MPMI Vol. 9, No. 5, 1996, pp. 364–372. Publication no. M-1996-0412-01R.

Leucaena leucocephala Nodules Formed by a Surface Polysaccharide Defective Mutant of *Rhizobium* sp. Strain TAL1145 Are Delayed in Bacteroid Development and Nitrogen Fixation

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Received 12 June 1995. Accepted 11 March 1996.

A Tn*5lacZ*-insertion mutant of the *Leucaena*-nodulating *Rhizobium* sp. strain TAL1145 defective in exopolysaccharide (EPS) and lipopolysaccharide (LPS) synthesis was characterized. This mutant, NP84, formed pseudonodules on *Phaseolus vulgaris* and *Gliricidia sepium* that did not show invasion by bacteria. On *Leucaena leucocephala* it formed nodules that appeared Fix$^-$ at 4 weeks but Fix$^+$ at 8 weeks after inoculation. Microscopic examination of the sections of the *L. leucocephala* nodules formed by NP84 after 4 weeks of inoculation showed fewer infected cells than in nodules formed by TAL1145. Ultrastructural differences were observed in the cells infected by NP84 compared with those infected by TAL1145. When NP84 was coinoculated with TAL1145 on *L. leucocephala*, all nodules were occupied by TAL1145 although some nodules showed double occupancy with NP84. None of the nodules were found to be infected by NP84 alone. A 3.8-kb DNA fragment of TAL1145 was identified and cloned that complemented NP84 for EPS, LPS, and symbiotic defects.

Additional keyword: nodulation competitiveness.

Rhizobia present in the soil interact with the roots of different legume hosts, induce nodule formation, invade these nodules through the formation of infection threads, differentiate into bacteroids, and finally fix atmospheric nitrogen. Development of the nodule and the nitrogen-fixing bacteroids is a complex process that requires signal exchanges between rhizobia and the plant cells. The cell surface polysaccharides of rhizobia, particularly exopolysaccharides (EPS) and lipopolysaccharides (LPS), have been shown to be involved in the symbiotic process with various legumes (for reviews, see Gray and Rolfe 1990; Leigh and Coplin 1992).

Generally, *Rhizobium* mutants defective in EPS have severe effects on the nodulation of indeterminate hosts while such mutants have little effects on the nodulation and nitrogen fixation of determinate hosts. *Rhizobium meliloti* mutants that fail to make succinoglycan induce nodules on alfalfa, but the nodules are devoid of bacteria and consequently fail to fix nitrogen (Finan et al. 1985; Leigh et al. 1985; Hynes et al. 1986; Long et al. 1988; Muller et al. 1988). Similarly, some EPS-defective mutants of the broad-host-range *Rhizobium* NGR234 formed calluslike pseudonodules on *Leucaena* (Chen et al. 1985). The nodulation and nitrogen-fixing abilities of three of these mutants were restored by the addition of EPS from NGR234 (Djordjevic et al. 1987). EPS-deficient mutants of *R. leguminosarum* bv. *viciae* did not form any nodules on peas but such mutants of *R. leguminosarum* bv. *phaseoli* formed nitrogen-fixing nodules on *Phaseolus vulgaris* (Borthakur et al. 1986). Similarly, EPS-defective mutants of *R. loti* formed nitrogen-fixing nodules on a determinate-nodulating host, *Lotus pedunculatus,* but formed only tumorlike growths on the indeterminate-nodulating host *Leucaena* (Hotter and Scott 1991).

As is the case with other Gram-negative bacteria, LPS is a unique component of the outer membrane of rhizobia and consists of a variable O-oligosaccharide linked to the highly conserved core oligosaccharide that is anchored to the membrane by glycolipids. Previous studies have shown the involvement of LPS in the specific attachment of rhizobia to the host roots (Lagares and Favelukes 1988; Kato et al. 1979, for review see Noel 1992). In determinate-nodulating hosts like *P. vulgaris* and *Glycine max*, LPS mutants lacking O-antigen failed to form normal infection threads or the bacteria were not released from the infection threads to invade the nodules (Cava et al. 1989, 1990; Maier and Brill 1978; Puvanesarajah et al. 1987; Stacey et al. 1991). The effects of LPS-defective *Rhizobium* mutants on indeterminate-nodulating hosts vary among different legumes. LPS-defective mutants of *R. leguminosarum* bv. *trifolii* formed small, white, nonfixing nodules on *Trifolium hybridum* (Brink et al. 1990). Such mutants of *R. leguminosarum* bv. *viciae* also formed small, ineffective nodules on *Vicia hirsuta*, *V. sativa,* and *Pisum sativum* (Priefer 1989; de Maagd et al. 1989; Goosen-de Roo et al. 1991). On the other hand, the LPS-defective mutants of *R. meliloti* are not defective in symbiosis (Clover et al. 1989).

Brink et al. (1990) suggested that LPS may be a signal molecule that is required at different times during nodule development in different hosts. It has also been proposed that

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Hawaii Institute of Tropical Agriculture and Human Resources Journal Series no. 4156.

LPS may protect rhizobia by suppressing the host defense mechanisms (Noel 1992). Studies of the *R. meliloti*–alfalfa symbiosis showed that the genes for EPS and LPS synthesis determine similar functions in the course of nodule development, and thus EPS and LPS provide equivalent information to the host plant (Putnoky et al. 1990). Williams et al. (1990) showed that *lpsZ*, a gene involved in LPS synthesis in *R. meliloti* Rm41, allowed the *exoB* and other *exo* mutants to form nitrogen-fixing nodules on various legume hosts. However, Reuhs et al. (1993) have shown that the presence of a component of the capsular-type polysaccharide in this strain, not the LPS, may substitute for the EPS in this system.

