Nodulation Mutants of *Rhizobium fredii* USDA257

D. S. Heron, T. Érsek, H. B. Krishnan, and S. G. Pueppke

Department of Plant Pathology, University of Missouri, Columbia, 65211 U.S.A.

Rhizobium fredii strain USDA257 nodulates cowpea, siratro, primitive soybean genotypes, and several other legume species. It does not nodulate the improved soybean cultivar McCall. By using Tn*5* mutagenesis, we have isolated two classes of symbiotically altered mutants of strain USDA257. Three mutants of one class failed to nodulate all tested legumes. Five mutants of the second class retained the nodulating ability of the parent strain, but acquired the capacity to nodulate McCall soybean. Each mutant contains a single copy of Tn*5*, but marker exchange experiments confirmed that the transposon is responsible for the altered phenotype of only two. In one, Tn*5* is in the Sym plasmid. Nodules produced by this mutant on McCall soybean are morphologically normal and reduce acetylene. In the other, the transposon is in the chromosome. On McCall soybean, this mutant produces a small number of abnormal nodules that fail to reduce acetylene.

Knowledge of the nodulation of soybean by nitrogen-fixing bacterial symbionts has been advanced significantly by the recent discovery of *Rhizobium fredii* (Keyser *et al.* 1982; Scholla and Elkan 1984). The ability of this bacterium to nodulate and fix nitrogen in association with soybean is strain-specific and highly variable. Eleven strains were originally described, but only USDA191 is generally Nod⁺Fix⁺ with both primitive and improved cultivars (Yelton *et al.* 1983; Hattori and Johnson 1984; DuTeau *et al.* 1986; Israel *et al.* 1986). The remaining strains exhibit varying degrees of incompatibility with improved cultivars, failing to nodulate under some circumstances and forming Fix nodules under others (Keyser *et al.* 1982; Jansen vanRensburg *et al.* 1983; Stowers and Eaglesham 1984; Heron and Pueppke 1984, 1987). *R. fredii* also nodulates other legumes, including wild soybean (*Glycine soja*), siratro (*Macroptilium atropurpureum*), phasey bean (*Macroptilium lathyroides*), mung bean (*Vigna radiata*), pigeon pea (*Cajanus cajan*), cowpea (*Vigna unguiculata*), bean (*Phaseolus vulgaris*), and *Sesbania cannabina* (Keyser *et al.* 1982; Scholla and Elkan 1984; Stowers and Eaglesham 1984; Sadowsky *et al.* 1988).

This paper describes initial stages of a genetic analysis of *R. fredii* USDA257, a strain that contains a single plasmid of about 300 Md (Heron and Pueppke 1984) that is also a Sym plasmid (Appelbaum *et al.* 1985b, 1988). In previous studies, we found that USDA257 nodulates cowpea and siratro, but not the improved soybean cultivars McCall and Viçoja (Heron and Pueppke 1984). There are two possible explanations for such nodulation specificity. In addition to genes generally required for nodulation, USDA257 may either lack a special gene or genes required to nodulate McCall and Vicoja soybean, or it may contain one or more genes that specifically function to block nodulation of these cultivars. Appelbaum *et al.* (1985a, 1985c) found that transfer of the Sym plasmid of *R. fredii* USDA191 to USDA257 expanded the host range to include soybean cultivars nodulated by the former. Although this provides evidence that USDA257 may lack genes required for nodulation of improved cultivars, the data are complicated by incompatibility between the Sym plasmids, which resulted in large deletions and the formation of cointegrates. We sought to identify negatively and positively acting nodulation genes of USDA257 by transposon mutagenesis and screening for mutants that had lost the ability to nodulate cowpea or had acquired the ability to nodulate McCall soybeans. Both classes of mutants were obtained, and we have begun to characterize them.

MATERIALS AND METHODS

Organisms. Wild-type *R. fredii* strains USDA191 and USDA257 (hereafter referred to as 191 and 257) were from H. H. Keyser (U. S. Department of Agriculture, Beltsville, MD). Strains were maintained as stock cultures in glycerol at −70° C. Bacteria were routinely cultured in YEM broth (Vincent 1970) at 30° C and 125 rpm.

Seeds of soybean (*Glycine max* (L.) Merr.) cultivars were from the following sources: Evans, Hodgson 78, and McCall from D. Whited (North Dakota State University, Fargo); TGM119 from A. Eaglesham (Boyce Thompson Institute, Cornell University, Ithaca, NY); Harosoy from J. Paxton (University of Illinois, Urbana); Viçoja from K. Hinson (USDA-ARS, University of Florida, Gainesville); Peking, Williams 82, and PI89772 from V. Luedders and V. Dropkin (University of Missouri, Columbia). Siratro (*M. atropurpureum* (DC) Urb.) seeds were from R. L. Weaver (Texas A & M University, College Station); cowpea (*V. unguiculata* (L.) Walp. 'Pink Eye Purple Hull') seeds were purchased from Hastings Seed Co. (Atlanta, GA). Seeds of white clover (*Trifolium repens* L. 'Ladino'), alfalfa (*Medicago sativa* L. 'Saranac'), and pea (*Pisum sativum* L. 'Laxton's Progress no. 9') were obtained from D. Hubbell (University of Florida, Gainesville), D. Barnes (University of

Minnesota, St. Paul), and Royal Seed Co. (Kansas City, MO), respectively.

