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Rhizobium phaseoli Symbiotic Mutants with Transposon Tn*5* Insertions

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Rhizobium phaseoli CFN42 DNA was mutated by random insertion of Tn*5* from suicide plasmid pJB4JI to obtain independently arising strains that were defective in symbiosis with *Phaseolus vulgaris* but grew normally outside the plant. When these mutants were incubated with the plant, one did not initiate visible nodule tissue (Nod$^-$), seven led to slow nodule development (Ndv), and two led to superficially normal early nodule development but lacked symbiotic nitrogenase activity (Sna$^-$). The Nod$^-$ mutant lacked the large transmissible indigenous plasmid pCFN42d that has homology to *Klebsiella pneumoniae* nitrogenase (*nif*) genes. The other mutants had normal plasmid content. In the two Sna$^-$ mutants and one Ndv mutant, Tn*5* had inserted into plasmid pCFN42d outside the region of *nif* homology. The insertions of the other Ndv mutants were apparently in the chromosome. They were not in plasmids detected on agarose gels, and, in contrast to insertions on indigenous plasmids, they were transmitted in crosses to wild-type strain CFN42 at the same frequency as auxotrophic markers and with the same enhancement of transmission by conjugation plasmid R68.45. In these Ndv mutants the Tn*5* insertions were the same as or very closely linked to mutations causing the Ndv phenotype. However, in two mutants with Tn*5* insertions on plasmid pCFN42d, an additional mutation on the same plasmid, rather than Tn*5*, was responsible for the Sna$^-$ or Ndv phenotype. When plasmid pJB4JI was transferred to two other *R. phaseoli* strains, analysis of symbiotic mutants was complicated by Tn*5*-containing deleted forms of pJB4JI that were stably maintained.

Interest in the symbiotic nitrogen fixation which occurs in the root nodules of leguminous plants has initiated genetic studies of the fast-growing nodulating bacteria, including *Rhizobium meliloti* (14, 25), *R. leguminosarum* (4, 24), and *R. trifolii* (26, 33). A relatively neglected fast grower, *R. phaseoli*, also merits attention. As is the case with other fast growers, genetic analysis is possible through transmission of indigenous plasmids (23) and chromosomal recombination mediated by R plasmids (20). On the other hand, several features of its symbiosis with *Phaseolus vulgaris* distinguish it from the genetically better characterized *Rhizobium* species. In development, morphology, and nitrogen assimilation (35), bean nodules are similar to those of soybean and cowpea, other members of the Phaseolae. Many concepts of nodule development and physiology based on studies of mutants of *R. meliloti* (17), *R. leguminosarum* (24), and *R. trifolii* (33) may not apply to the Phaseolae. For biochemical analysis a favorable attribute is the relatively large yield of bean nodule tissue. Beans are agriculturally important, and the demonstrated variability in the germ plasm (15) allows the possible enhancement of nitrogen fixation through breeding (6).

In *R. phaseoli*, as in other fast growers (3, 5, 14, 19, 24, 33), large plasmids determine at least some symbiotic functions (23), including the host range of nodulation (5, 11), and contain DNA homologous to the nitrogenase genes (*nif*) of *Klebsiella pneumoniae* (19, 30, 32). Whereas earlier studies of *R. phaseoli* were concerned exclusively with these plasmids, our approach in the present study was more general. We sought mutations in the entire genome which resulted in altered nodulation or symbiotic nitrogen fixation by insertion of transposon Tn*5* at random (2). Our screening technique was not intentionally biased toward either the indigenous plasmids or the chromosome.

When *Agrobacterium tumefaciens* receives a *nif* plasmid from *Rhizobium* strains, it initiates nodulation, but development is slow, incomplete, and does not result in nitrogen fixation (1, 20). *R. phaseoli* and *R. trifolii* recipients of the *nif* plasmid from *R. leguminosarum* generally nodulate peas in a similarly incomplete fashion (3). Although the latter cases have been attributed to incompatible plasmid functions (3, 5), a largely unexplored possibility is the importance of chromosomal genes. Our initial findings have indicated that symbiosis-specific mutations occur in the chromosome as frequently as in the *nif* plasmid. The chromosomal mutations predominantly affected nodule development in the intermediate stages between the initial induction of nodule growth (17), which is at least partially determined by plasmid genes, and the mature stage of the nodule in which *nif* expression yields nitrogen fixation (17).

MATERIALS AND METHODS

Media and growth of bacteria. *R. phaseoli* was grown at 30°C in rich medium (PY) containing 0.5% peptone of casein (Bioxon), 0.3% yeast extract (Bioxon), and 10 mM $CaCl_2$ or in minimal medium containing (grams per liter): K_2HPO_4, 0.22; $MgSO_4 \cdot 7H_2O$, 0.1; NH_4NO_3, 1.0; $CaCl_2 \cdot 2H_2O$, 0.15; $FeCl_3 \cdot 6H_2O$, 0.037; sucrose, 1.0. *Escherichia coli* and *A. tumefaciens* were grown on LB medium (27) at 37 and 30°C, respectively.

Tn*5* mutagenesis. *R. phaseoli* and *E. coli* 1830 carrying plasmid pJB4JI were grown in rich liquid to stationary phase and late-logarithmic phase, respectively. They were mated on Millipore filters (2) upon PY agar. After 8 h at 30°C, the cells on the filters were suspended in PY medium and plated on PY agar with 60 μg of kanamycin per ml and either 25 μg of rifampin per ml or 200 μg of streptomycin per ml for

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counterselection. Colonies appearing after 3 days at 30°C were purified once on selective media. Those with symbiotic defects were repurified at least once on nonselective medium (PY) and retested extensively.

Assay for nodulation and nitrogen-fixing capability. *P. vulgaris* cv. Negro Jamapa was grown in 24-ml vials with 20 ml of nitrogen-free plant nutrient (36) and 0.6% agar, modified after the method of Wacek and Brill (36). Instead of covering the vials individually with plastic bags, we placed 50 vials inside plastic food storage boxes (20 by 30 by 30 cm) perforated for gas exchange. The surface of the agar was inoculated with *R. phaseoli*, with either 0.5 ml of liquid culture or an agar culture transferred by a cotton swab. A surface-sterilized (36) 2-day-old seedling was planted such that the cotyledon was supported on an aluminum foil cap through which the root was inserted into the agar. After 13 days in a growth chamber, nitrogenase activity was measured by acetylene reduction (36), and nodulation was scored. Longer incubations for protein analysis were performed in growth pouches (28). To follow nodulation further, plants inoculated with liquid culture were grown in 0.6% agar with nitrogen-free nutrient in 250-ml flasks for 21 days.

