nodule and placed on 300-mesh copper grids. Grids were stained 10 min in 1% uranyl acetate in 50% (v/v) ethanol and 2 min in 0.4% alkaline lead citrate (Venable and Coggeshall 1965). Stained specimens were viewed with a Jeol 100B electron microscope.

effective combinations, the nucleus was centrally located, and the organelles were adjacent to the plasmalemma, although a few mitochondria were observed near the nucleus. Conspicuous starch granules were present in both infected and uninfected cells in the central area. Large starch deposits are typical of nodule tissue that is not actively fixing nitrogen. Hence, this observation was consistent with the observation of low levels of acetylene reduction at 31 dpi in this combination (Table 1). In general, the ultrastructure appearance of cells in the central portion of the nodule from this combination was similar to that of other ineffective combinations, notably soybean–*B. japonicum* strain 3122 (M. C. Huber, unpublished results).

Cells infected with *B. japonicum* mutant 5142 were greatly delayed in nodule development. In some samples, bacteroids were observed only in infection threads (Fig. 2E). In others, single, usually spherically shaped, bacteroids were present within structures resembling symbiosome membranes. As is typical of cells induced during ineffective combinations, infected cells were filled with numerous empty vesicles, and large starch granules were found at the periphery of both infected and uninfected cells.

These mutants of *B. japonicum,* isolated by their inability to bind the common soybean lectin, were found to induce determinate nodules on soybeans, but only after a marked time delay relative to the parent strain. It is not known whether the lectin screen selects a particular subset of EPS/CPS deficient mutants that show the delayed infection phenotype. The *exo*B strain of *B. japonicum* was previously reported to display a delayed nodulation phenotype (Parniske et al. 1993, 1994). P. Müller kindly provided the *exo*B strain of *B. japonicum* (Parniske et al. 1993) to test in the soybean lectin binding assay. The *exo*B mutant was also unable to bind soybean lectin, implying the binding assay may be useful for obtaining additional EPS/CPS mutants that result in a delayed nodulation phenotype. The results presented here provide additional evidence that EPS/CPS do indeed have a role in the formation of determinate nodules since four different mutants each with reduced amounts of EPS/CPS all affect nodulation. The differences between the mutants described here may be due to differences in the composition, structure, or regulation of the synthesis of the EPS/CPS.

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