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Isolation and Characterization of Transposon Tn5-Induced Symbiotic Mutants of *Rhizobium loti*

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Rhizobium loti **NZP2037 and NZP2213, each cured of its single large indigenous plasmid, formed effective nodules on *Lotus* spp., suggesting that the symbiotic genes are carried on the chromosome of these strains. By using pSUP1011 as a vector for introducing transposon Tn*5* into *R. loti* NZP2037, symbiotic mutants blocked in hair curling (Hac), nodule initiation (Noi), bacterial release (Bar), and nitrogen fixation (Nif/Cof) on *Lotus pedunculatus* were isolated. Cosmids complementing the Hac, Noi, and Bar mutants were isolated from a pLAFR1 gene library of NZP2037 DNA by in planta complementation and found to contain *Eco*RI fragments of identical sizes to those into which Tn*5* had inserted in the mutants. The cosmids that complemented the mutants of these phenotypic classes did not share common fragments, nor did cosmids that complemented four mutants within the Noi class, suggesting that these symbiotically important regions are not tightly linked on the *R. loti* chromosome.**

Rhizobium loti (27) is a group of fast-growing rhizobia that nodulate the agriculturally important pasture legume *Lotus* spp., as well as a variety of other legumes (27, 39, 41). Within this species are found strains, such as NZP2037, which form effective nitrogen-fixing (Nod^+ Fix^+) nodules on both *Lotus tenuis* and *Lotus pedunculatus*, and others, such as NZP2213, which form effective (Nod^+ Fix^+) nodules on *Lotus tenuis* but ineffective (Nod^+ Fix^-) tumor-like structures on *Lotus pedunculatus* (41). Because little is known about the molecular basis of these nodulation differences, we initiated a study on the nodulation genes of these strains. Although a single large indigenous plasmid was identified in *R. loti* strains NZP2037(pRlo2037) and NZP2213(pRlo2213) (40, 46), unlike the other fast-growing species of *Rhizobium* examined to date (1, 2, 24, 25, 38, 42, 47, 48, 49) *nif* structural genes were not localized on the *R. loti* plasmids (40). In addition, the ability to nodulate *Lotus* spp. could not be transferred when pRlo2037 was conjugated into other *Rhizobium* strains, suggesting that the genes for nodulation were not located on the plasmid (40).

The genetic analysis of *Rhizobium* symbiotic genes has been greatly facilitated in recent years by the development of plasmid vectors, such as pJB4JI, for the introduction of transposons into the genome of this genus (4). The plasmid pJB4JI (4) has been used successfully in the isolation of transposon Tn*5*-induced symbiotic mutants of *Rhizobium meliloti* (2, 6, 18, 34), *Rhizobium trifolii* (44, 51), *Rhizobium phaseoli* (37) and a slow-growing *Rhizobium* sp. (*Parasponia*) (9). However, attempts to use pJB4JI for Tn*5* mutagenesis in a number of other *Rhizobium* strains, including *R. loti*, have been unsuccessful (34, 40, 45, 50), and an added problem has been the insertion of Mu phage DNA sequences as well as Tn*5* into the genome (6, 34). The recent construction of a group of broad host range mobilizable vectors based on well-known *Escherichia coli*-amplifiable plasmids has provided an alternative system of Tn*5* mutagenesis in *Rhizobium* spp. and overcomes the problems associated with using pJB4JI (52).

We describe here the isolation of a range of Tn*5*-induced symbiotic mutants of NZP2037 using the pSUP1011 vector system. We also report on the isolation of plasmid-cured derivatives of strains NZP2037 and NZP2213 that still develop effective (Nod^+ Fix^+) nodules on *L. tenuis*.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. *R. loti* cultures were grown at 28°C in either TY medium (3) or S20 defined medium, which is M9 medium (35) containing 2.4 g of sucrose per liter and 0.2 mg of biotin per liter. *E. coli* cultures were grown at 37°C in either LB medium (35) or TY medium. S20 medium was supplemented as required after autoclaving with sterilized solutions of streptomycin (Str; 200 μg/ml), neomycin (Neo; 500 μg/ml), kanamycin (Kan; 200 μg/ml) or tetracycline (Tet; 2 μg/ml).

Curing experiments. A pRlo2037-cured derivative of NZP2037 was isolated by incubating strain PN4005 at 37°C for 7 days (36), followed by screening for kanamycin-sensitive survivors at 28°C. A pRlo2213-cured derivative of *R. loti* NZP2213 was isolated during an analysis of the plasmid content of the 1.0% survivors of a UV-mutagenized (15 s at 2,000 μW/cm^2) culture of PN183.

Crosses. Crosses were carried out by the patch plate method (29).

Nodulation tests and symbiotic phenotype. Nodulation tests were carried out with *L. pedunculatus* Cav. 'Grasslands Maku' and *L. tenuis* Waldst. et Kit. seedlings by previously described methods (50). After 6 weeks, plants were examined for the presence (Nod^+) or absence (Nod^-) of nodules and for symptoms of nitrogen starvation, which indicated an ineffective (Fix^-) symbiosis. In all nodulation tests, uninoculated plants and plants inoculated with the wild-type strain were included for comparison. To define the block in the symbiosis of the Tn*5*-induced symbiotic mutants, tumor-like structures and ineffective nodules induced by these mutants were examined by light and electron microscopy, and a phenotype was assigned on the basis of the system devised by Vincent (55).