Plasmid analysis. Bacterial cultures were gently lysed in gel slots before electrophoresis in agarose, as proposed by Eckhardt (12) and modified for *Rhizobium* spp. (21). After partial hydrolysis in 0.25 M HCl (37), DNA was transferred to nitrocellulose (34) and hybridized (9, 30) to purified plasmid DNA labeled with ^{32}P by nick translation (31).

Bacterial crosses. *R. phaseoli* strains were grown in liquid PY medium to late-exponential growth phase (donor strains) or to stationary phase (recipients). They were mixed and incubated on Millipore filters (4) upon PY agar at 30°C for 20 h. The cells were suspended in PY medium, and serial dilutions were plated on selective media.

In situ hybridization. *R. phaseoli* strains were patched onto nitrocellulose above PY agar and grown for 2 to 3 days. The cultures were lysed in place (16). Released DNA was fixed to the nitrocellulose (16) and probed with ^{32}P-labeled DNA.

Protein analysis. Nodules were harvested 3 weeks after inoculation. They were crushed and were fractionated by differential centrifugation into bacterial and soluble plant portions (28) in a buffer containing 10 mM Tris-chloride (pH 7.5), 5 mM $MgCl_2$, and 10 mM β-mercaptoethanol. Protein content was determined by the Bradford procedure (7). Samples were subjected to one-dimensional (22) or two-dimensional (29) polyacrylamide gel electrophoresis (28).

RESULTS

Tn*5* mutagenesis. The suicide Mu plasmid pJB4JI was used to deliver Tn*5* to *R. phaseoli*. A control experiment was performed first with plasmid pPH1JI, an IncP1 plasmid carrying the genetic markers of plasmid pJB4JI except for Tn*5* and phage Mu (2). Both plasmids were transferred from *E. coli* to *R. phaseoli* EP1 and CE3 (Table 1). Gm^r and Sp^r transconjugants arose 10^6-fold more frequently with pPH1JI transfer than with pJB4JI. Apparently, the presence of Mu interfered with plasmid replication or *R. phaseoli* survival, as is the case with other bacterial recipients of pJB4JI (2, 13, 14, 25). Of the Km^r transconjugants obtained with pJB4JI transfer, 99% were Gm^s and Sp^s, and 0.3% of the Gm^sSp^s colonies were auxotrophs, again in agreement with the results of pJB4JI transfer to other *Rhizobium* strains (2, 14, 25).

Five auxotrophs of strain CE3 were analyzed. One required arginine, two required tryptophan, and two required a combination of isoleucine, leucine, and valine. Prototrophic derivatives arose spontaneously at a frequency of 10^{-8} and were Km^s. Thus, precise excision of Tn*5* appeared to occur, as observed in some (2, 14), but not all (25) of the other *Rhizobium* strains examined.

TABLE 1. Strains and plasmids used in this study

Strain/plasmid	Relevant description	Reference[a]
R. phaseoli		
CFN1	Field isolate (Sym^+)[b]	30
EP1	CFN1 *rif-2* (Sym^+)	
EP102, EP103, EP107	Nodulation-defective mutants isolated after transfer of pJB4JI to strain EP1	
CFN42	Field isolate (Sym^+)	30
CE1	CFN42 *rif-1* (Sym^+)	
CE2	CFN42 *rif-1 cam-1* (Sym^+)	
CE3	CFN42 *str-1* (Sym^+)	
CE106, CE107, CE108, CE109, CE110, CE111, CE112, CE114, CE115, CE116, CE117	Mutants isolated after transfer of pJB4JI to strain CE3[c]	
A. tumefaciens GV3105	Ery^r Cml^r lacks Ti plasmid	18
Plasmid		
pJB3	R68.45 Km^s (IncP1 Cma^+ Ap Tc)	8
pJB4JI	IncP1 Gm Cm Sp Mu::Tn*5* (Km)	2
pPH1JI	IncP1 Gm Cm Sp	2
pBR322:Tn*5*	In vivo insertion of Tn*5*	

[a] Where no reference is shown, the strain was isolated in this study.
[b] A Sym^+ strain nodulates and reduces acetylene with wild-type proficiency.
[c] The symbiotic defects of the mutants of CE3 are described in Fig. 1, Table 2, and the text.

Symbiotic mutants. Plasmid pJB4JI was transferred to strain CE3 (CFN42 *str-1*). Prototrophic Km^r transconjugants were screened for nodulating and nitrogen-fixing (acetylene-reducing) capability with a 2-week assay on bean plants. Approximately 1.5% were consistently defective upon extensive retesting. Ten independently derived mutants (Fig. 1; Table 2) were chosen for further study. They all grew normally on rich or minimal medium with either 1.0% sucrose or 1.2% succinate as the sole carbon source.

Inoculation of 2-day-old bean seedlings with strain CE3 led to visible nodules within 7 days; 2 days later nitrogenase activity was detectable and nodules were pink, due to the presence of leghemoglobin (10). Although nodules of strains CE108 and CE112 had no nitrogenase activity (0.3% or less of normal acetylene reduction per nodule weight), during the first 2 weeks after inoculation they were similar in size and appearance to nodules of strain CE3, except for paler red color. However, after 3 weeks they were smaller than normal, and the majority were green. Nodules of strain CE116 formed more slowly. Two weeks after inoculation they were white, small, and without nitrogenase activity, but 1 week later they were pink and had 7% of the normal (CE3) nitrogenase activity (per nodule [fresh weight]). All but one of the other mutants induced nodular structures which