Plasmids used in this study are listed in Table 1. *Escherichia coli* strains were stored in 7.5% glycerol at −70° C and cultured at 37° C in Luria-Bertani broth (Maniatis *et al.* 1982). Antibiotics were supplied at the following concentrations: ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), gentamicin (50 μg/ml), kanamycin (100 μg/ml), spectinomycin (25 μg/ml), tetracycline (10 μg/ml), and trimethoprim (50 μg/ml).

Tn5-mutagenesis and screening. The suicide vector pSUP1011 was used to introduce Tn5 into strain 257 (Hom *et al.* 1984). Filters containing the mating mixtures were incubated at 30° C for 3 days, at which time bacteria were scraped from three well-separated areas of each filter with a sterile inoculation loop. Each mass of bacteria was suspended in 2 ml of phosphate-buffered saline (PBS; Pueppke 1983) containing 0.01% Tween 40, and 100-μl aliquots were plated on YEM containing kanamycin and trimethoprim.

Nod$^-$ mutants were sought by screening individual mutant colonies on cowpea seedlings. Twenty-four Km^R colonies from each mass of bacteria (a total of 72 colonies from each filter) were transferred to a fresh plate of antibiotic containing YEM and allowed to grow. Cowpea seedlings were germinated as described previously (Pueppke 1983). A seedling was grasped aseptically with forceps and the root touched to a colony. The seedling then was planted in a 70-ml urine-collection bottle (Fisher Scientific Co.) that had been filled with vermiculite, saturated with Jensen's N-free solution (Vincent 1970), and autoclaved. Water loss was minimized by affixing a plastic Whirl-Pak bag (Fisher Scientific Co.) to the mouth of each bottle. Each bag was slit to permit exchange of gases, and the bottles were incubated under fluorescent lights (400 μEinsteins/m^2/sec; 12-hr photoperiod) for 14–16 days. The plants then were harvested and scored for nodulation.

Nod$^+$ mutants were screened on McCall soybean, which is not nodulated by parental strain 257 (Heron and Pueppke 1984). The strategy was based on the en masse screening approach of Long *et al.* (1982), in which bacteria are rescued via nodulation from a mixed inoculum of Nod$^+$ and Nod$^-$ bacteria. In preliminary experiments with mixed inocula (Nod$^+$ and Nod$^-$ strains of *R. fredii*), the Nod$^+$ strain was able to nodulate McCall soybean when outnumbered by up to 10,000:1 by Nod$^-$ bacteria in the inoculum. Therefore, in screening for Nod$^+$ mutants of 257, we pooled 500–1,000 Km^R colonies from a single filter mating and adjusted the bacterial concentration to about 10^9 cells per milliliter of

PBS. McCall seedlings were germinated aseptically as described above and transferred to autoclaved plastic pouches (Northrup King Co., Minneapolis, MN), each of which contained 15 ml of Jensen's solution. Each pouch received three seedlings, which then were inoculated individually with 250 μl of bacterial suspension delivered to the root with a micropipette. Controls received autoclaved PBS. The seedlings were incubated as described above.

Bacteria were isolated from nodules as follows: individual nodules were surface-disinfested by immersion in 1% aqueous sodium hypochlorite for 10 min, and then in two changes of sterile water (1 min each). To check for surface contaminants, each nodule was rolled across one-half of a YEM agar plate containing kanamycin. The nodule was crushed and a portion of the contents streaked on the other half of the plate. Bacteria from uncontaminated nodules were retained for additional testing.

DNA libraries. Total DNA was isolated from parental strain 257 and from Tn5-containing mutants by the method of Jagadish and Szalay (1984). DNA was partially digested with *Eco*RI and fractionated on a linear 5–25% sucrose gradient (Friedman *et al.* 1982). Fragments of about 20 kb were ligated to *Eco*RI-digested cosmid pLAFR1 (Friedman *et al.* 1982) and packaged into phage lambda heads (Stratagene, San Diego, CA). Aliquots of the packaging mixture were used to infect *E. coli* HB101. Tetracycline-resistant colonies were transferred in arrays to master plates and screened by colony hybridization (Grunstein and Hogness 1975).

Molecular and genetic methods. Restriction endonucleases from Bethesda Research Laboratories and Promega Biotec were used according to the manufacturers' instructions. DNA was fractionated on horizontal 0.7% agarose gels in TBE buffer (89 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.0), denatured, and transferred to BA-85 nitrocellulose or Nytran nylon membranes (Schleicher and Schuell, Keene, NH) essentially as described by Maniatis *et al.* (1982). Large plasmids were isolated on vertical gels by the procedure of Heron and Pueppke (1984). Ethidium bromide-stained DNA in these gels was irradiated with UV light for 15 min and depurinated for 15 min with 0.25 M HCl before denaturation and transfer to filters.

Recombinant plasmids were prepared by the lysozyme procedure of Maniatis *et al.* (1982), restricted, and stored at −20° C. DNA probes were labeled by nick translation to about 5 × 10^7 cpm/μg and hybridized overnight to filters at 68° C. The hybridization solution consisted of 10× Denhardt's solution in 6× saline sodium citrate (SSC), and contained salmon sperm DNA (100 μg/ml) (Maniatis *et al.* 1982). Filters were washed before autoradiography at 68° C in 0.3× SSC containing 0.1% sodium dodecyl sulfate.