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TABLE 1. Bacterial strains and plasmids

Strain	Relevant characteristics	Source or reference
R. loti		
NZP2037	Nod$^+$ Fix$^+$ (*L. pedunculatus*, *L. tenuis*)	DSIR Culture Collection
PN184	Nod$^+$ Fix$^+$ *str-1*	This study; spontaneous mutant of NZP2037
PN233	Nod$^-$ *str-1*	This study; Tn*5*-induced mutant of PN184
PN234-PN246	Nod$^+$ Fix$^-$ *str-1*	This study; Tn*5*-induced mutants of PN184
PN4005	Nod$^+$ Fix$^+$ *str-2*	Pankhurst et al. (40)
PN4010	Nod$^+$ Fix$^+$ *str-2*	This study; PN4005 heat cured of pRlo2037::Tn*5*
NZP2213	Nod$^+$ Fix$^-$ (*L. pedunculatus*) Nod$^+$ Fix$^+$ (*L. tenuis*)	DSIR Culture Collection
PN183	Nod$^+$ Fix$^+$ (*L. tenuis*) *str-1*	This study; spontaneous mutant of NZP2213
PN225	Nod$^+$ Fix$^+$ (*L. tenuis*) *str-1*	This study; PN183 cured of pRlo2213
A. tumefaciens		
C58		C. Kado
E. coli		
HB101	*pro leu thi lacY* Str^r *recA*$^-$ *hsdR hsdM*	G. Ditta
SM10	*thi thr leu tonA lacY supE recA* [RP4.2 Tc::Mu] Ap^s Tc^s Mu2$^+$ Km^r Tra$^+$	Simon et al. (52)
BHB 2690	N205 *recA* [λ *imm*^{434} *c*Its *b2 red E*am *S*am/λ]	Hohn (22)
BHB 2688	N205 *recA* [λ *imm*^{434} *c*Its *b2 red D*am *S*am/λ]	Hohn (22)
PN440	HB101(pPN360)	This study: *R. loti* PN4005/pJB3JI × *E. coli* HB101
E. coli plasmids		
pSUP1011	Cm^r Km^r *oriT* RP4	Simon et al. (52)
RP4	Ap^r Tc^r Km^r	Holloway and Richmond (23)
pACYC184	Cm^r Tc^r	Chang and Cohen (10)
pKan2	pBR322 containing the 3.5-kb *Hin*dIII fragment of Tn*5*	Scott et al. (51)
pLAFR1	RK290 (Tc^r) containing λ cos	Friedman et al. (19)
pRK2013	Km^r	Ditta et al. (13)
pSA30	*Klebsiella pneumoniae Eco*RI fragment (*nifHDK*) cloned in pACYC184	Cannon et al. (8)
pPN360	pRlo2037::pJB3JI Ap^r Km^s Tc^r	This study
pJB3JI	Km^s derivative of R68.45 Ap^r Tc^r	A. Johnston

Analysis of plasmids in *Rhizobium* spp. The screening of plasmids present in *Rhizobium* strains was carried out by a modification of the Eckhardt procedure (16) as previously described (50).

Preparation of DNA. Amplifiable plasmids were isolated by the cleared lysate method, followed by cesium chloride density gradient centrifugation (11).

The indigenous plasmid of *R. loti* NZP2213(pRlo2213) was purified by a previously described alkaline lysis procedure (49), except that the temperature used was 65°C. After lysis, the plasmid DNA was concentrated by precipitation with 10% (wt/vol) polyethylene glycol (molecular weight, 6,000) at 4°C overnight. The DNA was pelleted by centrifugation (3,000 × *g*, 1.5 min at 4°C), suspended in TE buffer (10 mM Tris, 1 mM Na_2EDTA [pH 8]), and further purified by cesium chloride-ethidium bromide density ultracentrifugation in a Beckman rotor (type 65) at 250,000 × *g* for 40 h at 16°C. Plasmid DNA was collected, and ethidium bromide was removed by extraction with isopropanol saturated with 5 M NaCl. The DNA was dialyzed in TE buffer and used directly for DNA digests or preparation of a ^{32}P-labeled probe.

The *R. loti* NZP2037 cointegrate plasmid pPN360 (pRlo2037::pJB3JI) and pLAFR1 cosmids were isolated from *E. coli* by the method of Ish-Horowitz and Burke (26).

Total *Rhizobium* DNA was isolated by a method based on that of Fischer and Lerman (17). Cells (10 ml) grown in TY medium (absorbance at 600 nm of 1.0) were washed once in 0.1% Sarkosyl in TE buffer (50 mM Tris, 20 mM Na_2EDTA [pH 8.0]) and once in TE buffer alone and suspended in 10 ml of TE buffer. Lysozyme was added to a concentration of 300 μg/ml, and the cells were incubated for 30 min at 37°C. Proteinase K (Boehringer Mannheim Biochemicals) and Sarkosyl were added to final concentrations of 300 μg/ml and 10 mg/ml, respectively, and the mixture was incubated at 50°C overnight. The lysate was extracted twice with two volumes of phenol-$CHCl_3$ (50:50 [vol/vol]) and twice with $CHCl_3$, and the DNA was precipitated with 2.5 volumes of ethanol.

Restriction enzyme digestion of DNA and agarose gel electrophoresis. All DNA digestions were carried out in a standard reaction mixture containing 6 mM Tris (pH 7.6), 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol with the NaCl concentration adjusted for each restriction enzyme to a concentration recommended by the manufacturer (New England Biolabs) of the enzyme. Reaction mixtures were incubated for 1 to 2 h at 37°C and then stopped by either incubating the reaction mixture at 65°C for 10 min or by adding 0.25 volumes of a sodium dodecyl sulfate-dye mixture consisting of 20% (wt/vol) sucrose–5 mM Na_2EDTA–1% (wt/vol) sodium dodecyl sulfate–0.2% (wt/vol) bromophenol blue. DNA was separated by electrophoresis (1.5 V/cm) in a horizontal 0.7% (wt/vol) agarose gel (20 by 15.5 by 0.4 cm) with Tris-acetate buffer (40 mM Tris, 2 mM Na_2EDTA, 20 mM sodium acetate [pH 7.9]). Gels were stained and photographed as previously described (50).