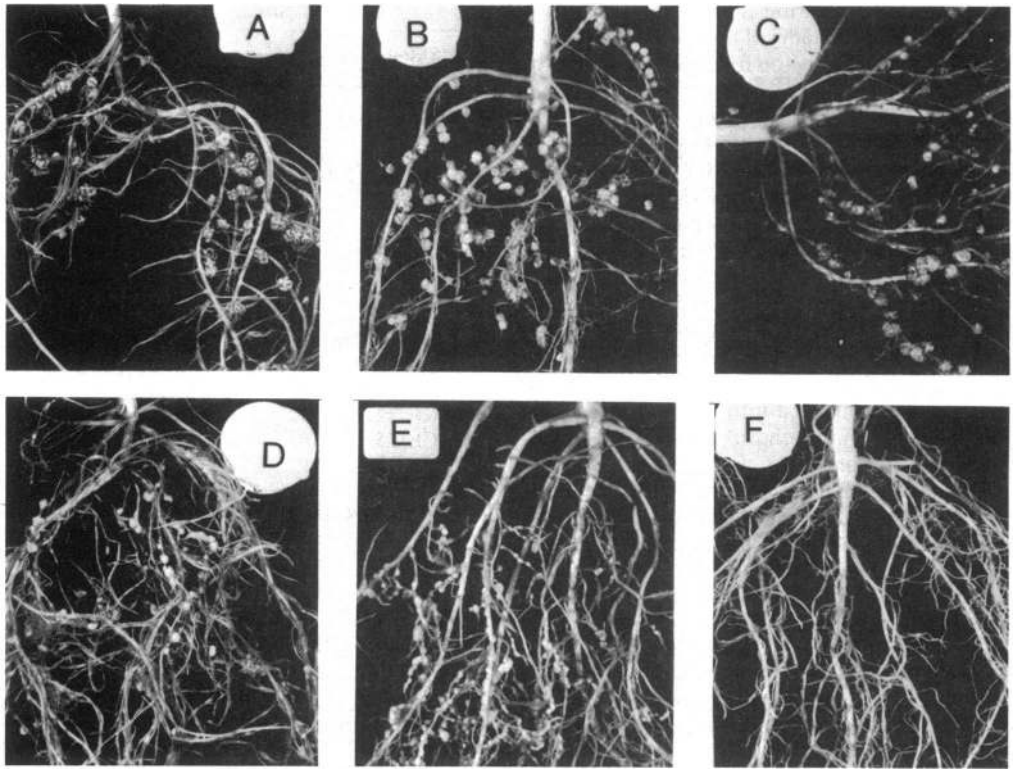

FIG. 1. Nodulation, 21 days after inoculation of bean seedlings, representative of these groups of strains derived from *R. phaseoli* CFN42: (A) CFN42, CE3, or CE117; (B) CE116; (C) CE108 or CE112; (D) CE109 or CE114; (E) CE106, CE107, CE110, or CE115; (F) CE111.

developed even more slowly, at a rate that depended on the mutant (Fig. 1). None of these structures had any nitrogenase activity or red color, even after 3 weeks. The number of these growths was generally about half the number of genuine nodules produced by strain CE3. Even very small nodule-like growths of this sort were never observed with strain CE111 (Nod^-).

Strain CE117 was characterized originally as partially active, although repeated testing did not bear this out. In the end it was saved because its Tn*5* had inserted in an indigenous plasmid.

Protein analysis. Analysis of nodule proteins (28) provided a molecular basis for distinguishing between the three mutants with larger nodules (CE108, CE112, and CE116) and one representative of the class whose nodules developed slowly (CE106). The plant nodule proteins were analyzed by sodium dodecyl sulfate-gel electrophoresis (Fig. 2). Only the nodules of strain CE106 lacked protein, which probably was leghemoglobin (10), based on its low molecular weight and high concentration in normal nodules. The nodules of this mutant exhibited other differences in plant proteins (Fig. 2) and extremely low bacteroid protein content (Table 3).

Bacteroid proteins of strains CE108, CE112, and CE116 were mainly identical to those of parent strain CE3. Differences were confined to a certain region of the two-dimensional gels (Fig. 3). Each mutant showed unique changes, but some pleiotropic changes were common to all three.

Plasmid and restriction enzyme analysis. The Eckhardt procedure (12) was used to analyze the plasmid content of these strains (Fig. 4 and 5). The wild type exhibits five plasmid bands, the lower of which is due to two plasmids, pCFN42a and pCFN42b (J. Leemans, G. Soberon, and L.

TABLE 2. *R. phaseoli* mutants crossed with wild type

Strain[a]	Symbiotic defect[b]	Tn*5*[c]	Transmission of Tn*5*[d]		Co-inheritance of symbiotic defect[e]
			+pJB3	−pJB3	
CE3	None	None			
CE106	Ndv Sna^-	C	8×10^{-7}	1×10^{-7}	100
CE107	Ndv Sna^-	C	NM	NM	100
CE108	Sna^-	p42d	4×10^{-6}	3×10^{-6}	100
CE109	Ndv Sna^-	C	NM	NM	100
CE110	Ndv Sna^-	C	6×10^{-7}	9×10^{-8}	97
CE111	Nod^-	C	5×10^{-7}	2×10^{-7}	14
CE112	Sna^-	p42d	5×10^{-6}	4×10^{-6}	87
CE114	Ndv Sna^-	p42d	4×10^{-6}	4×10^{-6}	75
CE115	Ndv Sna^-	C	NM	NM	100
CE116	Ndv Sna	C	8×10^{-7}	2×10^{-7}	98
CE117	None	p42a	5×10^{-4}	8×10^{-4}	

[a] Strain CE3 is CFN42 *str-1*. All other strains were derived from strain CE3.

[b] See text for further explanation. Nod^-, no nodule-like tissue; Ndv, slow nodule development (Fig. 1); Sna, Sna^-, weak (Sna) or no (Sna^-) symbiotic nitrogenase activity as measured by acetylene reduction, after a 3-week incubation with plant.

[c] Site of Tn*5* insertion according to hybridization of Eckhardt-type gels. C, Chromosome; p42a, plasmid CFN42a; p42d, plasmid CFN42d.

[d] Mutant donors (*str* Km), with (+pJB3) or without (−pJB3) plasmid pJB3JI, were crossed with strain CE2 (CFN42 *rif*). Value given is frequency per donor of Rif^r Km^r Str^s transconjugants. NM, Not measured.

[e] At least 30 transconjugants (Rif^r Km^r Str^s) from each cross were tested with plants by the 2-week agar assay. Value given is percentage that showed the donor mutant phenotype.