In *R. meliloti* the *exoB* and *exoC* mutants are defective in both EPS and LPS synthesis (Leigh and Lee 1988). Diebold and Noel (1989) described two *R. leguminosarum* bv. *phaseoli* mutants that were defective in both EPS and LPS synthesis and nodule development on beans. We describe here a mutant of the *Leucaena*-nodulating *Rhizobium* sp. strain TAL1145 that is defective in both EPS and LPS synthesis and forms nodules on *Leucaena leucocephala* that are delayed in bacteroid development and nitrogen fixation. TAL1145 forms effective nodules on *Leucaena* and other tree legumes such as *Gliricidia sepium* and *Calliandra spp.* (Turk and Keyser 1992). It also forms nitrogen-fixing nodules on *P. vulgaris* (Pooyan et al. 1994a) and is able to catabolize the *Leucaena*-toxin mimosine (Soedarjo et al. 1994). On the basis of 16S RNA sequences it was shown to be different from but closely related to *R. leguminosarum* and *R. tropicii* (George et al. 1984). Previously, we isolated two cosmid clones, pUHR182 and pUHR183, by complementing an EPS-defective double-insertion mutant, NP54, for EPS production (Parveen and Borthakur 1994). The polysaccharide mutant described here was isolated by homogenotization of the Tn*5lacZ* insertion in the plasmid pUHR182 to the chromosome of the wild-type strain TAL1145. The Tn*5lacZ* insertion in this mutant was mapped on a 6.3-kb *Eco*RI fragment of TAL1145 (Parveen and Borthakur 1994). In the present study, we report that the mutant NP84 is also defective in LPS synthesis and is complemented by cloned DNA in cosmids pUHR182 and pUHR183. We have shown that a 3.8-kb fragment subcloned from pUHR183 complements NP84 for EPS, LPS, and symbiotic defects. We tested the mutant NP84 for infection, nodule formation, and bacteroid development on both indeterminate-nodule-forming (*Leucaena*) and determinate-nodule-forming (*Gliricidia*) tree legumes.

RESULTS

Mutant NP84 is defective in both EPS and LPS synthesis.

Mutant NP84 forms small, opaque colonies on YEM medium compared with the large, translucent, mucoid colonies formed by the wild-type strain TAL1145. These colonies show bluish fluorescence on Calcafluor-containing YEM agar similar to TAL1145. The smaller colony size of NP84 compared with that of TAL1145 was not due to a slower growth rate, as both strains had similar growth rates in YEM broth (data not shown). In glutamic acid–mannitol–salt (GMS) broth NP84 produced less than half the amount of EPS produced by TAL1145 (Table 1). When NP84 was complemented with cosmid pUHR182, the transconjugants synthesized EPS in the same manner as the wild-type strain (Table 1). Size fractionation of the EPS made by TAL1145 and NP84 showed that EPS of both strains contains a high and a low molecular weight fraction (Fig. 1). Thus, the EPS produced by the two strains could not be distinguished by size fractionation. Analysis of the sugar composition of the mutant and the wild type by GLC also did not show any detectable differences (Fig. 2B). NMR spectra of the mutant NP84 and the wild type (Fig. 2A) did not show major differences in the carbohydrate ring protons, as shown in the 3.5 to 4.5 ppm region of the spectra. However, a triplet representing a succinyl substituent observed in the wild type between 2.4 and 2.8 ppm was absent in the mutant.

Mutant NP84 was defective for LPS also. Crude LPS extracted from TAL1145, NP84, and three transconjugants of

Table 1. Quantification of exopolysaccharide (EPS) produced by TAL1145 and NP84 and the effect of inoculation by these strains on the growth of *Leucaena leucocephala* plants

Strain	Dry weight of EPS/ml (mg) (mean ± SD)[a]	Shoot dry weight of *Leucaena* plants (mg) (mean ± SD)[b]	
		4 weeks	8 weeks
TAL1145	11.84 ± 0.28	251 ± 48	488 ± 72
NP84	5.71 ± 0.12	132 ± 48	477 ± 72
NP84(pUHR182)	10.3 ± 1.03	244 ± 36	ND
Control	. . .	114 ± 2	142 ± 10

[a] EPS in culture supernatant precipitated using 3 volumes of ethanol and dried. Mean of three replicates shown.
[b] Eight replicates were used for each treatment.
[c] Not determined.

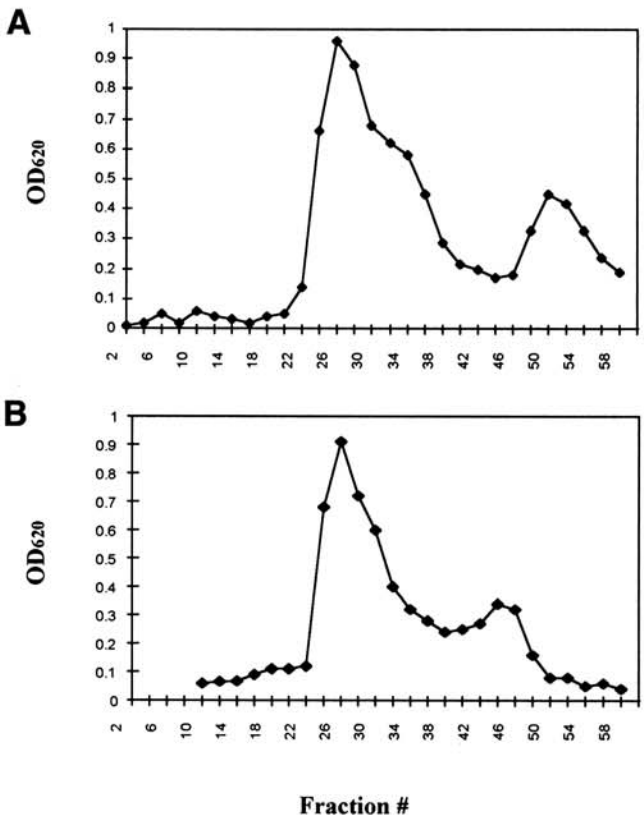

Fig. 1. Gel filtration of the culture supernatant of **(A)** TAL1145 and **(B)** mutant NP84 through a Bio-Gel A-5 m column. TAL1145 (20 mg) and NP84 (0.2 g) lyophilized culture supernatants were loaded on the column. The hexose content of the samples was determined by the procedure mentioned in Materials and Methods.