Construction of a pLAFR1 gene library to *R. loti* NZP2037. Total DNA from *R. loti* NZP2037 was partially digested with *Eco*RI to an average size of 25 kilobases (kb) and then separated by electrophoresis (1.5 V/cm for 16 h) in a horizontal 1% Seaplaque agarose (Marine Colloids) gel. DNA in the size range of 15 to 30 kb was electroeluted (120 V for 4 h) from the agarose, extracted with phenol-chloroform (50:50 [vol/vol]), and concentrated by ethanol precipitation. The DNA was suspended in distilled water and used directly for cloning. The electroeluted DNA (5 μg) was

mixed with 0.5 μg of *Eco*RI-cut pLAFR1 (19) DNA and ligated overnight at 4°C in a 10-μl reaction mixture containing 6 mM Tris (pH 7.6), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM ATP, and 400 units of T4 DNA ligase. A 2.5-μl portion of the ligation mixture was packaged by the method of Hohn (22) with packaging extracts prepared from the λ lysogenic strains BHB2690 and BHB2688 as described by Maniatis et al. (33). Packaged DNA was used to infect *E. coli* HB101 (22), and the cells were plated on LB plates containing 15 μg of tetracycline per ml. After overnight incubation at 37°C the Tet^r colonies were washed off with 50% glycerol and stored at −20°C.

In planta complementation of *R. loti* symbiotic mutants. To isolate pLAFR1 cosmids capable of complementing the Tn5-induced symbiotic mutants of *R. loti*, the gene library was crossed en masse (31) with each *R. loti* Tn5-induced symbiotic mutant in a triparental mating with *E. coli* HB101(pRK2013) as a source of helper plasmid (13). The Tc^r transconjugants from the cross were used in batches of about 200 colonies to inoculate *L. pedunculatus* seedlings, and after 6 weeks the plants were examined for complementation (31). In those cases in which complementation occurred, nodules were removed and surface sterilized, and bacteria were isolated by previously described methods (50). The *R. loti* derivatives isolated were retested on plants to confirm the complementation results and then used as donors in an additional triparental mating with *E. coli* HB101 and *E. coli* HB101(pRK2013) to transfer the cosmid responsible for the complementation back into *E. coli* HB101. Each of the cosmids was transferred back to the corresponding *R. loti* mutant in an additional triparental mating to confirm that bacteria isolated from the nodules were not revertants.

Hybridization procedures. DNA was labeled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp.) by primed synthesis with DNA polymerase I (Klenow fragment) and denatured herring sperm DNA primers as previously described (56). DNA digests were separated by electrophoresis (1.5 V/cm for 16 h) in a 0.7% agarose (type I; Sigma Chemical Co.) horizontal gel with Tris-acetate buffer (40 mM Tris, 20 mM acetic acid, 2 mM Na_2EDTA [pH 7.9]) and transferred to nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) by the method of Southern (53). Hybridization was carried out at 65°C for 16 to 24 h as previously described (56). Filters were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 20°C and exposed to Kodak XS-5 X-ray film in the presence of Cronex Intensifying screens for 1 to 5 days at −70°C.

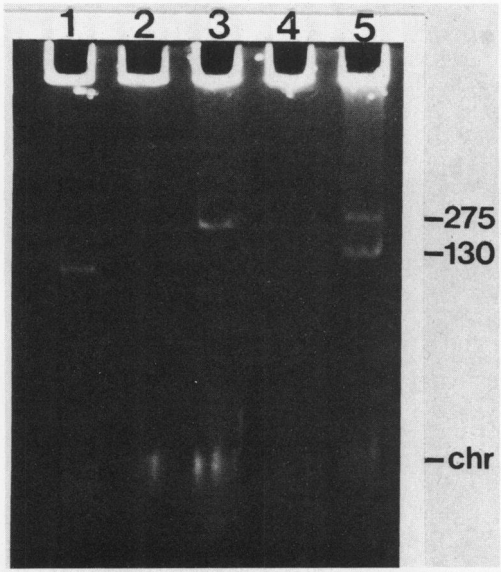

FIG. 1. Eckhardt gel of wild-type and plasmid-cured derivatives of *R. loti*. Lane 1, *R. loti* NZP2213; lane 2, *R. loti* PN225; lane 3, *R. loti* NZP2037; lane 4, *R. loti* PN4010; lane 5, *A. tumefaciens* C58. Numbers indicate molecular size of plasmids in megadaltons; chr, chromosomal DNA.

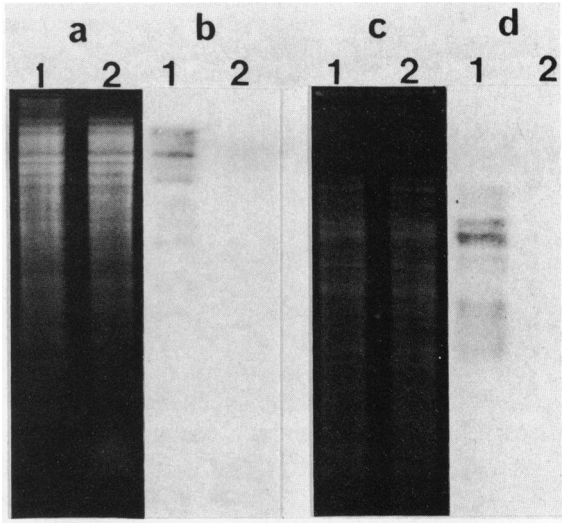

FIG. 2. Hybridization of *R. loti* plasmid DNA to total DNA from *R. loti* wild-type and plasmid-cured derivatives. (a) Agarose gel of *Eco*RI digests of NZP2213 (lane 1) and PN225 (lane 2). (b) Autoradiograph of (a) hybridized with ^{32}P-labeled pRlo2213. (c) Agarose gel of *Eco*RI digests of NZP2037 (lane 1) and PN4010 (lane 2). (d) Autoradiograph of (c) hybridized with ^{32}P-labeled pPN360.