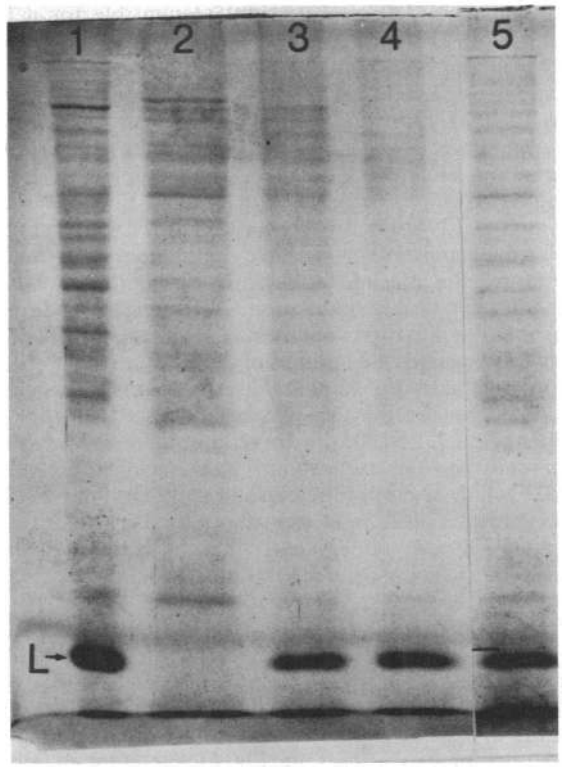

FIG. 2. Nodule plant proteins of beans inoculated with strains CE3 (lane 1), CE106 (lane 2), CE108 (lane 3), CE112 (lane 4), and CE116 (lane 5). The plant fractions soluble at 15,000 × *g* were applied to sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis they were stained with Coomassie blue. Band L is a polypeptide with the molecular weight and relative concentration expected of leghemoglobin (10).

Fernandez, unpublished data). By this nomenclature the middle band is pCFN42d, the plasmid which has regions homologous to the nitrogenase genes of *K. pneumoniae* (30). Strain CE111 (Nod$^-$) was missing this plasmid band (Fig. 4), but the other mutants yielded the normal pattern of five bands (Fig. 4 and 5).

The DNA in the gels was transferred to nitrocellulose and hybridized to ^{32}P-labeled pBR322::Tn*5* (Fig. 4 and 5). In mutants CE108, CE112, and CE114, Tn*5* had inserted into plasmid pCFN42d, whereas in strain CE117 it was in the smallest plasmid, pCFN42a. In the other strains, Tn*5* was absent from plasmid DNA; therefore, it was presumed to have inserted in the chromosome (Fig. 4; Table 2).

*Eco*RI digests of total DNA from strains CE108, CE112, and CE114 yielded a single fragment that hybridized to the Tn*5* probe. This fragment measured 12, 10, and 15 kilobases for strains CE108, CE112, and CE114, respectively. In no case was there a change in the *Eco*RI fragments homologous to *K. pneumoniae* nitrogenase genes (30).

Genetic analysis. Wide-host-range plasmid R68.45 and a

TABLE 3. Fresh weight and protein content of nodules[a]

Inoculant strain	Nodule fresh wt per plant (g)[b]	Protein (mg/g of fresh nodule)	
		Bacteroid[c]	Soluble plant[d]
CE3	0.18	5.3	5.8
CE106	0.05	0.3	1.1
CE108[e]	0.14	5.0	1.8
CE112[e]	0.23	4.1	1.8
CE116	0.15	3.0	2.9

[a] Three weeks after inoculation.
[b] Average of four plants.
[c] After rupture by sonic oscillation.
[d] After dialysis against extraction buffer.
[e] Only red (pink) nodules of CE108 and CE112 were analyzed for protein.

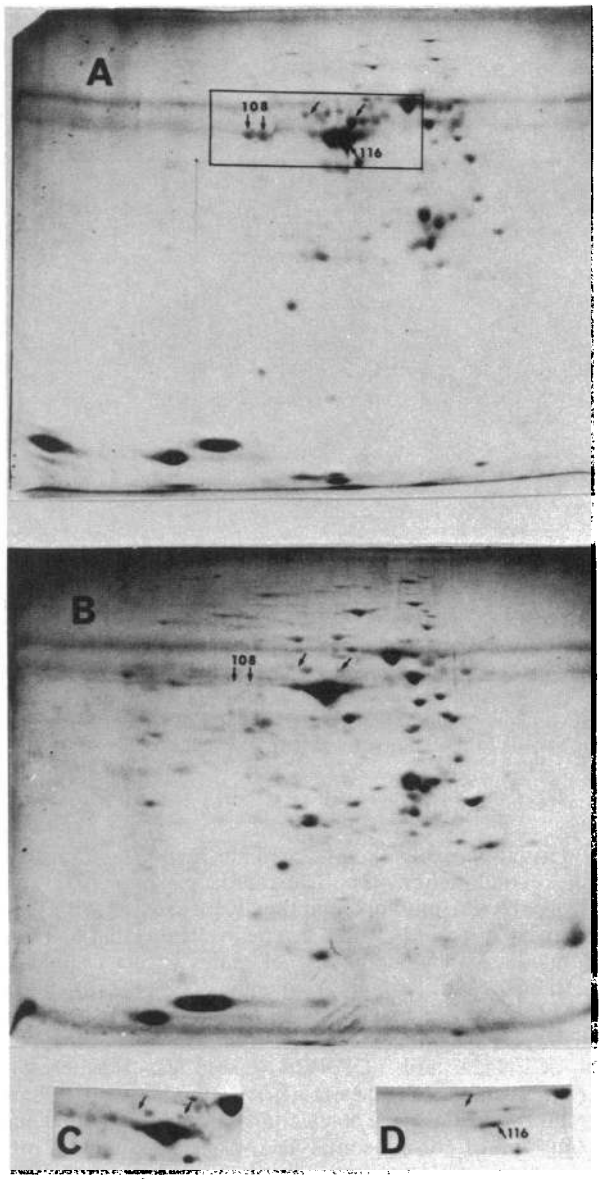

FIG. 3. Differences in the major bacteroid proteins of strains CE3 (A), CE108 (B), CE112 (C), and CE116 (D). After two-dimensional electrophoresis of the nodule bacterial fractions, the gels were stained with Coomassie blue. In panels C and D only the regions of the gels corresponding to the boxed area of panel A are shown. Spots missing or greatly decreased from strain CE108 or CE116 are indicated by arrows. Two arrows without an accompanying number point to proteins missing or decreased in all three mutants. The CE116 sample had one-third the protein concentration of the other samples.