NP84 containing the plasmids pUHR182, pUHR251, and pUHR252 was analyzed by sodium dodecyl sulfate–deoxycholate–polyacrylamide gel electrophoresis (SDS-DOC-PAGE) and stained with alcian blue-silver (Fig. 3). Similar results were obtained when the gels were stained with the Bio-Rad silver stain without alcian blue, except that the bands were golden rather than brown (data not shown). Two major banding regions were observed in LPS gels, LPS I and LPS II. A series of bands (LPS III) was observed between LPS I and LPS II, and above LPS I. LPS I was severely affected in NP84. The LPS bands for the three transconjugants of NP84 were similar to those for TAL1145, indicating complementation of NP84 by the cloned TAL1145 DNA. Immunochemical analysis of the LPS blots using rabbit polyclonal antibodies against TAL1145 cells (provided by the NifTAL Center, Maui, Hawaii) showed reaction only with LPS I of the wild type and three complemented mutants but not with the mutant (data not shown).

Symbiotic phenotype of NP84 on *L. leucocephala*.

NP84 and TAL1145 formed similar numbers of nodules on *Leucaena*, but those formed by NP84 were smaller than those formed by TAL1145 4 weeks after inoculation. However, the size and weight of these nodules became very similar to those formed by TAL1145 after 8 weeks. The *Leucaena* plants inoculated with NP84 were yellow and stunted 1 month after inoculation and showed a Fix$^-$ phenotype. Acetylene reduction assays of nodules harvested from these plants did not show detectable nitrogenase activity. The dry weight of these plants was similar to that of plants in the uninoculated control (Table 1). However, when the plants nodulated with NP84 were grown for more than 5 weeks they started to fix nitrogen, as judged from the growth of the plants and the color of their leaves. After 8 weeks these plants became as green as those nodulated by TAL1145. At this stage the total dry weight of these plants was virtually identical to that of plants nodulated by TAL1145, showing that normal nodule development occurred during the 4- to 8-week period (Table 1). Bacteria isolated from the nodules of these 8-week-old plants were kanamycin resistant and EPS-defective as judged from colony morphology on YEM agar. The transconjugant NP84 (pUHR182) formed effective nodules on *Leucaena,* as judged from the acetylene reduction assay and the dry weights of the plants, which were similar to the weights of plants inoculated with TAL1145. This experiment was conducted three times, with similar results each time.

Even the largest nodule formed by NP84 after 4 weeks showed a much smaller number of infected cells than did nodules formed by TAL1145 (Fig. 4A and B). However, normal-looking, bacteroid-filled cells and peripheral vascular bundles were seen in the nodules formed by the mutant. The complemented mutant had the same nodule phenotype as the wild type (Fig. 4C). Electron micrographs of nodules formed by NP84 showed many cells with fibrillar material, which may be an indication of bacterial degeneration. Bacteroids were more tightly packed than those in nodules formed by TAL1145 (Fig. 5A). Bacteroids in nodules formed by NP84 contained smaller poly-β-hydroxy butyrate granules and more electron-dense cytoplasm than did TAL1145 bacteroids (Fig. 5B). The peribacteroid membrane and peribacteroid space enclosing the bacteroids were clearly observed in the core region of the nodules formed by TAL1145 but they were not discernible in the nodules formed by the mutant.

Mutant NP84 is defective in nodulation competitiveness.

Because NP84 forms nodules on *Leucaena* at the same time that TAL1145 does, we wanted to determine whether NP84 can invade *Leucaena* nodules when coinoculated with the wild-type strain. Data on competitive nodule occupancies by

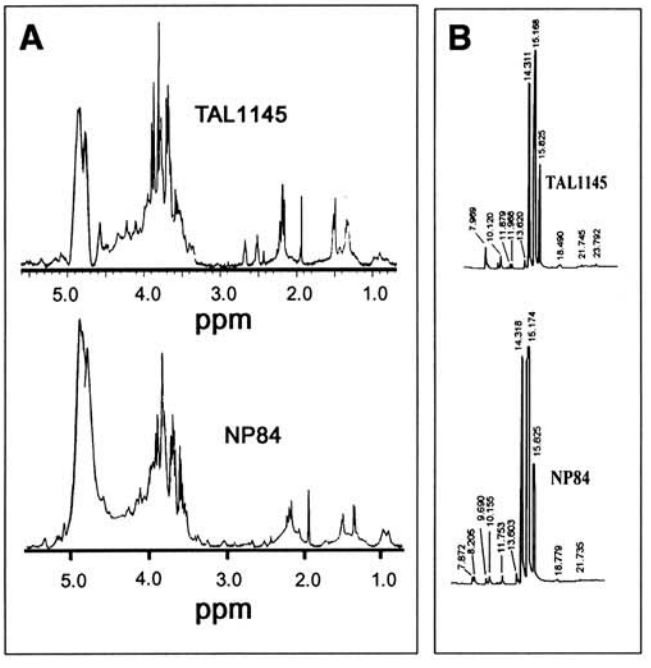

Fig. 2. A, ^{1}H nuclear magnetic resonance spectra of ethanol-precipitated exopolysaccharide (EPS) of TAL1145 and NP84. Samples were dissolved in D_2O and spectra were obtained at 500 Mhz. The triplets between 2.4 and 2.7 ppm represent the methylene protons of the succinyl group (absent in NP84). **B,** Gas liquid chromatography analyses of alditol acetate derivatives of trifluoroacetic acid hydrolyzed EPS of TAL1145 and NP84.