Light and electron microscopy. Light and electron microscopy of nodule sections was carried out as previously described (41).

RESULTS

Curing of plasmids in *R. loti*. To determine whether the single large indigenous plasmid of *R. loti* NZP2213 (Fig. 1, lane 1) and NZP2037 (Fig. 1, lane 3) contained genes necessary for the nodulation of *Lotus* species, plasmid-cured derivatives of these strains (Fig. 1, lanes 2 and 4) were isolated as described above. Plasmid analysis of *Agrobacterium tumefaciens* C58 (Fig. 1, lane 5) confirmed that the megaplasmid known to be present in this strain was readily detected by the methods used here to analyze the *R. loti* strains.

The symbiotic response of the plasmid-cured derivatives of NZP2037 and NZP2213 on *Lotus* spp. was identical to that of the wild-type strains, with strain PN4010 forming effective nodules on both *L. pedunculatus* and *L. tenuis*, whereas strain PN225 formed effective nodules on *L. tenuis* and ineffective tumor-like structures on *L. pedunculatus*. An analysis of the *Eco*RI chromosomal DNA digest patterns of the plasmid-cured derivatives confirmed that they were derived from the respective wild-type strains (Fig. 2). To confirm that the plasmid DNA sequences had indeed been lost from strains NZP2213 and NZP2037, plasmids pRlo2213 and pPN360 (pRlo2037::pJB3JI) were purified as described above and used as hybridization probes against Southern blots of *Eco*RI digests of total DNA from the wild-type and

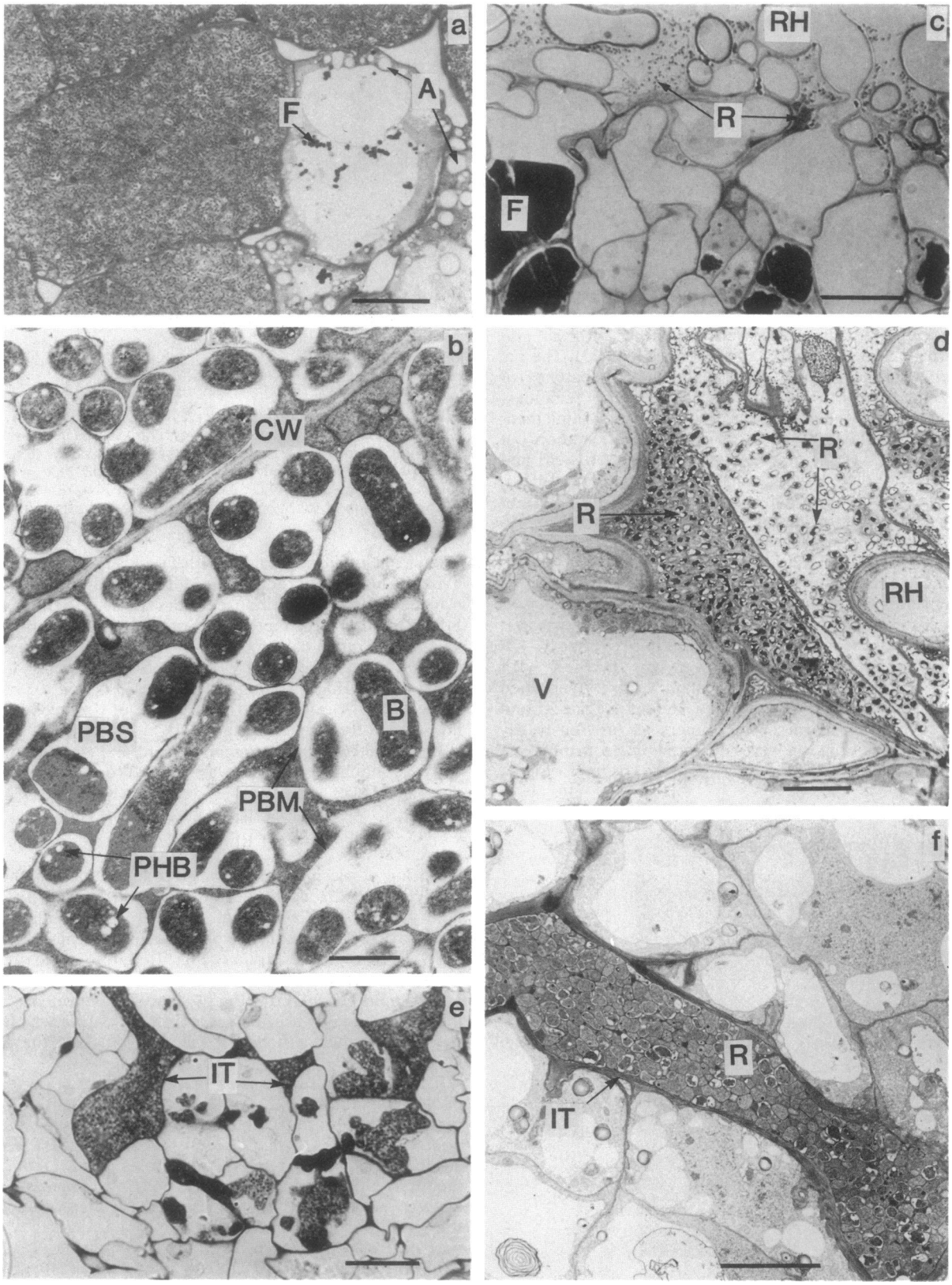

FIG. 3. Micrographs of 30-day-old nodules and tumor-like structures formed on *L. pedunculatus* by *R. loti*. (a) Light micrograph showing infected and uninfected cells in a nodule formed by wild-type strain NZP2037. (b) Electron micrograph of bacteroids in an infected cell of a NZP2037 nodule. (c) Light micrograph showing cortical and root hair cells at the periphery of a tumor-like structure formed by the Noi mutant PN238. (d) Electron micrograph showing a large mass of PN238 rhizobia on the surface and penetrating between epidermal cells of a