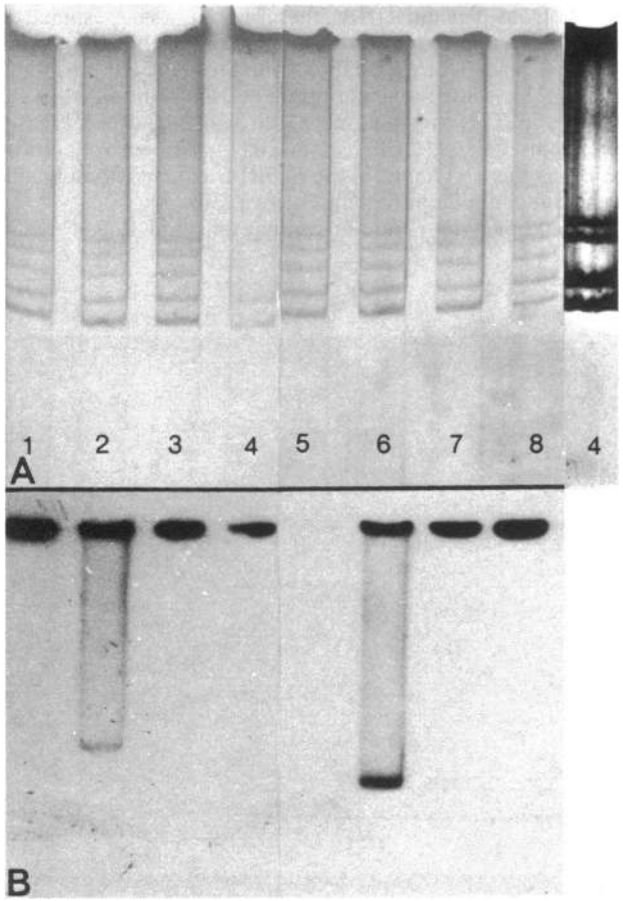

FIG. 4. (A) Plasmid DNA of strains CE115 (lane 1), CE114 (lane 2), CE110 (lane 3), CE111 (lane 4, shown twice), CE3 (lane 5), CE117 (lane 6), CE106 (lane 7), and CE116 (lane 8) separated on agarose gels and stained with ethidium bromide. (B) Localization of Tn*5*. The DNA of the gels in (A) was transferred to nitrocellulose and hybridized to plasmid pBR322::Tn*5* labeled with ^{32}P. The order of the strains in the autoradiograms is the same as in (A).

Km^s derivative, pJB3 (8, 14), mediate *R. phaseoli* chromosomal recombination (3). Therefore, plasmid pJB3 was transferred to the mutants, and they were crossed with strain CE2 (CFN42 *rif cam*) (Table 2). The insertions that had been designated as chromosomal by gel analysis were transferred and established at the same frequency as CFN42 *trp* and *cys* genes (A. Sanchez and D. Noel, unpublished data). This frequency was significantly lower than transmission of plasmids pCFN42a and pCFN42d (Table 2). Furthermore, whereas plasmid pJB3 did not increase the native transfer of indigenous plasmids pCFN42a and pCFN42d, it did enhance transmission of chromosomal Tn*5* insertions (and auxotrophic markers), although even chromosomal insertions were transmitted without it (Table 2).

The Tn*5* insertion of strain CE106, CE107, CE108, CE109, CE110, CE115, or CE116 was the same as or very closely linked to the mutation responsible for its Ndv or Sna$^-$ phenotype (Table 2). In strains CE112 and CE114, the corresponding mutation and the Tn*5* insertion were separable, but the co-inheritance frequency (Table 2) suggested that both were on plasmid pCFN42d.

Strain CE111 had undergone at least two genetic changes, a chromosomal Tn*5* insertion not responsible for it being Nod$^-$ (Table 2) and loss of plasmid pCFN42d. This plasmid and nodulating ability are both missing from another strain, CFN2001, reported previously (R. Palacios, C. Quinto, H. de la Vega, M. Flores, L. Fernandez, M. Hernandez, and G. Soberon, Proc. First Int. Symp. Mol. Gen. Bacteria-Plant Interaction, Bielefeld, Germany, 1982). Being Km^s and Rif^r, strain CFN2001 was crossed with strains CE108 and CE112, in which pCFN42d had been marked by Tn*5* insertion. Rif^r Km^r transconjugants were able to induce large nodules. Therefore, the plasmid apparently has at least one gene necessary for visible nodulation. As expected from indications that the mutations of strains CE108 and CE112 also lie along this plasmid, the nodules of the transconjugants lacked nitrogenase activity. Another discovery in the experiment was that transfer of the plasmid occurred at the same frequency to CFN2001 as to strains already carrying it. Apparently, no self-exclusionary mechanisms are conferred by pCFN42d, or part of the plasmid still exists in CFN2001.

Plasmid pCFN42d was transmissible also to *A. tumefaciens* GV3105. *A. tumefaciens* transconjugants which had received Tn*5* from strain CE108 or CE112 also carried the plasmid (Fig. 5).