Cosmids were transferred from *E. coli* to rhizobia by triparental mating with pRK2013 as the helper plasmid (Ditta *et al.* 1980). The presence of each cosmid was verified by modified Eckhardt gels (Heron and Pueppke 1984). *Inc*P1 plasmid pPH1JI was used in marker-exchange experiments to force recombination of cosmid inserts into the *Rhizobium* genome (Ruvkun *et al.* 1982). Homologous recombination was confirmed by hybridization analysis of restricted total genomic DNA.

Plant tests. The capacity of bacteria to form nodules was assessed by inoculating replicate sets of pregerminated seedlings (Pueppke 1983). Nodulation rates of pouch-grown

Table 1. Bacterial plasmids

Plasmid	Characteristics	Reference
pSUP1011	Tn5 suicide vector, Km^{R}Cm^R	Simon *et al.* 1983
pRK2013	Mobilizing plasmid, Km^R	Ditta *et al.* 1980
pPH1JI	*Inc*P1, Gm^{R}Spc^R	Beringer *et al.* 1978
pLAFR1	Cosmid cloning vector, Tc^R	Friedman *et al.* 1984
pRmSL42	pBR325 with 2.2-kb *Bam*HI-*Hind*III insert containing *nodAB* and portions of *nodCD* of *R. meliloti* 1021	Egelhoff *et al.* 1985
pUC19::Tn5	3.3-kb *Hind*III fragment of Tn5 in pUC19	This study
pACYC184	Cloning vector, Cm^{R}Tc^R	Chang and Cohen 1978

plants were determined as described previously (Pueppke and Payne 1987). Nitrogen fixation *in planta* was measured by acetylene reduction (Schwinghamer *et al.* 1970).

Miscellaneous methods. The preparation of antibodies directed against strain 257 and their use in immuno-fluorescence microscopy has been described (Heron and Pueppke 1987). Auxotrophy of bacterial mutants was assessed by the method of Davis *et al.* (1980). Bacterial generation times were measured as follows: minimal medium (Maier and Brill 1976) or YEM broth (25 ml per 250-ml flask) was inoculated with bacterial cells (10^7 cells per milliliter) obtained from liquid starter cultures in the same medium. The flasks were incubated at 125 rpm and 30° C. Bacterial growth was measured turbidimetrically, beginning when growth was first evident, and at 3-hr intervals thereafter.

RESULTS

Screening for mutants. A total of 4,056 presumptive Tn5-containing mutants of *R. fredii* 257 were tested individually on cowpea. Three mutants, designated 257B3, 257F3, and 257M5, failed to nodulate in the primary screening experiment. Each was purified by single-colony isolation and confirmed to be a derivative of strain 257 by its plasmid profile and by immunofluorescence microscopy with antibodies directed against parental strain 257. The Nod⁻ phenotype subsequently was confirmed by repeated tests with cowpea.

Slightly more than 22,000 presumptive mutants were tested on McCall soybean. Five bacterial isolates were obtained from nodules that appeared on these plants (controls remained nodule-free). These Nod⁺ mutants were designated 257DH1, 257DH2, 257DH3, 257DH4, and 257DH5. Each was purified as above by single-colony isolation and confirmed to be a derivative of strain 257. Each was inoculated onto plants; bacteria were reisolated from the resultant nodules, and they were confirmed to be Km^R and derivatives of strain 257.

Biological characterization of mutants. Although strain 257 and all eight mutants grew on minimal medium, growth of 257F3 was markedly slower than that of the others. Addition of adenosine to the medium permitted 257F3 to grow at the wild-type rate, indicating that this mutant is a partial adenosine auxotroph. There were no differences in the generation times of the mutants in YEM broth; the doubling time of each was 3–4 hr.

The three mutants that failed to nodulate cowpea appear to be uniformly Nod⁻. They did not nodulate siratro, Peking soybean, and PI89772 soybean (all nodulated by parental strain 257) or McCall soybean (not nodulated by strain 257). Addition of 50 mM adenosine to the bacterial inoculum did not permit 257F3 to nodulate cowpea. Pairwise mixtures of 257B3, 257F3, and 257M5 in all three combinations also failed to nodulate McCall soybean. Also, pRmSL26, which contains the common *nod* genes from *R. meliloti* 1021 (Long *et al.* 1982), was transferred into each mutant. After confirming by gels (Heron and Pueppke 1984) that the cosmid was present in each mutant, we inoculated each onto cowpeas. Again, no nodules formed.

All five Nod⁺ mutants retained the ability of parental strain 257 to nodulate cowpea, siratro, and the soybean lines Peking, TGM119, and PI89772. These mutants were addi-

tionally tested on a series of soybean lines not nodulated by the parental strain (Table 2). All were Nod⁺, with the exception of 257DH5, which failed to nodulate Harosoy. None of the mutants nodulated pea, white clover, or alfalfa.

Figure 1 shows the rates with which the Nod⁺ mutants nodulate McCall soybean in growth pouches. Wild-type *R. fredii* 191, which unlike strain 257 nodulated McCall soybean, was included as a positive reference. Nodules were first detected on the eighth day after inoculation, and more than 98% of the plants inoculated with strain 191 were nodulated by day 12. The mutants were separated into two classes, neither of which produced as many nodules as the reference strain. The nodulation capacities of mutants 257DH1, 257DH2, 257DH3, and 257DH4 were similar. Nodules first appeared on day 8, and 80–90% of the plants was nodulated when the experiments were terminated on day 20 (Fig. 1). In contrast, the nodulating ability of mutant 257DH5 was meager. The mean number of nodules per plant was <1, and only about 25% of the plants ever formed nodules.