TABLE 2. Characterization of *R. loti* symbiotic mutants

Strain	Phenotype	Block in symbiosis[a]	Size of *Eco*RI fragment (kb) containing Tn5[b]	Predicted size of *Eco*RI fragment (kb) in wild-type strain[c]	Presence of pACYC184 sequences[d]
PN233	Nod$^-$	Hac	12.8	7.1	−
PN234	Nod$^+$ Fix$^-$	Noi	13.2	7.5	−
PN235	Nod$^+$ Fix$^-$	Nif/Cof	7.2	1.5	−
PN236	Nod$^+$ Fix$^-$	Noi	17.3	11.6	−
PN237	Nod$^+$ Fix$^-$	Noi	17.3	11.6	−
PN238	Nod$^+$ Fix$^-$	Noi	7.8	2.1	−
PN239	Nod$^+$ Fix$^-$	Bar	13.7	8.0	−
PN240	Nod$^+$ Fix$^-$	ND	Multiple bands		+
PN241	Nod$^+$ Fix$^-$	ND	Multiple bands		+
PN242	Nod$^+$ Fix$^-$	ND	Multiple bands		+
PN244	Nod$^+$ Fix$^-$	Noi	12.1	6.4	−
PN245	Nod$^+$ Fix$^-$	Nif/Cof	13.1	7.4	−
PN246	Nod$^+$ Fix$^-$	Nif/Cof	14.2	8.5	−

[a] Nomenclature based on that of Vincent (55). Hac, Hair curling; Noi, nodule initiation (all such mutants formed ineffective tumor-like structures); Bar, bacterial release; Nif, nitrogen fixation; Cof, complementary functions.
[b] Determined by hybridization of ^{32}P-labeled pKan2 (51) to Southern blots of *Eco*RI digests of total DNA.
[c] Predicted size of *Eco*RI fragments carrying the wild-type gene(s) in *R. loti* NZP2037 as Tn5 is 5.7 kb in size (28).
[d] Determined by hybridization of ^{32}P-labeled pACYC184 (10) to Southern blots of *Eco*RI digests of total DNA.

plasmid-cured strains (Fig. 2). In both cases, the plasmid probe hybridized to wild-type DNA sequences (Fig. 2b and d, lanes 1) but not to the plasmid-cured derivatives (Fig. 2b and d, lanes 2).

Transposon mutagenesis of *R. loti* NZP2037. To isolate symbiotically defective mutants of *R. loti* NZP2037, Tn5 mutagenesis was carried out with the broad host range mobilizable vector pSUP1011 (52). *E. coli* SM10(pSUP1011) was crossed with *R. loti* PN184, and Neo^r Str^r transconjugants were obtained at a frequency of 10^{-4}. Selection was carried out on S20 defined medium containing neomycin and streptomycin to exclude the growth of auxotrophic mutants. A total of 1,060 Neo^r Str^r *R. loti* transconjugants were single colony purified on the same medium and then inoculated onto *L. pedunculatus* seedlings to test their symbiotic phenotype. One isolate, strain PN233, was completely defective in nodulation (Nod$^-$), and 12 others, strains PN234 to PN246, were blocked at various stages of nodule development (Table 2).

Physical analysis of presumptive Tn5-induced mutants. To confirm the presence of Tn5 sequences in the mutants, Southern blots of *Eco*RI digests of total DNA from each of the mutants were probed with ^{32}P-labeled pKan2 DNA (51). For 10 of the 13 mutants, hybridization was found to a single *Eco*RI fragment (Table 2), but for three of the Fix$^-$ mutants several *Eco*RI bands hybridized; these strains all contained pACYC184 vector DNA sequences (Table 2). These strains, as well as strain PN245, which showed a high frequency of reversion, were not studied further.

Light and electron microscopy of Tn5-induced mutants. To define more precisely the stage in nodule development at which the mutants were blocked, root nodules and nodule-like structures formed on *L. pedunculatus* were examined by light and electron microscopy. Because nodule development on *L. pedunculatus* by effective and ineffective *R. loti* strains has been described previously (41), only details pertinent to the description of the mutant phenotypes are presented here.

Nodules formed by wild-type strain NZP2037 appeared 10 to 12 days after inoculation of *L. pedunculatus* seedlings. After 6 weeks, fully developed spherical nodules (diameter, 1.5 to 2.0 mm) were observed and these contained a central zone of enlarged cells filled with bacteria (Fig. 3a), surrounded by a nodule cortex. Within the infected cell, the rhizobia released from infection threads had differentiated into bacteroids and were enclosed within a membrane envelope, the peribacteroid membrane (43) (Fig. 3b). The bacteroids generally lacked inclusions, although small deposits characteristic of β-polyhydroxybutyrate (PHB) were often observed (Fig. 3b).

An examination of the root hairs of *L. pedunculatus* seedlings inoculated with strain PN233 (Table 2) showed that this mutant was unable to induce root-hair curling (Hac) (55). In contrast, strains PN234, PN236, PN237, PN238, and PN244 (Table 2) all induced root hair curling and the formation of nodule-like structures on the roots of *L. pedunculatus*. These structures varied in size and shape from those resembling small nodules (diameter, 0.5 to 1 mm) to large irregular tumor-like structures (diameter, 1 to 2 mm). In transverse section, these structures contained a mass of meristematic cells (initiated from cells of the inner root cortex) surrounded by cortical cells, many of which contained flavolans (condensed tannins; 41) (Fig. 3c). There was some vascular differentiation within these structures, but they contained no *Rhizobium*-infected plant cells. We classified these mutants to be of the Noi (nodule initiation) phenotype (55). Despite the absence of rhizobia within the cells, rhizobia were readily observed on the surface of root hairs and epidermal cells and were often seen to have penetrated a short distance between the epidermal and outer cortical cells (Fig. 3c and d). Rhizobia in outer cortical cells were surrounded by copious quantities of an electron-dense material that was probably polysaccharide.