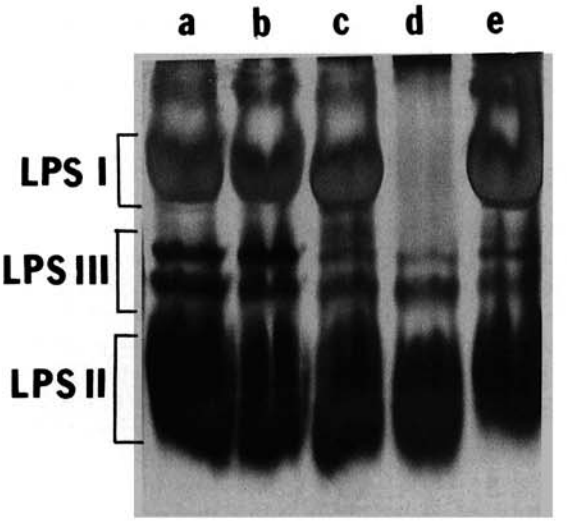

Fig. 3. Alcian blue-silver stained polyacrylamide gel (18% acrylamide containing, 10% sodium dodecyl sulfate and 0.5% deoxycholic acid) of crude LPS preparations from different strains: a, NP84(pUHR252); b, NP84 (pUHR251); c, NP84(pUHR182); d, NP84; and e, TAL1145.

NP84 and TAL1145 are shown in Table 2. None of the nodules from plants coinoculated with both strains showed single occupancy by NP84 even when its inoculum was nine times that of TAL1145. However, in all coinoculated plants a few nodules were occupied by both TAL1145 and the mutant. In nodules where double occupancy was observed, the proportion of the mutant in the ex-nodule rhizobia was estimated to be 1 per 10^6 CFU. Double occupancy was observed in less than 2% of the nodules when the proportion of the mutant in the inoculum was 10 or 50%. In these experiments 8 to 20% of the nodules showed double occupancy when the proportion of the mutant was 90% in the inoculum. When these experiments were repeated, similar results were obtained.

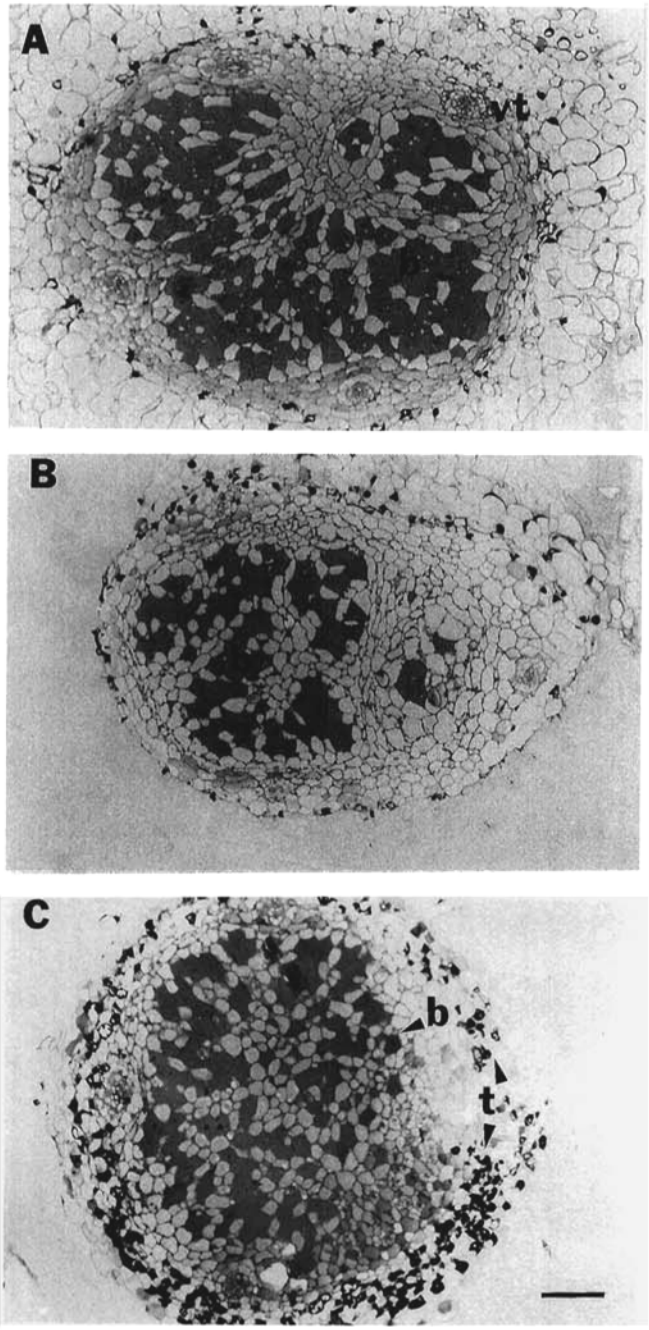

Fig. 4. Light microscopy of 1-µm-thick sections of 5-week-old *Leucaena leucocephala* root nodules **(A)** TAL1145, **(B)** NP84, and **(C)** NP84(pUHR182). Vascular tissue (vt) and densely stained bacteroid-filled cells (b) and tannins (t) in outer cortex are shown. Bar represents 100 µm.

Symbiotic phenotypes of NP84 on other legumes.

Mutant NP84 formed calluslike pseudonodules on *G. sepium* (Fig. 6B) and *P. vulgaris* (Fig. 6E) compared with the