Nodules produced on McCall soybean by mutants 257DH1, 257DH2, 257DH3, and 257DH4 were pink and apparently morphologically normal. Acetylene reduction rates by nodules harvested 24 days after inoculation were 19.0, 22.8, 9.2, and 28.6 μmoles/hr/g of fresh nodule weight for mutants 257DH1, 257DH2, 257DH3, and 257DH4,

Table 2. Nodulation of soybean lines by *Rhizobium fredii* 257 and its mutants

| Mutant | Nodulation[a] of: | | | | |
	Evans	Harosoy	Hodgson 78	Viçoja	Williams 82
257DH1	+	+	+	+	+
257DH2	+	+	+	+	+
257DH3	+	+	+	+	+
257DH4	+	+	+	+	+
257DH5	+	−	+	+	+
257 (control)	−	−	−	−[b]	−

[a] + = nodules formed; − = no nodules formed.
[b] Plants not nodulated, but tumor-like structures appeared.
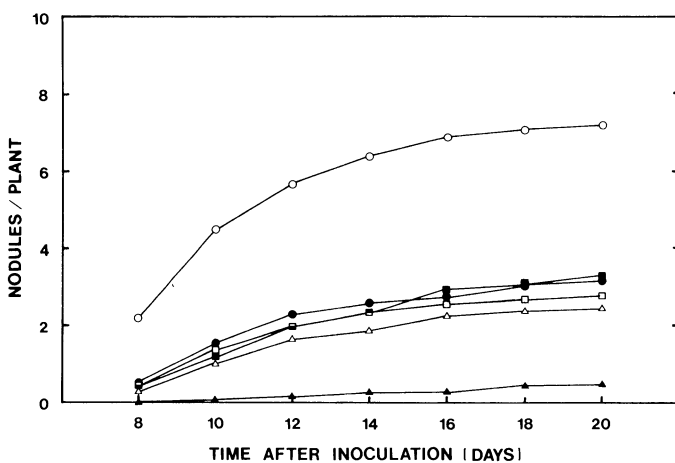

Fig. 1. Nodulation of McCall soybean by mutants of *R. fredii* 257. Pregerminated seedlings were inoculated as described previously (Pueppke 1983) and transferred to plastic growth pouches. Nodules were counted on the eighth day and alternate days thereafter. The results are means from three repeat experiments. The total number of plants per treatment was 56–82. o = strain 191; ● = 257DH1; △ = 257DH2; □ = 257DH3; ■ = 257DH4; ▲ = 257DH5.

respectively. Nodules produced by mutant 257DH5 were white, and they lacked detectable acetylene reduction activity.

Molecular characterization of mutants. Total DNA from each mutant was isolated, digested with either *Bam*HI or *Eco*RI, electrophoresed and blotted to nitrocellulose, and probed with the internal *Hin*dIII fragment of Tn5 (Fig. 2). Each mutant contains a single hybridizing *Eco*RI fragment and two hybridizing *Bam*HI fragments. Tn5 has a single *Bam*HI site and no *Eco*RI sites, and thus each mutant contains a single copy of the transposon. The band patterns of each mutant are different.

nodDABC of parental strain 257 reportedly is on a large Sym plasmid (Appelbaum *et al.* 1985b), and thus it was of interest to determine if the Tn5 insertions also are plasmid-borne. Figure 3 shows that, as expected, the parent and each mutant has a Sym plasmid of about 300 Md (Heron and Pueppke 1984). The common *nod* genes of *R. meliloti* hybridize to this Sym plasmid. Tn5, in contrast, only hybridizes to the Sym plasmids of mutant 257DH4 and the positive control 257S1.

*Eco*RI-digested total DNA was probed with a *Bam*HI-*Hin*dIII fragment of pRmSL42 of about 2.3 kb, which contains *nodAB* and portions of *nodCD* from *R. meliloti* (Egelhoff *et al.* 1985). Parental strain 257 and six of the mutants contain three hybridizing fragments of about 6.8, 5.2, and 2.7 kb (Fig. 4). This indicates that the Sym plasmid

insertion of Tn5 in mutant 257DH4 is not in *nodDABC* and that this region is not detectably altered in the other mutants. Mutant 257B3 lacks the 6.8-kb *Eco*RI fragment with homology to *nodDABC*, and 257DH3 contains a pair of additional fragments of about 7.0 and 5.0 kb (Fig. 4). These observations prompted us to search for integrated vector sequences by probing *Eco*RI digests of total DNA with pACYC184, the basic replicon of the suicide vector used for mutagenesis. There was homology only to the fragments of 257DH3 of about 7.0 and 5.0 kb (data not shown), indicating that vector sequences have been maintained in this mutant.