Problems with suicide plasmid pJB4JI. Plasmid pJB4JI was conjugated into two other *R. phaseoli* strains, EP1 and CE1. Approximately 2% of the resulting Km^r colonies were defective in symbiosis. Examination of the plasmids of three such mutants of EP1 revealed that an extra small plasmid with pJB4JI homology was present (Fig. 6), even though only one mutant had inherited the drug-resistance markers (Cm, Gm, Sp) of pJB4JI. The plasmids with pJB4JI homology varied in size and were not transmissible to other *R. phaseoli* strains. Only one Tn*5* copy was present in *Eco*RI digests of total

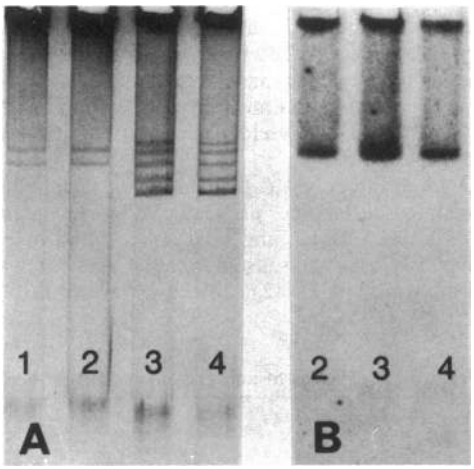

FIG. 5. Plasmid pCFN42d transfer to *A. tumefaciens* GV3105. *R. phaseoli* CE108 (*str* Km) and CE112 (*str* Km) were crossed with *A. tumefaciens* GV3105 (Ery^r Cml^r). (A) Agarose gel separation and ethidium bromide staining of the plasmids of a transconjugant (Ery^r Cml^r Km^r) from each cross (lanes 1 and 2) alongside the plasmids of CE108 and CE112 (lanes 3 and 4). The upper plasmid of the transconjugants is the cryptic pATC58 of strain GV3105 (18). (B) Localization of Tn*5* in one of the transconjugants and the two donor strains by autoradiography of nitrocellulose replicas of lanes 2, 3, and 4 of (A) which had been hybridized to pBR322::Tn*5* ^{32}P-labeled DNA.

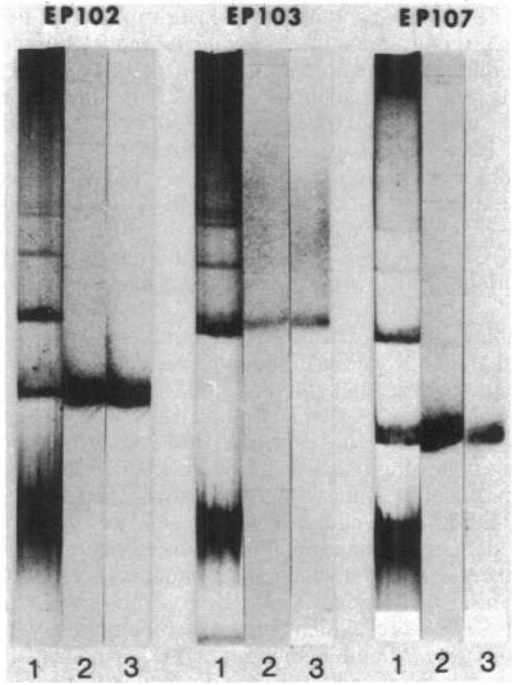

FIG. 6. Plasmid content of nodulation-defective mutants that arose after suicide plasmid pJB4JI was transferred to *R. phaseoli* CFN1. Plasmids of strains EP102, EP103, and EP107 were separated on agarose gels and analyzed in three ways (lanes 1 to 3). After being stained with ethidium bromide (lanes 1), parallel lanes were hybridized to ^{32}P-labeled pBR322::Tn*5* (lanes 2) or plasmid pPH1JI (lanes 3), a probe for pJB4JI sequences. The analysis shows that the lowest plasmid band (not present in wild-type CFN1) in each case has a different size and has sequences homologous to both Tn*5* and other regions of pJB4JI.

DNA. It resided in each case in the new plasmid (Fig. 6). These plasmids have not been examined for possible content of *Rhizobium* DNA, nor for Mu content.

The other symbiotic mutants were screened for pJB4JI sequences by in situ hybridization (16), using plasmid pPH1JI as the probe. Although fewer than 3% of Km^r colonies chosen at random after mutagenesis of strains EP1 (CFN1) or CE3 (CFN42) contained such sequences, the incidence of pJB4JI fragments in symbiotically defective clones of EP1 (CFN1) and CE1 (CFN42) was 70 and 84%, respectively. Of the strains with pJB4JI sequences, 39% had none of the drug resistance markers (Cm, Gm, Sp) of the plasmid aside from Tn*5*. Thus, absence of these markers is not necessarily reliable evidence of pJB4JI absence.

Three of the EP1-derived mutants having deleted forms of pJB4JI were characterized further. Strain EP107 was phenotypically similar to strain CE116 (Ndv Sna), whereas strains EP102 and EP103 were Nod$^-$. In these Nod$^-$ strains, a deletion had eliminated *nif* homology as well as nodulating ability, although migration of the 400-kilobase *nif* plasmid on agarose gels was not detectably altered (Fig. 7). The region of *nif* homology was still present in strain EP107.

None of the mutants derived from strain CE3 described in the previous sections exhibited deleted forms of pJB4JI when probed in situ with pPH1JI or when plasmid content was analyzed. Nor was pJB4JI DNA found in any of the Tn*5*-containing auxotrophs derived from strain CE1 or EP1.

DISCUSSION

Previous analysis of *R. phaseoli* has emphasized the role of indigenous plasmids in the symbiosis with *P. vulgaris* (5, 11, 19, 23, 30). Plasmid pCFN42d, for instance, carries homology to nitrogenase genes of *K. pneumoniae* (30). In this study, nodulating ability was restored to previously isolated Nod$^-$ strain CFN2001 by restoring this plasmid, and three mutants with mutations in the plasmid were isolated. One mutation led to abnormal nodule development, and two mutations outside the immediate region of *nif* homology eliminated nitrogenase activity. Moreover, the only mutants completely unable to induce nodule tissue, whether from strain CFN42 or CFN1, had suffered a loss or a deletion in the *nif* plasmid. Therefore, these plasmids carry genes necessary for nodule development and nitrogen fixation, as do certain plasmids of other *Rhizobium* species (3, 5, 11, 14, 19, 24, 33).