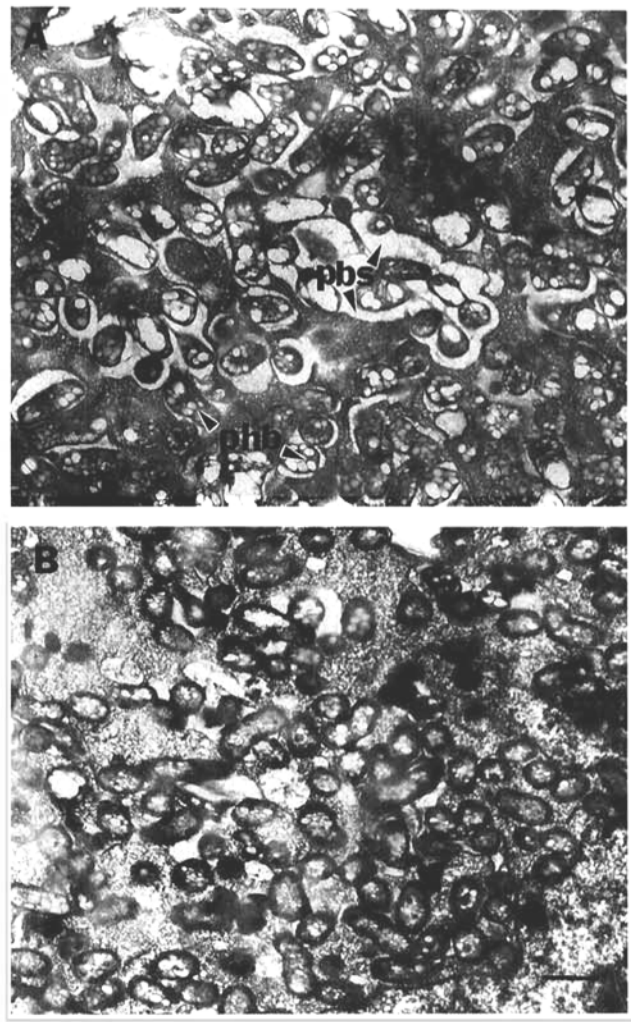

Fig. 5. Transmission electron microscopy of *Leucaena leucocephala* nodules showing infected cells. **A,** TAL1145. **B,** NP84. Bar indicates 4.5 µm. The ininfected cells (uic), poly-β-hydroxy butyrate (phb), and peribacteroid space (pbs) are shown.

Table 2. Nodule occupancy by strains TAL1145 and NP84 in paired inoculations of *Leucaena leucocephala*

Experiment	Strain ratio TAL1145/ NP84	Nodules with double occupancy (%) (mean ± SD)[a]	Nodules occupied by TAL1145 only (%) (mean ± SD)[a]
1	9 : 1	1.7 ± 2.6	98.3 ± 2.6
	1 : 1	0.7 ± 0.9	99.3 ± 0.9
	1 : 9	19.9 ± 6.0	80.1 ± 6.0
2	9 : 1	0.6 ± 1.4	99.6 ± 1.4
	1 : 1	1.8 ± 1.5	98.2 ± 1.5
	1 : 9	7.2 ± 4.8	92.8 ± 4.8

[a] Mean values of plants from eight Leonard jars were taken.

nitrogen-fixing nodules formed by TAL1145 on these legumes (Fig. 6A and D, respectively). The nodules formed by TAL1145 on *G. sepium* were at least three times larger in diameter than those formed on *P. vulgaris*. Similarly, the pseudonodules formed by NP84 on *G. sepium* were a little larger than those formed on *P. vulgaris*. Bacteria could not be recovered from the pseudonodules of either host. No bacteroid zones or bacteroid-filled cells were visible in either host but vascular tissue was observed in the center of some pseudonodules. Small, meristematic-like cells filled with dense cytoplasm and prominent nuclei were observed in the pseudonodule sections of both hosts (Fig. 6F). *P. vulgaris* and *G. sepium* inoculated with the transconjugant NP84(pUR182) formed normal, nitrogen-fixing nodules (Fig. 6C).

NP84 is complemented by a 3.8-kb DNA fragment from TAL1145.

Previously, we complemented NP84 with cloned DNA from TAL1145 in the cosmid pUHR182 and showed that a 6.3-kb *Eco*RI fragment in this cosmid hybridized with the Tn*5lacZ*-