A cosmid clone containing Tn5 was obtained from a genomic library in pLAFR1 of each mutant except 257DH3. Each cosmid was mobilized into parental strain 257, and marker exchange was forced by plasmid incompatibility with pPH1JI. Three to eight Km^{R}Spc^{R}Tc^S colonies from each mating mixture were retained. DNA was isolated from each and subjected to Southern analysis with Tn5 as a probe to confirm that marker exchange had been successful (data not shown). The marker-exchange mutants then were inoculated separately onto plants. Presumptive Nod$^-$ mutants were tested on cowpea, and presumptive Nod$^+$ mutants were tested on McCall soybean. All three of the former retained the Nod$^+$ phenotype of strain 257. Marker-exchange mutants derived from the 257DH1 and 257DH2 cosmids also retained the parental phenotype (Nod$^-$ on
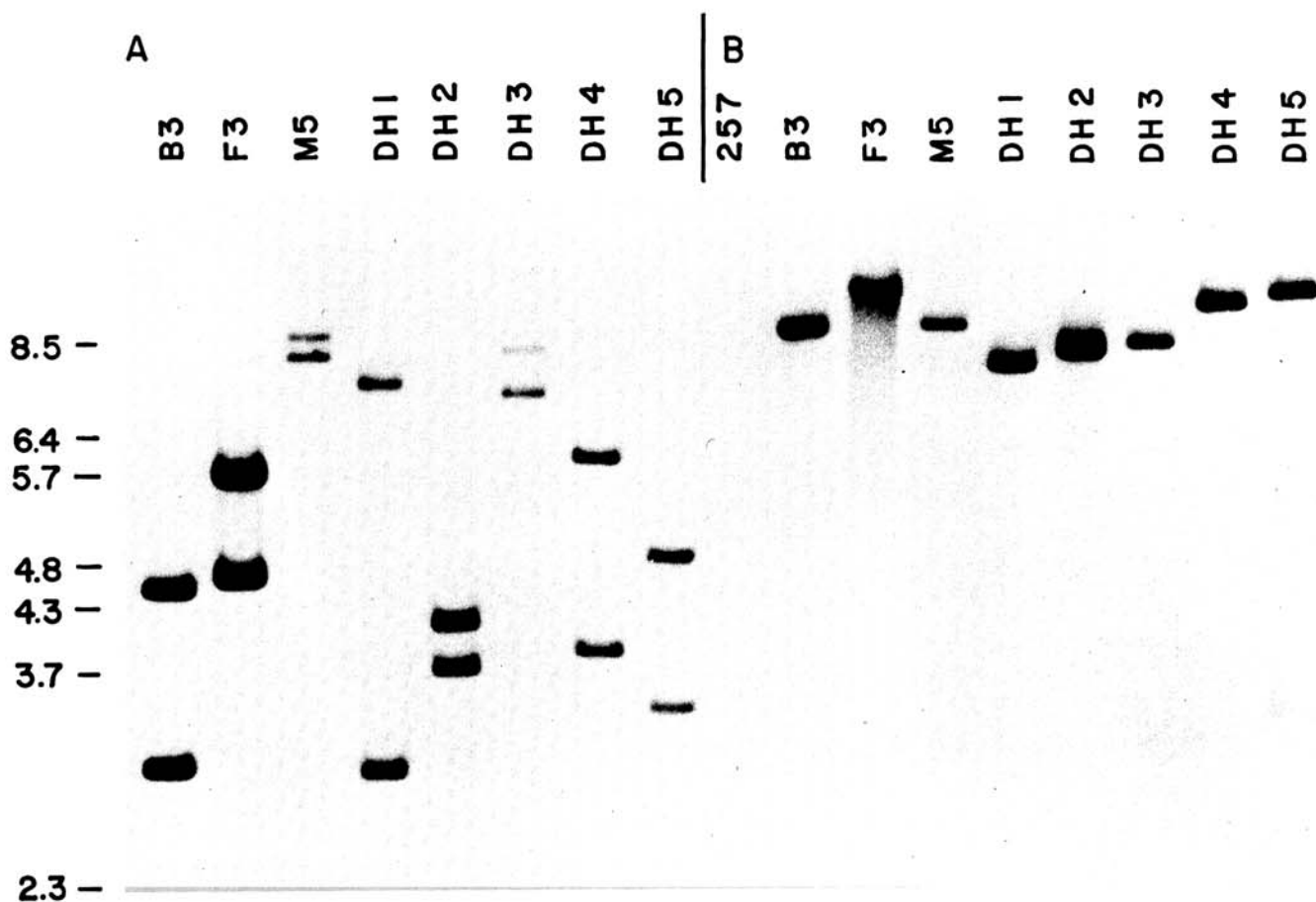

Fig. 2. Hybridization of Tn5 to total DNA from *R. fredii* 257 and eight mutants derived from it. DNA was digested with *Bam*HI (**A**) or *Eco*RI (**B**). The hybridization probe was the internal *Hin*dIII fragment of Tn5, which had been isolated from pUC19::Tn5. The numbers on the left give the sizes in kb of molecular weight markers.

McCall). The phenotype of marker-exchange mutants of 257DH4 and 257DH5, however, was Nod$^+$ on McCall soybean.

DISCUSSION

Although *R. fredii* 257 readily nodulates a number of legume species, it is markedly cultivar-specific on soybean (Keyser *et al.* 1982; Heron and Pueppke 1984; Stowers and Eaglesham 1984). These characteristics make strain 257 useful for identification of a broad range of genes with diverse functions in the nodulation process. McLoughlin *et al.* (1987) obtained transposon mutants of strain 257 and identified several with normal nodulating abilities, but defects in competitiveness. By using random transposon mutagenesis, we have identified two additional categories of symbiotically interesting mutants.