Nevertheless, more than half of the symbiosis-specific mutations of strain CFN42 appeared to be chromosomal. All affected nodule development to some degree, rather than nodule initiation or bacteroid nitrogen fixation, per se. Potentially six genes were mutated, if a different one was hit in each case, resulting in at least two discernible phenotypic classes. Although auxotrophic mutations that affect nodulation or nitrogen fixation are well documented (3), symbiosis-specific chromosomal mutations in *Rhizobium* prototrophs seldom have been reported. Recently, however, five were mapped to widely separated sites of the chromosome of *R. meliloti* 41 (14). The resulting phenotypes were not described explicitly, but apparently at least some affected nodule development (14). The existence of chromosomal genes crucial to post-initiation development of the nodule would be a reason why *A. tumefaciens* carrying *Rhizobium* plasmids

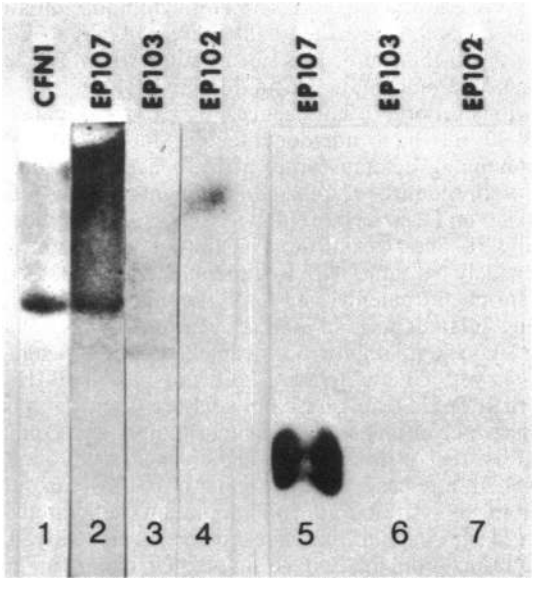

FIG. 7. Nodulation-defective mutants of *R. phaseoli* CFN1 probed for *nif* DNA. Plasmid DNA (lanes 1 to 4) and total DNA digested with *Eco*RI (lanes 5 to 7) were separated on agarose gels and hybridized with ^{32}P-labeled cloned DNA of strain CFN42 homologous to the nitrogenase genes of *K. pneumoniae* (30). The intensely reacting plasmid band of strains CFN1 and EP107 (lanes 1 and 2) corresponds to one of the triplet plasmids of highest molecular weight (lanes 1 of Fig. 6).

leads to incompletely developed nodules lacking nitrogen-fixing activity (1, 20).

In many cases after random Tn*5* mutagenesis of *R. meliloti* strains, the Tn*5* insertion of a symbiotic mutant has not been responsible for the symbiotic defect (14, 25). Such was the case with 3 of the 10 mutants of *R. phaseoli* CFN42 described here. In the other seven, Tn*5* insertion seemed to be responsible for the Ndv or Sna^- phenotype, but caution is warranted since loci separated by few genes are poorly resolved by R68.45 conjugation (3).

Theoretically, plasmid genes are more poorly resolved by conjugation than chromosomal genes since a plasmid is more likely than a chromosome to be transferred and established intact, thereby obviating recombination. However, Tn*5* insertions seemingly were resolved from linked mutations on plasmid pCFN42d in crosses with strains CE112 and CE114. Apparently, the incidence of recombination was nearly equal to the chance of the incoming plasmid replacing the original plasmid.

Strain CE111 is most probably Nod^- because it lacks plasmid pCFN42d. Thus, the observed 14% "co-inheritance" of the chromosomal Tn*5* and inability to nodulate deserves comment. This number may reflect the presence of another mutation in the chromosome, but two circumstances argue that it is not co-inheritance (i.e., cotransmission) at all. First, at low frequency (about 4% or less), plants at random did not nodulate even with wild-type inoculation. Second, selection in the cross was for Rif^r Str^s Km^r progeny from Str^r Km^r (CE111)/pJB3 and Rif^r (CE2) parents. Independently, it was noted that transmission of chromosomal markers occurred without pJB3 at 10 to 20% of the level of pJB3-mediated transmission. If *rif* genes from CE2 were transmitted by this mechanism to CE111, the highly-linked *str* gene (3) would become str^+ and the resulting strain would be Rif^r Str^s Km^r/pJB3, the same as CE2 having received Tn*5* by pJB3-mediated transmission. This type of artifact is magnified by very low linkage of Tn*5* and symbiotic mutation; a true linkage of 95% is artifactually increased only to 95.6% in this way. Furthermore, it is not a factor when Tn*5* is on a transmissible indigenous plasmid (e.g., CE114).

Previous reports of mutagenesis by pJB4JI suicide (2, 13, 14, 23–25, 33) have not documented stable Gm^s Sp^s Cm^s Tn*5*-containing deleted forms of the plasmid, as observed with two of the three *R. phaseoli* recipients (strains CE1 and EP1). Several aspects of this *R. phaseoli* behavior were puzzling. (i) The sizes of the transmitted portions of pJB4JI were widely variable. (ii) Recipients with pJB4JI content were enriched tremendously for symbiotic defects, whereas carrying related plasmid R68.45 was not deleterious. (iii) Strain CE3 should differ from strain CE1 in only two loci (*str* and *rif*); yet, CE3 derivatives did not have pJB4JI DNA other than Tn*5*. Strains EP1 and CE1 are both Rif^r; perhaps that character or an aspect of using rifampin as a counterselection is responsible for a higher incidence of pJB4JI survival. In any case, since variants of pJB4JI can survive, at least in some strains, *Rhizobium* mutants generated by use of this plasmid should be checked for its presence. The in situ hybridization method is simple; checking for genetic markers (Gm, Sp, Cm, Tra) also is simple, but is not sufficient. Mainly because of other anomalies (14, 25) with pJB4JI mutagenesis, current mutagenesis is being performed with alternative transposon vectors, such as ones based on ColE1 plasmid derivatives (33a). Testing for the presence of vector DNA is warranted before extensive analysis of a mutant, particularly the first ones derived from a given strain.

Nodule protein content is a measure of nodule development. Certain mutants of *R. japonicum* induce nodules that are devoid of leghemoglobin, other nodule-specific plant proteins, and most bacteroid proteins (28). Electron microscopic examination of these nodules (28) indicates a problem during release of bacteria from infection threads or in bacteroid survival. *R. phaseoli* CE106 is similar, at least with respect to greatly decreased nodule-specific plant protein synthesis and bacteroid content. On the other hand, strains CE108, CE112, and CE116 allowed normal nodule protein production, with few exceptions among the abundant proteins.