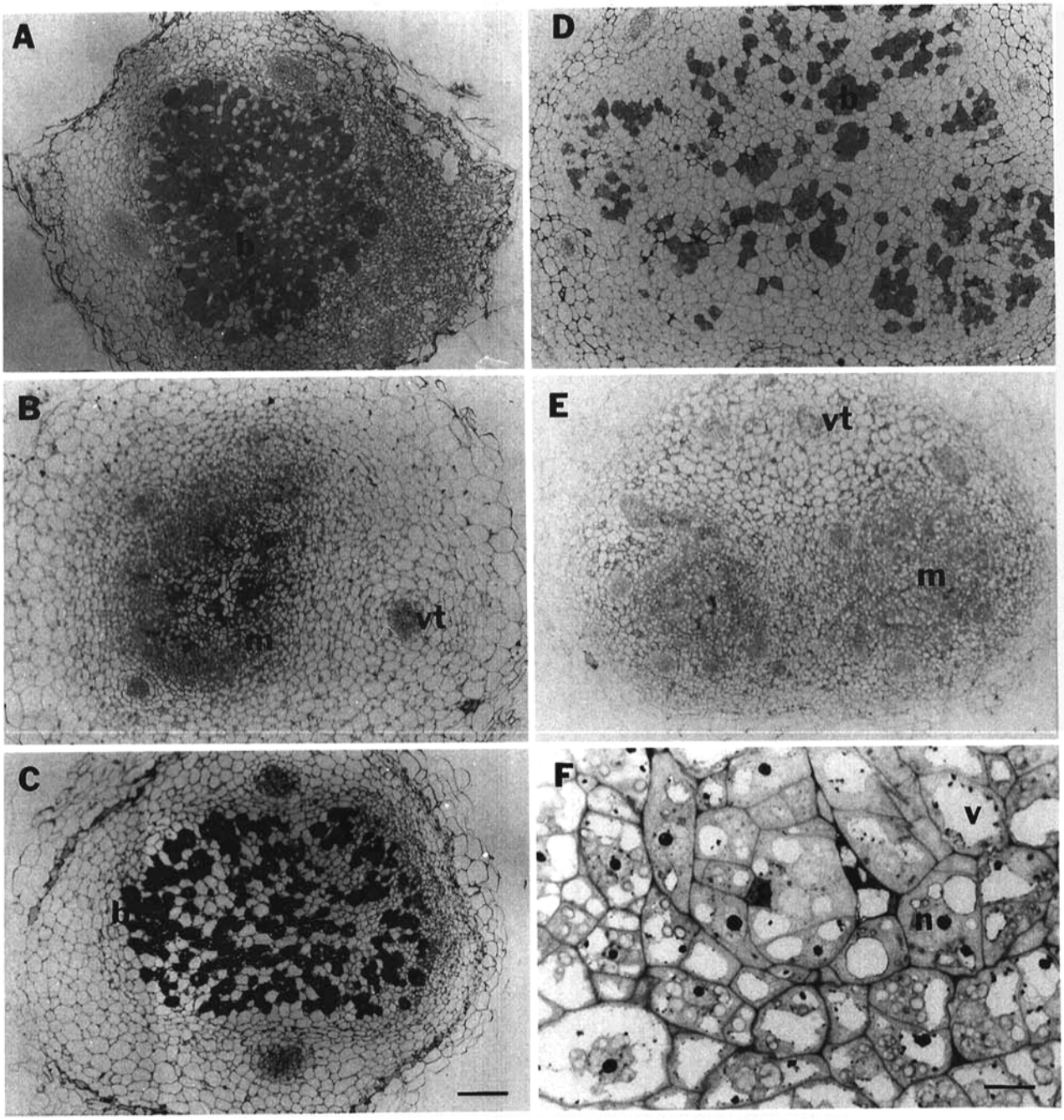

Fig. 6. Light micrographs of 1-μm-thick sections of *Gliricidia sepium* nodules inoculated with **(A)** TAL1145, **(B)** NP84, and **(C)** NP84(pUHR182), and *P. vulgaris* nodules inoculated with **(D)** TAL1145 and **(E)** NP84. **F,** Pseudonodules formed by NP84 on beans. Bars represent 110 μm (**A** to **E**) and 10 μM (**F**). Bacteroid-filled cells (b), vascular tissue (vt), meristematic-like cells (m), vacuoles (v), and plant cell nucleus (n) are shown.

inserted fragment in NP84 (Parveen and Borthakur 1994). We isolated another cosmid, pUHR183, by complementing NP84 for the EPS defect on YEM agar. Cosmid pUHR183 had the same *Eco*RI fragments as pUHR182 except that it had a 3.8-kb *Eco*RI fragment instead of a 6.3-kb *Eco*RI fragment. Restriction mapping and further Southern analysis showed that the 3.8-kb fragment is a part of the 6.3-kb *Eco*RI fragment. One *Eco*RI site in this fragment comes from the multiple cloning site of the cosmid vector pLAFR3. The cosmid clone library of TAL1145 was constructed by cloning approximately 25 kb of partial *Sau*III fragments into the *Bam*HI site of pLAFR3 (George et al. 1994). Cosmid pUHR183 contained the 3.8-kb fragment as one of its end fragments. The 6.3-kb fragment of pUHR182 and the 3.8-kb fragment of pUHR183 were cloned in the broad-host-range cloning vector pRK404 to obtain plasmids pUHR251 and pUHR252, respectively. The transconjugants of NP84 containing either pUHR251 or pUHR252 produced EPS in the same way as TAL1145 was produced and formed normal nitrogen-fixing nodules on *P. vulgaris*.

DISCUSSION

In this study we found that the transposon-insertion mutant NP84 has alterations in both EPS and LPS. Nodule development by this mutant is more severely affected in the determinate hosts, *P. vulgaris* and *G. sepium,* than in the indeterminate host *L. leucocephala.* We have also shown that a 3.8-kb DNA fragment cloned from TAL1145 complements this mutant. The mutation in NP84 has a greater effect on LPS than on EPS synthesis. LPS I synthesis is most severely affected and reduced amounts of EPS are produced in media containing mannitol. The differences in the low mobility bands in SDS-DOC-PAGE are due to differences in the LPS and may not be due to polysaccharides such as Kdop (Reuhs et al. 1993) because a similar banding pattern was observed after both silver and alcian blue-silver staining procedures. In the EPS of NP84 a noncarbohydrate succinyl substituent was absent. However, we know that the mutated gene in NP84 is not *exoH*-encoding succinyl transferase because we have isolated and sequenced *exoH* from strain TAL1145 and found that it is located in another cluster of genes. The product of the gene mutated in NP84 may encode a protein involved in the synthesis or transport of certain components of LPS. It may also interact with the ExoH protein, which is located in the inner membrane and is involved in succinyl group transfer to EPS.

Results of the plant experiments with determinate-nodule-forming hosts *P. vulgaris* and *G. sepium* suggest that specific oligosaccharide motifs of LPS may be required for normal development of determinate nodules because NP84 formed pseudonodules on these hosts. These results are supported by the findings of other workers (Carlson et al. 1987, 1992). Reduction of LPS I or its absence in *R. leguminosarum* bv. *phaseoli* mutants had also been shown to inhibit nodule development of beans (Cava et al. 1989; Noel et al. 1986; Vandenbosch et al. 1985). We observed the presence of central vascular bundles in addition to the normal peripheral conducting tissues in both beans and *Gliricidia.* The presence of central instead of peripheral branched vascular bundles was also reported in bean nodules formed by LPS mutants (Noel et al. 1986).

Bacteroid development and nitrogen fixation are delayed in *Leucaena* nodules formed by NP84. This may be due to some modification of the EPS or the absence of LPSI in the LPS or both. In *R. meliloti* a capsular polysaccharide, known as the Kdo polysaccharide, which is rich in 3-deoxy-D-manno-2-octulosonic acid, was shown to perform the same function as EPS in nodule development on alfalfa (Reuhs et al. 1993). It is possible that the EPS produced by NP84 compensated for the absence of some LPS components in *Leucaena* nodules at later stages of development. Leakiness of Exo phenotype (the presence of a few nitrogen-fixing nodules) was observed earlier in *R. meliloti* (Klein et al. 1988). LPS defects in *R. meliloti* do not affect nodule development in alfalfa, which also forms indeterminate nodules like *Leucaena* (Clover et al. 1989). On the other hand, LPS mutants of *R. leguminosarum* bv. *viciae* formed white Fix$^-$ mutants on indeterminate hosts such as *Vicia hirsuta, Pisum sativum,* and *Lens culinaris* (Priefer 1989). These nodules remained small and senesced prematurely. The symbiotic phenotype of NP84 on *Leucaena* in the present study is different from those of *R. meliloti* and *R. leguminosarum.* The *Leucaena* nodules formed by NP84 are Fix$^-$ 4 weeks after inoculation and slowly became similar to those formed by the wild-type strain in size, weight, and nitrogen fixation. The small Fix$^-$ *V. hirsuta* nodules formed by the LPS mutants of *R. leguminosarum* contained infection threads from which bacteria were released into the host cells and became enclosed in peribacteroid membranes (Priefer 1989). Similarly, in the *Leucaena* nodules formed by NP84, bacteria were released from the infection threads and bacteroids formed slowly, but unlike the *V. hirsuta* nodules, *Leucaena* nodules did not senesce prematurely. TAL1145 bacteroids had clear peribacteroid membranes and they were surrounded by distinct peribacteroid space in *Leucaena* nodules while the bacteroids formed by NP84 were more tightly packed and the peribacteroid space was not prominent. It is possible that LPS or EPS or both may be involved in the separation of the peribacteroid membrane from the bacteroids in the nodules formed by the wild-type. However, there is no direct evidence for the presence or absence of polysaccharide components in the peribacteroid space.