The three mutants of the first class uniformly lack the capacity to nodulate. A single copy of Tn5 is present, presumably in the chromosome of each, but marker-exchange experiments confirm that bona fide insertion of the transposon is not responsible for the Nod$^-$ phenotype of any of these mutants. Similar complications are commonly

encountered during transposon mutagenesis of rhizobia, including *R. meliloti* (Buikema *et al.* 1983), *R. phaseoli* (Noel *et al.* 1984), and *R. fredii* (Appelbaum *et al.* 1985b; McLoughlin *et al.* 1987). Although we are uncertain of the precise nature of the defects in Nod$^-$ mutant 257M5, 257F3 is a purine auxotroph, and such mutants often are symbiotically defective (Djordjevic *et al.* 1988; Kim *et al.* 1988). 257B3 lacks the 6.8-kb *Eco*RI fragment with homology to *nodDABC* of *R. meliloti* (Egelhoff *et al.* 1985), indicating that a Sym plasmid deletion has occurred.

The second class of symbiotically defective mutants are more unusual than the first, because they nodulate soybean cultivars not nodulated by the parental strain. This implies that strain 257 has genes that function to prevent nodulation of some plant genotypes. Phenotypically similar genes have been detected in *R. trifolii* and *R. meliloti*. Insertion of Tn5 into a region of about 1.5 kb of the Sym plasmid of *R. trifolii* simultaneously impairs nodulation of white clover and extends nodulation to two cultivars of pea (Djordjevic *et al.* 1985). Mutants with Tn5 inserted in *hsnD* (= *nodH*) of *R. meliloti* are Nod$^-$ on alfalfa and delayed Nod$^+$ on white sweet clover (both normal hosts of *R. meliloti*), but at low frequency they induce nodule-like structures on roots of *Vicia sativa* and *V. villosa* (Horvath *et al.* 1986).

The Nod$^+$ mutants of 257 are, however, fundamentally different from the *R. trifolii* and *R. meliloti* mutants. Nodulation ability apparently is extended only to additional cultivars of a normal host species and not to new legume species. Moreover, acquisition of the ability to nodulate new cultivars is not accompanied by any detectable reduction in symbiotic competency on normally nodulated plants such as cowpea and Peking soybean (Gibbons and Pueppke, unpublished data). In this regard, the strain X cultivar compatibility of the Nod$^+$ mutants of strain 257 is similar to that of *avr* mutants of plant pathogenic bacteria (Keen and Staskawicz 1988).

Insertion of Tn5 is responsible for the mutant phenotype of two of the five Nod$^+$ mutants. The insertion is in the Sym plasmid of mutant 257DH4, but is not in *nodDABC*. This is to be expected, because mutations in these genes reduce or
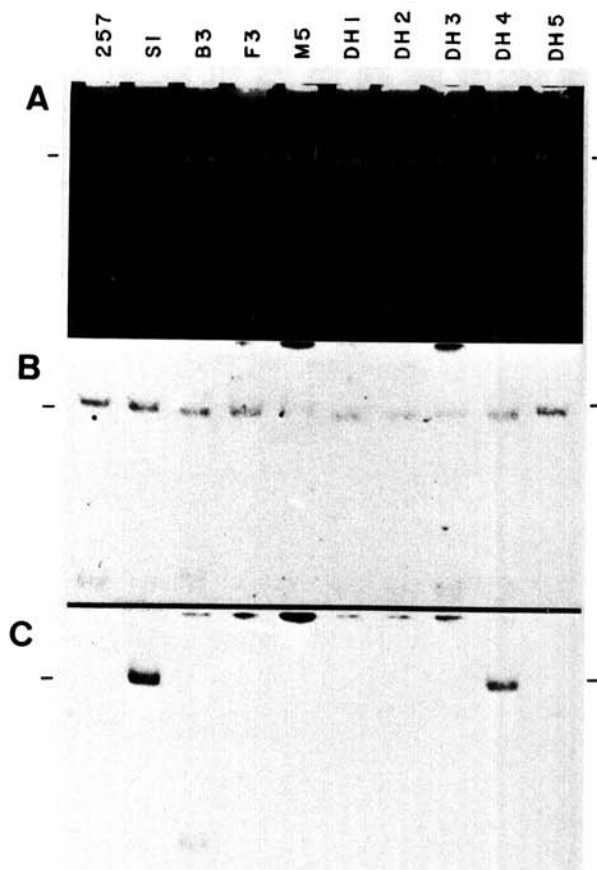

Fig. 3. Plasmids of *R. fredii* 257 and nine mutants derived from it. Plasmids were isolated according to Heron and Pueppke (1984). Panel **A**, Gel stained with ethidium bromide. Panel **B**, Southern blot probed with the 2.3-kb *Bam*HI-*Hind*III fragment of pRmSL42, which contains parts of *nodDABC* region of *R. meliloti*. Panel **C**, Southern blot reprobed with the internal *Hind*III fragment of Tn5. 257S1 is a reference mutant that contains a silent insertion of Tn5 in its Sym plasmid (Pueppke, unpublished).
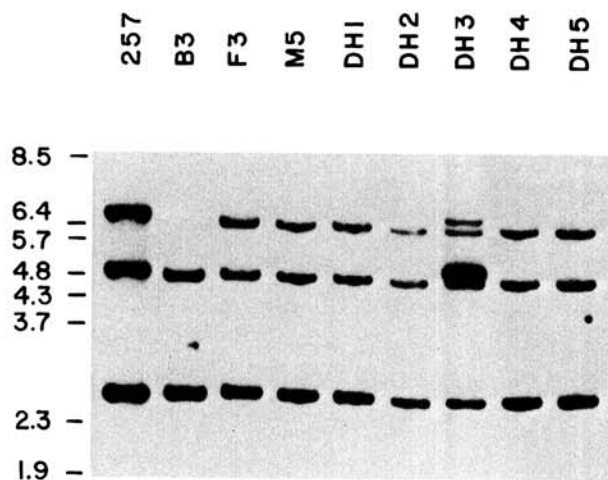