One mutant, strain PN239 (Table 2), formed spherical nodules of normal external morphology, but when transverse sections of these nodules were examined by light microscopy, the rhizobia were found to be confined to infection threads with no evidence of bacterial release (Bar) into the cytoplasm of the plant cells (Fig. 3e). The infection threads in nodules formed by PN239 were tightly packed with rhizobia (Fig. 3f) and were considerably larger than infection threads formed by the wild-type strain (cf. reference 41, Fig. 1c).

Two mutants, PN235 and PN246 (Table 2), formed nodules which contained bacteroids but were completely blocked in nitrogen fixation (Nif) or some complementary functions (Cof) necessary for nitrogen fixation. Neither of these mu-

tumor-like structure. (e) Light micrograph showing the presence of enlarged infection threads in the infected cells of a nodule formed by the Bar mutant PN239. (f) Electron micrograph of an enlarged infection thread containing many PN239 rhizobia. The bars in (a), (c), and (e) correspond to 10 μm; in (d) and (f) they correspond to 5 μm; and in (b) the bar corresponds to 1 μm. Abbreviations: A, amyloplast; B, bacteroids; CW, plant cell wall; F, flavolan; IT, infection thread; PBM, peribacteroid membrane; PBS, peribacteroid space; R, rhizobia; RH, root hair; V, plant cell vacuole.

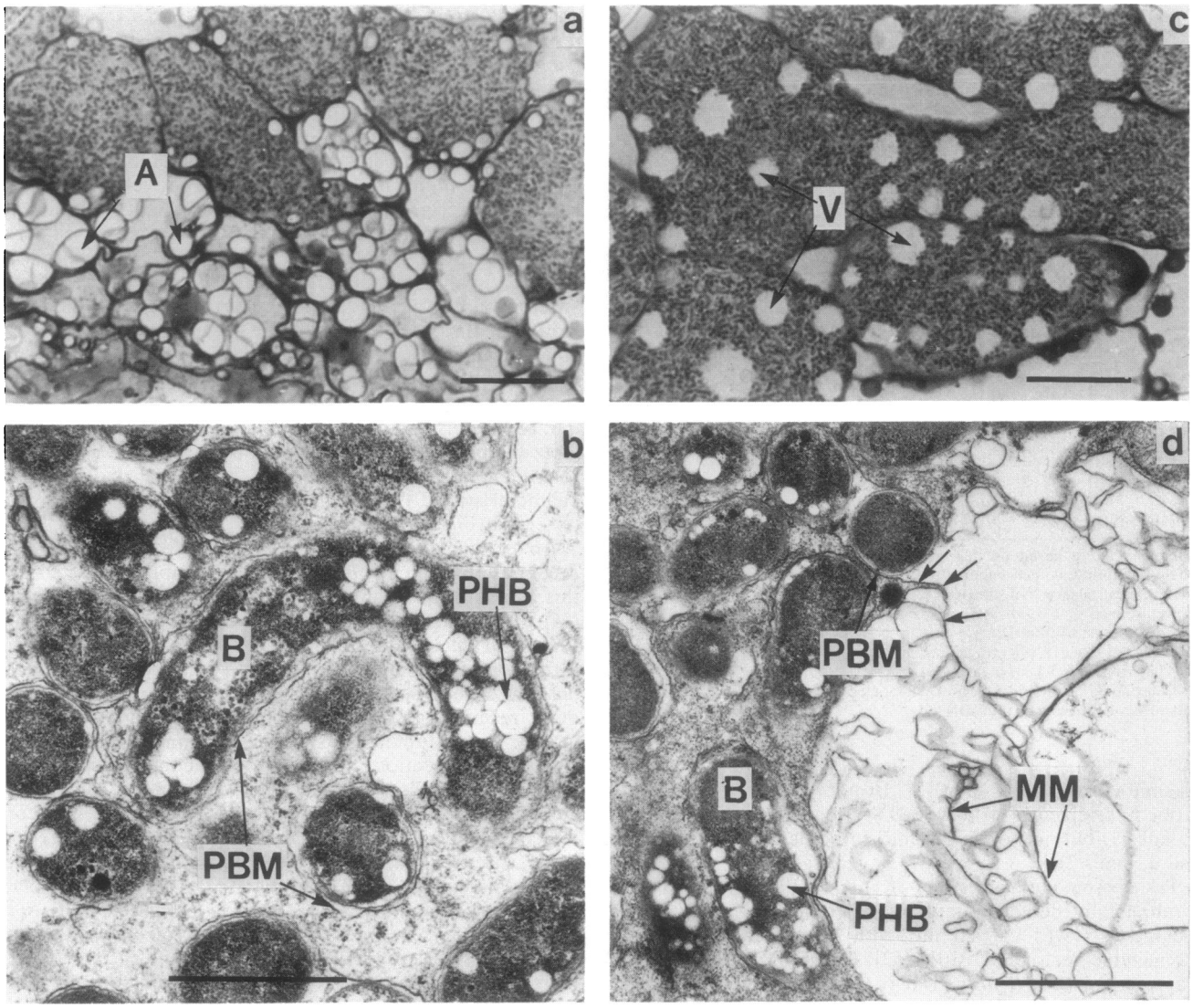

FIG. 4. Micrographs of 30-day-old nodules formed on *L. pendunculatus* by *R. loti* mutants. (a) Light micrograph showing infected plant cells and cortical cells with many amyloplasts in a nodule formed by mutant PN235. (b) Electron micrograph showing PN235 bacteroids. (c) Light micrograph showing infected plant cells in a nodule formed by mutant PN246. (d) Electron micrograph showing PN246 bacteroids bordering a vacuole in an infected plant cell; the arrows indicate the apparent continuity between the peribacteroid membrane and the membrane material filling the vacuole. The bars on (a) and (c) correspond to 10 μm, and on (b) and (d) they correspond to 1 μm. Abbreviations: A, amyloplast; B, bacteroid; MM, membrane material; PBM, peribacteroid membrane; V, vacuole.

tants contained Tn*5* in the *nifHD* structural genes, because ^{32}P-labeled pSA30 hybridized to a single 5-kb *Eco*RI fragment in both mutant (PN235, PN246) and wild-type (NZP2037) strains (data not shown), whereas pKan2 hybridized to *Eco*RI fragments of 7.2 and 14.2 kb in PN235 and PN246, respectively (Table 2).