Inoculation of *Leucaena* plants with NP84 and TAL1145 separately resulted in the appearance of nodules simultaneously. However, NP84 was less competitive than the wild type. The competition defects of NP84 may be due to the EPS or the LPS defects or both. Bhagwat et al. (1991) suggested that the reduction of EPS synthesis by a *Bradyrhizobium* mutant was responsible for its defect in the competitive nodule occupancy. Reduced competitiveness of *B. japonicum exoB* mutants that show alterations in both EPS and LPS for nodulation of *Glycine max* has also been reported by Parniske et al. (1993).

The wild-type DNA fragment homologous to the Tn*5lacZ*-inserted fragment in NP84 has been cloned. A 3.8-kb fragment cloned in plasmid pUHR252 complemented NP84 for both EPS and LPS defects and allowed the transconjugant of NP84 to nodulate *Gliricidia* and *P. vulgaris*. Since *exoB* mutants of *R. meliloti* are also defective in both EPS and LPS synthesis (Leigh and Lee 1988) we wanted to determine if the cloned 3.8-kb fragment contains the *exoB* gene of TAL1145. Previously, the *exoB* gene of NGR234 was also thought to be involved specifically in nodulation of *Leucaena* (Zhan et al. 1990). However, unlike NP84, the *exoB* mutants of NGR234 are defective in EPS but not in LPS synthesis (Gray et al. 1991). Also, we used the 3.8-kb fragment in plasmid

pUHR252 in Southern hybridization with plasmids pD56 and pEX312 containing the *exoB* gene of *R. meliloti* in an 1.4-kb *Eco*RI fragment (Long et al. 1988) and found that it did not hybridize with this fragment, suggesting that the 3.8-kb cloned DNA in pUHR252 did not contain a gene equivalent to *exoB*. However, under low stringency conditions the 3.8-kb fragment hybridized with the 7.8-kb *Eco*RI fragment in the *exo* gene cluster, suggesting that this fragment may contain partial homology with one of the other *exo* genes in this cluster. We also used the 3.8-kb fragment as a probe against 16 of the *Leucaena*-nodulating strains and found that it strongly hybridized with all of these, indicating that the genes contained in this fragment are highly conserved in *Leucaena*-nodulating rhizobia. Phylogenetically, TAL1145 was identified as a different species and was placed close to *R. tropici, R. leguminosarum,* and *R. meliloti* (George et al. 1994). In conclusion, we have identified a 3.8-kb DNA fragment of TAL1145 containing gene(s) involved in both LPS and EPS synthesis that is essential for the infection of nodules in determinate hosts, and is required for normal bacteroid development as well as competitiveness in *Leucaena* nodules.

MATERIALS AND METHODS

Bacterial strains, plasmids and media.

Strains and plasmids used in this work are listed in Table 3. Rhizobia were grown on yeast extract mannitol (YEM) (Vincent 1970) or tryptone yeast extract (TY) medium (Beringer 1974) at 28°C and *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989). The EPS phenotype of the wild type and the mutant was also checked on YEM medium containing 0.02% Calcofluor. Rhizobia were grown in 10 × GMS medium for the production of EPS (Breedveld et al. 1990). Antibiotics were used at the following concentrations (per liter): Rifampicin (Rif), 30mg; Streptomycin (Str), 100 mg; Kanamycin (Kan), 25 mg; Tetracycline (Tet), 10 mg.

DNA manipulations.

Genomic DNA was prepared as previously described (Pooyan et al. 1994b). Recombinant plasmids were isolated from *Rhizobium* by the alkaline-lysis method (Sambrook et al. 1989). Restriction enzymes were obtained from Promega (Madison, WI).

Southern hybridization.

Electrophoresis of DNA in 0.7% agarose gel was followed by transfer of DNA to a Gene Screen Plus membrane (NEN, Dupont, Boston, MA) by the alkaline transfer procedure (Reed and Mann 1985). Probes for hybridization were prepared by the random priming method (Feinberg and Vogelstein 1983). Hybridization was done at 65 and 55°C for high and low stringency, respectively.

EPS analysis.

Seven-day-old liquid culture of rhizobia grown in Breedveld's 10 × GMS medium was centrifuged at 16,000 × *g* and the supernatant was transferred to a beaker. EPS for quantification was extracted with three volumes of ethyl alcohol at 4°C overnight. The EPS pellet was removed by centrifugation at 10,000 × *g*, dried under vacuum, and weighed. For size fractionation of EPS, the culture supernatant was concentrated by rotary evaporation, filtered (0.45 μm pore size, Millipore Corp.), and dried by lyophilization. After redissolving in sodium phosphate buffer, the EPS sample was loaded on a Bio-Gel A-5m column (39 × 4 cm). EPS was eluted with 10 mM sodium phosphate buffer pH 7.0 at a flow rate of approximately 0.5ml/min. Fractions were collected and their hexose contents were determined by anthrone analysis (Trevelyan and Harrison 1952).