Fig. 4. Hybridization of the *Bam*HI-*Hind*III fragment of pRmSL42, which contains the common *nod* region of *R. meliloti*, to *Eco*RI-digested total DNA from *R. fredii* 257 and eight mutants derived from it. The numbers on the left give the positions and sizes in kb of molecular weight markers.

prevent nodulation (Johnston *et al.* 1988). In contrast, the insertion in mutant 257DH5 apparently is chromosomal. Similar chromosomal mutations have not been detected in rhizobia, and mutant 257DH5 is thus a unique tool to study positive regulation of nodulation. Wild-type sequences (about 20 kb) flanking the sites of transposon insertion in mutants 257DH4 and 257DH5 already have been cloned into pLAFR1, and their characterization is in progress. Genetic analysis of the remaining three mutants will be more challenging. Straightforward cloning of wild-type genes by complementation of the mutations (Long *et al.* 1982) will be difficult because selection will be for a negative change, from Nod$^+$ to Nod$^-$ on McCall soybean.

To our knowledge, this the first report of negatively acting genes that block nodulation of specific host cultivars. A gene of the opposite sort is known to exist and has been cloned from the Sym plasmid of *R. leguminosarum* bv. *viciae* strain TOM (Hombrecher *et al.* 1984; Götz *et al.* 1985; Davis *et al.* 1988). This gene is required for nodulation of the pea cultivar Afghanistan by strain TOM and its transfer to other strains of *R. leguminosarum* bv. *viciae* extends their host ranges to Afghanistan pea. The mechanisms by which positively and negatively acting genes regulate the cultivar specificity of pea and soybean rhizobia, however, are unknown.

LITERATURE CITED

Appelbaum, E. R., Chartrain, N., Thompson, D., Johansen, K., O'Connell, M., and McLoughlin, T. 1985a. Genes of *Rhizobium japonicum* involved in development of nodules. Pages 101-107 in: Nitrogen Fixation Research Progress. H. J. Evans and P. H. Bottomley, eds. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Appelbaum, E. R., Johansen, E., and Chartrain, N. 1985b. Symbiotic mutants of USDA 191, a fast-growing *Rhizobium* that nodulates soybeans. Mol. Gen. Genet. 201:454-461.

Appelbaum, E. R., McLoughlin, T. J., O'Connell, M., and Chartrain, N. 1985c. Expression of symbiotic genes of *Rhizobium japonicum* USDA 191 in other rhizobia. J. Bacteriol. 163:385-388.

Appelbaum, E. R., Thompson, D. V., Idler, K., and Chartrain, N. 1988. *Rhizobium japonicum* USDA 191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. 170:12-20.

Beringer, J. E., Beynon, J. L., Buchanan-Wollaston, A. V., and Johnston, A. W. B. 1978. Transfer of the drug-resistance transposon Tn5 to *Rhizobium*. Nature 276:633-634.

Buikema, W. J., Long, S. R., Brown, S. E., vandenBos, R. C., Earl, C., and Ausubel, F. M. 1983. Physical and genetic characterization of *Rhizobium meliloti* symbiotic mutants. J. Molec. Appl. Genet. 2:249-260.

Chang, A. C. Y., and Cohen, S. N. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 124:1141-1156.

Davis, E. O., Evans, I. J., and Johnston, A. W. B. 1988. Identification of *nodX*, a gene that allows *Rhizobium leguminosarum* biovar *viciae* strain TOM to nodulate Afghanistan peas. Mol. Gen. Genet. 212:531-535.

Davis, R. W., Botstein, D., and Roth, J. R. 1980. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Ditta, G., Standfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Djordjevic, M. A., Schofield, P. R., and Rolfe, B. G. 1985. Tn5 mutagenesis of *Rhizobium trifolii* host-specific nodulation genes result in mutants with altered host-range ability. Mol. Gen. Genet. 200:463-471.

Djordjevic, S. P., Ridge, R. W., Chen, H., Redmond, J. W., Batley, M., and Rolfe, B. G. 1988. Induction of pathogenic-like responses in the legume *Macroptilium atropurpureum* by a transposon-induced mutant of the fast-growing, broad-host-range *Rhizobium* strain NGR234. J. Bacteriol. 170:1848-1857.

DuTeau, N. M., Palmer R. G., and Atherly, A. G. 1986. Fast-growing *Rhizobium fredii* are poor nitrogen-fixing symbionts of soybean. Crop Sci. 26:884-889.

Egelhoff, T. T., Fisher, R. F., Jacobs, T. W., Mulligan, J. T., and Long, S. R. 1985. Nucleotide sequence of *Rhizobium meliloti* 1021 nodulation genes: *nodD* is read divergently from *nodABC*. DNA 4:241-248.

Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. M. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene 18:289-296.

Götz, R., Evans, I. J., Downie, J. A., and Johnston, A. W. B. 1985. Identification of the host-range DNA which allows *Rhizobium leguminosarum* strain TOM to nodulate cv. Afghanistan peas. Mol. Gen. Genet. 201:296-300.