Infected plant cells in nodules formed by strain PN235 appeared to be normal but contained numerous amyloplasts in the peripheral cytoplasm. Amyloplasts were also present in the uninfected cells of the nodule central zone and in the cells of the nodule cortex (Fig. 4a). The bacteroids within the infected cells contained many PHB granules. The peribacteroid membrane surrounding the bacteroids was often disrupted, and the peribacteroid space was much smaller (Fig. 4b) than in normal NZP2037 nodules (Fig. 3b). The plant cell cytoplasm was also less electron dense than normal, suggesting that it may have begun to senesce.

Examination of transverse sections of nodules formed by strain PN246 suggested a different block to that observed for PN235. In these nodules, the bacteroid-containing cells were very elongated and contained many small vacuoles (Fig. 4c). The vacuoles were usually located toward the periphery of the infected cells (Fig. 4c). A common feature of these vacuoles was the presence of large quantities of membranous material within them (Fig. 4d), and on numerous occasions this membrane material was seen to be part of the peribacteroid membrane (Fig. 4d). As with the PN235 bacteroids, the PN246 bacteroids contained significant quantities of PHB, and the peribacteroid space was much reduced (Fig. 4d).

Complementation of Tn*5*-induced mutants. To isolate the complementary DNA sequences to the seven Hac, Noi, and Bar mutants described above, the NZP2037(pLAFR1) gene library was crossed en masse with each mutant, and the

cross was used to inoculate *L. pedunculatus* seedlings to select by in planta complementation *Rhizobium* derivatives containing wild-type sequences. By this method, all seven mutants were complemented on *L. pedunculatus*. Rhizobia were isolated from these nodules and single colony purified, and the resident pLAFR1 cosmid was then transferred to *E. coli* HB101 in a triparental mating. A further transfer of the pLAFR1 cosmid back to the *R. loti* mutant was carried out, and these transconjugants were tested on *L. pedunculatus* to confirm that bacteria isolated from the nodules were not revertants. When *Eco*RI digests (Fig. 5) of the representative cosmids found to complement the mutants PN233 (lane 1), PN234 (lane 2), PN236 (lane 3), PN239 (lane 4), PN238 (lane 5), and PN244 (lane 6) were analyzed on an agarose gel, each cosmid was found to contain an *Eco*RI fragment of the size predicted in Table 2. Strain PN237 was found to be complemented by an identical cosmid to that shown for strain PN236 in lane 3 (Fig. 5). The six cosmids shown in Fig. 5 were purified as described above, and each was used as a hybridization probe against *Eco*RI digests of the other cosmids. No homology was observed between these cosmids. Furthermore, no in planta cross complementation was observed when each cosmid was individually transferred into the five other *R. loti* mutants.

DISCUSSION

All of the fast-growing strains of *Rhizobium* examined to date (*R. meliloti*, *R. leguminosarum*, *R. phaseoli*, and *R. trifolii*) have been shown to carry genes for nodulation and nitrogen fixation on large indigenous plasmids (reviewed by Denarie et al. [12]). In contrast, the isolation here of plasmid-cured derivatives of *R. loti* NZP2037 and NZP2213 that retained the capacity to form effective nodules on *Lotus* spp., together with the fact that *nif* structural genes are not found on the single large indigenous plasmid found in these strains (40), suggests that in this species the symbiotic genes

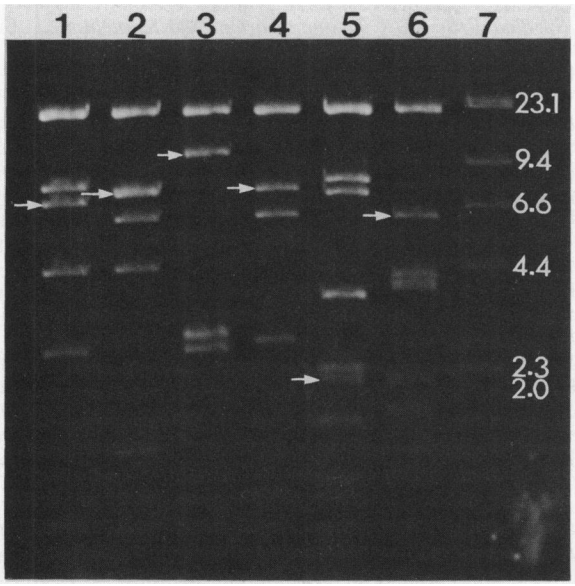

FIG. 5. Agarose gel of *Eco*RI digests of pLAFR1 cosmids found to complement various *R. loti* symbiotic mutants. *Eco*RI digests of cosmids found to complement (lane 1) PN233, (lane 2) PN234, (lane 3) PN236, (lane 4) PN239, (lane 5) PN238, and (lane 6) PN244. The molecular sizes (kb) of λ *Hin*dIII standards are also indicated.

are carried on the chromosome. Although the presence of an additional undetected plasmid in these strains cannot be totally excluded, the technique used here for screening plasmids in *R. loti* was satisfactory for the detection of the 300-megadalton cryptic plasmid in *A. tumefaciens* (Fig. 2) and the >300-megadalton megaplasmid present in *R. meliloti* (50). In addition, Kondorosi et al. (30) have detected the 1,000-megadalton *R. meliloti* megaplasmid (7) after it was transferred to *R. loti* NZP2037, but did not detect any further plasmids in NZP2037.