Ethanol-precipitated EPS of TAL1145 and the mutant NP84 was dialyzed and lyophilized. After hydrolysis of EPS with 2N trifluoroacetic acid the sugars were converted into their respective alditol acetate derivatives by the method of Albersheim et al. (1967). Gas liquid chromatography (GLC) was done using a SP-2380 fused silica capillary column (15 m × 0.53 mm ID) with 0.2-μm-thick film from Sopelco on a Hewlett Packard 5890 GLC instrument with a Hewlett Packard 7673 injector and FID detector. ^1H nuclear magnetic resonance (proton NMR) spectra of total EPS prepared as mentioned above were recorded at 500 Mhz in D_2O using a General Electric GN Omega 500 spectrometer at the NMR Facility of the Department of Chemistry, University of Hawaii, Honolulu.

Table 3. Bacterial strains and plasmids

Strains/plasmids	Relevant characteristics	Reference
Rhizobium		
TAL1145	Tree-nodulating wild type strain, EPS$^+$, Rif^r, Str^r	George et al. 1994
NP84	Tn*5lacZ* insertion mutant of TAL1145, EPS and LPS defective	Parveen and Borthakur 1994
R. tropici	Wild-type strains of Type A and Type B	Martinez-Romero et al. 1991
Escherichia coli		
DH5αmcr	*recA1, lacU*169,φ80d *lacZ*ΔM15	GIBCO-BRL Life Technologies, Gaithersburg, MD
Plasmids		
pUHR182	pLAFR3, TAL1145 cosmid complementing NP84	Parveen and Borthakur 1994
pUHR183	pLAFR3, TAL1145 cosmid complementing NP84 and overlapping pUHR182	This work
pUHR251	6.3-kb *Eco*RI fragment from pUHR182 cloned in pRK404, complements NP84	This work
pUHR252	3.8-kb *Eco*RI fragment from pUHR183 cloned in pRK404, complements NP84	This work
pD56	pLAFR1, *R. meliloti exoBF*-complementing plasmid	Long et al. 1988
p'3222	Plasmid containing *Rhizobium* sp. strain NGR234 *exo* genes, Tet^r	Gray et al. 1991
pRK404	Tet^r IncP	Ditta et al. 1985
pRK2013	RK2 derivative Kan^r, Tra$^+$	Figurski and Helinski 1979

Extraction and purification of LPS.

LPS was extracted by a hot phenol-water method (Johnson and Perry 1976) with some modifications. Bacteria were grown in 50 ml of medium (TY for *Rhizobium* and LB for *Salmonella typhimurium)* for 48 h, centrifuged at 16,000 × *g*, and washed three times with 0.5 M NaCl to remove EPS. The cell pellets were resuspended by vortexing in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA and 0.05% (wt/vol) sodium azide. The bacteria were lysed by sonication, 1 ml of hen egg white lysozyme (10 mg/ml) was added, and the suspension was stirred at 4°C for 16 h. After incubation the suspension was placed at 37°C for 30 min and vortexed. Ribonuclease and deoxyribonuclease were added to final concentrations of 100 µg/ml and 50 µg/ml, respectively. The suspension was incubated at 37°C for 30 min and enzymes were inactivated at 60°C for 10 min. Proteinase K was added to a final concentration of 100 µg/ml and after 1 h incubation at 37°C the conventional phenol (70°C) extraction method was followed. The aqueous phases after three phenol extractions were pooled and dialyzed against distilled water until no detectable odor of phenol remained. Crude LPS so obtained was then lyophilized.

SDS-DOC-PAGE of LPS.

LPSs were analyzed by PAGE using 18% acrylamide gels with 10% SDS and 0.5% deoxycholic acid as detergents. The gels were stained with Alcian blue-silver (Corzo et al. 1991).

Plant tests.

Nodulation assays on all three legumes, *G. sepium* (accession number NFTA 604), *L. leucocephala* (cultivar K-8), and *P. vulgaris* (cultivar Brazil 2), were performed in growth pouches as described in George et al. (1994). Two-day-old seedlings were inoculated with an approximately 10^7 CFU/ml rhizobial culture suspension. Nodules for microscopic studies were harvested 4 to 5 weeks after inoculation.

Plant experiments to study effectiveness of the strains and competition experiments to determine nodule occupancy were conducted in Leonard jars. Eight replicates were used for each treatment with each Leonard jar containing 3 plants. Three inoculant ratios, 1:1, 1:9, and 9:1, between TAL1145 and NP84 were used for competition studies. Plate counts of the rhizobia were taken and a minimum of 10^6 CFU of rhizobia per ml were used in each case. The plants were harvested after 6 weeks for nodule typing. Nodules were excised from the roots, surface sterilized with 20% bleach, and washed 5 to 6 times with sterile water. Individual nodules were crushed in 0.1 ml of sterile 0.25M NaCl in 96-well microtiter plates with a multiple inoculator. Nodule occupancy was determined on the basis of antibiotic resistance markers of the exnodule rhizobia by replica plating on YEM agar containing appropriate antibiotics.

Microscopic studies.

Nodules were excised from the roots and fixed in fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 6.8), followed by post-fixation in buffered 2% osmium tetraoxide. Specimens were dehydrated in ethanol and polypropylene oxide, and then embedded in Spurr's resin (Spurr 1969). Semithin (1 µm) sections were cut with a Sorval microtome and stained with toluidine blue for light microscopic examination of the nodules. Thin sections (90 to 100 nm) of *Leucaena* nodules were cut with an RMC6000 microtome and sections on copper grids were stained with 5% methanolic uranyl acetate and 0.5% lead citrate for observation by transmission electron microscopy.

ACKNOWLEDGEMENTS

We thank Esperanza Martinez-Romero for providing *R. tropici* strains, T. Lynn Reuber for providing the plasmids containing *R. meliloti* genes, and Steven P. Djordjevic for providing plasmid R'3222 of NGR234. We also wish to thank Leslie R. Berger for a critical review of the manuscript. This work was supported partially by a subgrant SM-CRPS-023 under the grant # DAN-1311-G-00-1049-00 from USAID to the NifTAL Project and partially by USDA grant # 92-37305-7875.

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