Grunstein, M., and Hogness, D. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci USA 72:3961-3965.

Hattori, J., and Johnson, D.A. 1984. Fast-growing *Rhizobium japonicum* that effectively nodulates several commercial *Glycine max* L. Merrill cultivars. Appl. Environ. Microbiol. 48:234-235.

Heron, D. S., and Pueppke, S. G. 1984. Mode of infection, nodulation specificity, and indigenous plasmids of 11 fast-growing *Rhizobium japonicum* strains. J. Bacteriol 160:1061-1066.

Heron, D. S., and Pueppke, S. G. 1987. Regulation of nodulation in the soybean-*Rhizobium* symbiosis. Strain and cultivar variability. Plant Physiol. 84:1391-1396.

Hom, S. S. M., Uratsu, S. L., and Hoang, F. 1984. Transposon Tn5-induced mutagenesis of *Rhizobium japonicum* yielding a wide variety of mutants. J. Bacteriol. 159:335-340.

Hombrecher, G., Götz, R., Dibb, N. J., Downie, J. A., Johnston, A. W. B., and Brewin, N. J. 1984. Cloning and mutagenesis of nodulation genes from *Rhizobium leguminosarum* TOM, a strain with extended host range. Mol. Gen. Genet. 194:293-298.

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Török, I., Györgypal, Z., Barabas, I., Wieneke, U., Schell, J., and Kondorosi, A. 1986. Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell 46:335-343.

Israel, D. W., Mathis, J. N., Barbour, W. M., and Elkan, G. H. 1986. Symbiotic effectiveness and host-strain interactions of *Rhizobium fredii* USDA 191 on different soybean cultivars. Appl. Environ. Microbiol. 51:898-903.

Jagadish, M. N., and Szalay, A. A. 1984. Directed transposon Tn5 mutagenesis and complementation in slow-growing, broad host range cowpea *Rhizobium*. Mol. Gen. Genet. 196:290-300.

Jansen vanRensburg, H., Strijdom, B. W., and Otto, C. J. 1983. Effective nodulation of soybeans by fast-growing strains of *Rhizobium japonicum*. S. Afr. J. Sci. 79:251-252.

Johnston, A. W. B., Firmin, J. L., and Rossen, L. 1988. On the analysis of symbiotic genes of *Rhizobium*. Pages 439-455 in: The Nitrogen and Sulfur Cycles. J. A. Cole and S. J. Ferguson, eds. Cambridge University Press, Cambridge.

Keen, N. T., and Staskawicz, B. 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. 42:421-440.

Keyser, H. H., Bohlool, B. B., Hu, T. S., and Weber, D. F. 1982. Fast-growing rhizobia isolated from root nodules of soybean. Science 215:1631-1632.

Kim, C. H., Kuykendall, L. D., Shah, K. S., and Keister, D. L. 1988. Induction of symbiotically defective auxotrophic mutants of *Rhizobium fredii* HH303 by transposon mutagenesis. Appl. Environ. Microbiol. 54:423-427.

Long, S. R., Buikema, W. J., and Ausubel, F. M. 1982. Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod mutants. Nature 298:485-488.

Maier, R. J., and Brill, W. J. 1976. Ineffective and non-nondulating mutant strains of *Rhizobium japonicum*. J. Bacteriol. 127:763-769.

Maniatis, T., Fritsch E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McLoughlin, T. J., Merlo, A. O., Satola, S. W., and Johansen, E. 1987. Isolation of competition-defective mutants of *Rhizobium fredii*. J. Bacteriol. 169:410-413.

Noel, K. D., Sanchez, A., Fernandez, L., Leemans, J., and Cevallos, M. A.

1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. 158:148-155.

Pueppke, S. G. 1983. *Rhizobium* infection threads in root hairs of *Glycine max* (L.) Merr., *Glycine soja* Sieb. & Zucc., and *Vigna unguiculata* (L.) Walp. Can. J. Microbiol. 29:69-76.

Pueppke, S. G., and Payne, J. H. 1987. Responses of Rj$_1$ and rj$_1$ soybean isolines to inoculation with *Bradyrhizobium japonicum*. Plant Physiol. 84:1291-1295.

Ruvkun, G. B., Sundaresan, V., and Ausubel, F. M. 1982. Directed transposon Tn*5* mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell 29:551-559.

Sadowsky, M. J., Cregan, P. B., and Keyser, H. H. 1988. Nodulation and nitrogen fixation efficiency of *Rhizobium fredii* and *Phaseolus vulgaris* genotypes. Appl. Environ. Microbiol. 54:1907-1910.

Scholla, M. H., and Elkan, G. H. 1984. *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybean. Int. J. Syst. Bacteriol. 34:484-486.

Schwinghamer, E. A., Evans, H. J., and Dawson, M. D. 1970. Evaluation of effectiveness in mutant strains of *Rhizobium* by acetylene reduction relative to other criteria of N$_2$ fixation. Plant Soil 33:192-212.

Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. Bio/Technology 1:784-791.

Stowers, M. D., and Eaglesham, A. R. J. 1984. Physiological and symbiotic characteristics of fast-growing *Rhizobium japonicum*. Plant Soil 77:3-14.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell Scientific Publications, Oxford.

Yelton, M. M., Yang, S. S., Edie, S. A., and Lim, S. T. 1983. Characterization of an effective salt-tolerant, fast-growing strain of *Rhizobium japonicum*. J. Gen. Microbiol. 129:1537-1547.