Symbiotically defective *Rhizobium* mutants are often classified as either Nod^-, if no nodules are formed, or Fix^-, if nodule tissue forms but cannot reduce acetylene (3). A fairly clear consensus has emerged on this basis for the phenotype Nod^-; therefore, we have used it. However, we regard Fix^- as being confusing because the word implies that bacterial nitrogen-fixing machinery has been altered, when that may or may not be true, and also because scientists vary widely in the manner of experimentally characterizing *Rhizobium* mutants. We wish to distinguish mutants such as CE106 (Ndv Sna^-) from mutants like CE108 (Sna^-). Nodules of mutant CE108 are similar to those of mutants with Tn*5* insertions in *nif*-homologous genes (17, 24). The nodules seemingly develop well at first and produce leghemoglobin, but show degeneration sooner than normal (17, 24), perhaps only because the abundant bacteroids cannot fix nitrogen. On the other hand, nodules of strain CE106 delay in appearing and subsequently grow much slower than normal; bacteroid content is very low and leghemoglobin is absent. Since we view the defects of these mutants as being fundamentally different, we have assigned separate composite traits, Ndv (nodule development) and Sna (symbiotic nitrogenase activity), to describe them.

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LITERATURE CITED

1. **Banfalvi, Z., G. S. Randhawa, E. Kondorosi, A. Kiss, and A. Kondorosi.** 1983. Construction and characterization of R-prime plasmid carrying symbiotic genes of *Rhizobium meliloti*. Mol. Gen. Genet. **189**:129–135.
2. **Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of drug-resistance transposon Tn5 to *Rhizobium*. Nature (London) **276**:633–635.
3. **Beringer, J. E., N. J. Brewin, and A. W. B. Johnston.** 1980. The genetic analysis of *Rhizobium* in relation to symbiotic nitrogen fixation. Heredity **45**:161–186.
4. **Beringer, J. E., S. A. Hoggan, and A. W. B. Johnston.** 1978. Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J. Gen. Microbiol. **104**:201–207.
5. **Beynon, J. L., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J. Gen. Microbiol. **120**:421–429.
6. **Bliss, F. A.** 1982. The inbred backcross line method for improving quantitative traits of self-pollinated crops. Hortic. Sci. **17**:503–507.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the

quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
8. **Brewin, N. J., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmid-mediated transfer of host-specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120:413–420.
9. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23:641–646.
10. **Dilworth, M. J., and C. A. Appleby.** 1979. Leghemoglobin and *Rhizobium* hemoproteins, p. 691–764. *In* R. W. F. Hardy, F. Bottomley, and R. C. Burns (ed.), A treatise on dinitrogen fixation, sections I–II. John Wiley & Sons, Inc., New York.
11. **Downie, J. A., G. Hombrecher, Q.-S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston.** 1983. Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. Mol. Gen. Genet. 190:359–365.
12. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584–588.
13. **Ely, B., and R. H. Croft.** 1982. Transposon mutagenesis in *Caulobacter crescentus*. J. Bacteriol. 149:620–625.
14. **Forrai, T., E. Vincze, Z. Bánfalvi, G. B. Kiss, G. S. Randhawa, and A. Kondorosi.** 1983. Localization of symbiotic mutations in *Rhizobium meliloti*. J. Bacteriol. 153:635–643.
15. **Graham, P. H.** 1981. Some problems of nodulation and symbiotic nitrogen fixation in *Phaseolus vulgaris* L.: a review. Field Crops. Res. 4:93–112.
16. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. 72:3961–3965.
17. **Hirsch, A. M., M. Bang, and F. M. Ausubel.** 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*::Tn*5* mutants of *Rhizobium meliloti*. J. Bacteriol. 155:367–380.
18. **Holsters, M., B. Silva, F. Van Vliet, C. Genetello, M. DeBlock, P. Ohaese, A. Depicker, D. Inze, G. Engler, R. Villaroel, M. Van Montagu, and J. Schell.** 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTi C_{58}. Plasmid 3:212–230.
19. **Hombrecher, G., N. J. Brewin, and A. W. B. Johnston.** 1981. Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. Mol. Gen. Genet. 182:133–136.
20. **Hooykaas, P. J. J., A. A. N. van Brussel, H. den Dulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort.** 1981. Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature (London) 291:351–353.
21. **Kondorosi, A., Z. Banfalvi, V. Sakanyan, C. Koncz, I. Dusha, and A. Kiss.** 1981. Location of nodulation of nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*, p. 407. *In* A. H. Gibson and W. E. Newton (ed.), Nitrogen fixation in perspective. Australian Academy of Science, Elsevier, Amsterdam.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
23. **Lamb, J. W., G. Hombrecher, and A. W. B. Johnston.** 1982. Plasmid-determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli*. Mol. Gen. Genet. 186:449–452.
24. **Ma, Q.-S., A. W. B. Johnston, G. Hombrecher, and J. A. Downie.** 1982. Molecular genetics of mutants of *Rhizobium leguminosarum* which fail to fix nitrogen. Mol. Gen. Genet. 187:166–171.
25. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. 149:114–122.
26. **Megias, M., M. A. Caviedes, A. J. Palomares, and J. Perez-Silva.** 1982. Use of plasmid R68.45 for constructing a circular linkage map of the *Rhizobium trifolii* chromosome. J. Bacteriol. 149:59–64.
27. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Noel, K. D., G. Stacey, S. R. Tandon, L. E. Silver, and W. J. Brill.** 1982. *Rhizobium japonicum* mutants defective in symbiotic nitrogen fixation. J. Bacteriol. 152:485–494.
29. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007–4021.
30. **Quinto, C., H. de la Vega, M. Flores, L. Fernandez, T. Ballado, G. Soberon, and R. Palacios.** 1982. Nitrogen fixation genes are reiterated in *Rhizobium phaseoli*. Nature (London) 299:724–726.
31. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.
32. **Ruvkun, G. B., and F. M. Ausbel.** 1980. Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. U.S.A. 77:191–195.
33. **Scott, K. F., J. E. Hughes, P. M. Gresshoff, J. E. Beringer, B. G. Rolfe, and J. Shine.** 1982. Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. J. Mol. Appl. Genet. 1:315–326.
33a. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technol. 1:784–791.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
35. **Sprent, J. I.** 1980. Root nodule anatomy, type of export product and evolutionary origin in some Leguminosae. Plant Cell Environ. 3:35–43.
36. **Wacek, T., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen fixing ability in soybean. Crop Sci. 16:519–522.
37. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. 76:3683–3687.