As a first step toward studying the structural organization of symbiotic genes in *R. loti* NZP2037, generalized transposon mutagenesis was carried out by the vector system developed by Simon et al. (52), and the Tn*5*-induced symbiotic mutants isolated were used to select the wild-type DNA sequences from an NZP2037(pLAFR1) gene library by the technique of in planta complementation. The 13 symbiotic mutants isolated contained Tn*5* sequences, but three of the Fix^- mutants (Table 2) also had vector sequences, suggesting that pSUP1011 (52) had integrated into the genome of *R. loti* in these cases. The mutants carrying only Tn*5* sequences were characterized further by light and electron microscopy, using the nomenclature developed by Vincent (55) to define the phenotype of the block in *L. pedunculatus* nodule development.

An examination of the root hairs of *L. pedunculatus* seedlings inoculated with strain PN233 (Table 2) showed that this mutant was blocked at the hair curling (Hac) stage. Cosmids found to complement *R. loti* PN233 all contained a 7.1-kb *Eco*RI fragment (e.g., Fig. 5, lane 1), and this fragment is identical to the cloned Tn*5*-containing *Eco*RI fragment (Table 2) found in PN233 (Scott et al., manuscript in preparation). Transposon-induced Hac mutants also have been isolated from *R. meliloti* (18, 21, 34), *R. leguminosarum* (5, 32) and *R. trifolii* (14, 44), and it is possible that the *nod* gene region identified here shares some common functions with these previously cloned *nod* regions.

Strains PN234, PN236, PN237, PN238, and PN244 (Table 2) all induced ineffective tumor-like structures on the roots of *L. pedunculatus*. These contained meristematic cells and some vascular tissue, but there was no bacterial invasion into the host cells. Therefore, these five mutants were classified to be of the Noi (nodule initiation) phenotype (55). The structures induced by these mutants resemble the ineffective (Nod^+ Fix^-) structures formed by *R. loti* NZP2213 on *L. pedunculatus* (41), the ineffective nodules formed on *Medicago sativa* by *A. tumefaciens* transconjugants harboring the pSym megaplasmid of *R. meliloti* 2011 (54), and the gall-like structures formed on *M. sativa* by the reactive Nod^- mutants of *R. meliloti* (21). However, in the latter case, intracellular invasion of the epidermal cells was observed (21). All the *R. loti* Noi mutants (Table 2) were complemented in planta, and the cosmids isolated from these derivatives contained *Eco*RI fragments of the sizes (Fig. 5) predicted from the *Eco*RI fragments shown to contain Tn*5* (Table 2). Surprisingly, only the cosmids that complemented PN236 and PN237 were found to share common fragments, and this was confirmed by Southern hybridization with the purified cosmids used as probes. This lack of linkage suggests that at least four different loci in *R. loti* can give rise to a Noi phenotype. Although these mutants were originally selected on a defined medium to exclude symbiotically defective auxotrophic mutants (for a review, see reference 55), mutant PN244 grew poorly on succinate-containing media, suggesting that at least this Noi mutant was blocked in an important metabolic pathway. However,

widely scattered *fix* alleles have also been identified on the chromosome of *R. meliloti* (18) and *R. phaseoli* (37).

The next developmental stage for which a mutant was isolated was bacterial release (Bar) from the infection thread (55). Strain PN239 induced ineffective nodules that contained large numbers of bacteria in the infection threads, but there was no release of these bacteria into the plant cells (Fig. 3e and f). A viomycin-resistant *R. trifolii* mutant and an *R. meliloti* leucine auxotroph have also been described with this phenotype (for a review, see reference 55). Downie et al. (15) have also described *R. leguminosarum nif* mutants that form nodules with a partial block at this developmental stage, but 5% of the plant cells still contained bacteria surrounded by a peribacteroid membrane. By using the in planta complementation technique, cosmids were isolated that complemented the Bar^- mutant strain PN239, and each contained an *Eco*RI fragment of 8.5 kb (e.g., Fig. 5, lane 4) which was identical in size to the fragment into which Tn*5* had inserted (Table 2).

The fact that the seven Hac, Noi, and Bar mutants were complemented by cosmids that contained an *Eco*RI fragment of a size identical to that into which Tn*5* had inserted would suggest that the symbiotic phenotype of these mutants was probably caused by Tn*5* insertion, and not to insertion of an indigenous sequence as was often found in *R. meliloti* (6).

The final two mutants characterized by electron microscopy (PN235 and PN246) formed nodules containing bacteroid-filled plant cells (Fig. 4a and c) but were unable to fix N_2. These mutants are blocked at a late stage of nodule development, possibly in the functioning of nitrogenase (20) or in some important ancillary process. Differences seen in the structure of the infected plant cells (Fig. 4b and d) suggest that the block in PN235 and PN246 is different. In addition, Tn*5* has inserted into an *Eco*RI fragment of different sizes in each mutant (Table 2). Cosmids able to complement these two mutants could not be isolated by in planta complementation, because this technique relies on the complemented mutant cells (wild type) being able to outcompete other cells. For mutants blocked at a late stage in nodule development, this plant selectivity does not occur.

In conclusion, we isolated a range of Tn*5*-induced symbiotic mutants of *R. loti* NZP2037. Tn*5* insertions resulting in blocks in the early stages of nodule development (Hac, Noi, and Bar phenotypes) are not clustered, suggesting that a relatively large number of genes are involved in the development of an N_2-fixing nodule by *R. loti*. The isolation of wild-type sequences that complement these mutants will allow us to define more precisely the number of complementation groups involved and the function of the gene products.

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