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Isolation and Characterization of Symbiotic Mutants of *Bradyrhizobium* sp. (*Arachis*) Strain NC92: Mutants with Host-Specific Defects in Nodulation and Nitrogen Fixation†

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Random transposon Tn*5* mutagenesis of *Bradyrhizobium* sp. (*Arachis*) strain NC92, a member of the cowpea cross-inoculation group, was carried out, and kanamycin-resistant transconjugants were tested for their symbiotic phenotype on three host plants: groundnut, siratro, and pigeonpea. Two nodulation (Nod^- phenotype) mutants were isolated. One is unable to nodulate all three hosts and appears to contain an insertion in one of the common nodulation genes (*nodABCD*); the other is a host-specific nodulation mutant that fails to nodulate pigeonpea, elicits uninvaded nodules on siratro, and elicits normal, nitrogen-fixing nodules on groundnut. In addition, nine mutants defective in nitrogen fixation (Fix^- phenotype) were isolated. Three fail to supply symbiotically fixed nitrogen to all three host plants. Surprisingly, nodules elicited by one of these mutants exhibit high levels of acetylene reduction activity, demonstrating the presence of the enzyme nitrogenase. Three more mutants have partially effective phenotypes ($Fix^\pm$) in symbiosis with all three host plants. The remaining three mutants fail to supply fixed nitrogen to one of the host plants tested while remaining partially or fully effective on the other two hosts; two of these mutants are Fix^- in pigeonpea and $Fix^\pm$ on groundnut and on siratro, whereas the other one is Fix^- on groundnut but Fix^+ on siratro and on pigeonpea. These latter mutants also retain significant nodule acetylene reduction activity, even in the ineffective symbioses. Such bacterial host-specific fixation (Hsf) mutants have not previously been reported.

Specific strains of rhizobia enter into nitrogen-fixing symbioses with specific host plants, almost exclusively legumes. Successful recognition between compatible bacterial and plant partners elicits the development of a novel plant organ, the root nodule. The bacteria invade the developing nodule and there differentiate into the nitrogen-fixing "bacteroid" form (reviewed in references 21 and 73).

There are two major groups of rhizobia, commonly known as the fast- and the slow-growing species. These two groups differ by many biochemical and physiological criteria and have recently been placed in two separate genera, *Rhizobium* and *Bradyrhizobium*, respectively (36). The two groups also differ in several symbiotic properties. For example, *Bradyrhizobium* strains can be induced to fix nitrogen in free-living culture (42, 47, 54), whereas *Rhizobium* species will only fix nitrogen symbiotically; fast-growing species generally infect only a few, closely related legumes, whereas *Bradyrhizobium* species of the cowpea cross-inoculation group can infect a broad range of diverse legume hosts (25); fast-growing species almost invariably infect their hosts via the root hairs, whereas a single slow-growing species may infect one host, such as pigeonpea, by the root hairs, and another, such as groundnut, by direct intercellular penetration between the epidermal cells—so-called crack entry (17, 21, 37).

Significant progress has been made in the identification of bacterial genes that are required for the induction of nodules (*nod* genes) and the fixation of nitrogen (*fix* genes) in the fast-growing *Rhizobium* species (reviewed in references 4 and 41). In contrast, considerably less is known about the bacterial genes required for the establishment of the symbiosis by slow-growing *Bradyrhizobium* species. To date, the most successful approach used in the study of *Bradyrhizobium* symbiotic genes has been to look for genes which share structural or functional homology with previously identified *Rhizobium* symbiotic genes. Thus, common nodulation genes, the nitrogenase structural genes, and the homologs of other *Rhizobium* genes required for nitrogen fixation have been identified in *Bradyrhizobium* species (28, 32, 46, 51, 62, 63). However, this approach is unlikely to identify all genes that are responsible for the differences in host range or for other symbiotic differences between the two groups of rhizobia. An alternative approach to the identification of such genes is random mutagenesis followed by screening for symbiotic mutants on one or more host plants. There are several reports of *Bradyrhizobium japonicum* (the endosymbiont of soybean) mutants which were isolated by chemical or by UV mutagenesis (20, 44, 69); more recently, transposon Tn*5* has been used both for random (16, 34, 58) and for localized (30, 35, 46) mutagenesis in several *Bradyrhizobium* species. However, the majority of these reports concern *B. japonicum*, and in each case mutants were only screened on a single host plant.

In this paper we describe the random Tn*5* mutagenesis of *Bradyrhizobium* sp. (*Arachis*) strain NC92, a member of the cowpea cross-inoculation group, and the screening of putative symbiotic mutants on three NC92 host plants, siratro (*Macroptilium atropurpureum*), pigeonpea (*Cajanus cajan*),

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TABLE 1. Plasmids used in the experiments

Plasmid	Relevant characteristics	Source or reference	Probe	NC92[a]
pGS9	Tn*5* suicide vector	64		
pRK2013	Helper plasmid for pRmSL26 conjugation	23		
pRmSL26	Cosmid clone carrying *R. meliloti nodABCD*	43		
Hybridization probes				
pACYC184	Probe for pGS9 vector sequences	19		
pRH232D	*Bradyrhizobium* sp. (*Vigna*) *nifH* in pBR322	35	1-kb *Sma*I-*Hin*dIII fragment	3.5
pBN187	*Bradyrhizobium* sp. (*Parasponia*) 1.5-kb *Eco*RI fragment containing N-terminal 600 base pairs of *nifD* in pUC13	B. T. Nixon	Plasmid	2.5
pRH231	*Bradyrhizobium* sp. (*Vigna*) *nifK* in pBR322	35	2.6-kb *Bam*HI-*Hin*dIII fragment	5.0, 14.0
pBJ152	*B. japonicum nifA*-like gene in pBR328	B. K. Chelm	Plasmid	15.0
pBN384	*Bradyrhizobium* sp. (*Parasponia*) *nifB* in pUC13	B. T. Nixon	Plasmid	7.0
pBJ135	*B. japonicum nifS* in pBR328	B. K. Chelm	Plasmid	7.0, 4.0
pBJ150	*B. japonicum fixA* and partial *nifA*-like gene in pBR328	B. K. Chelm	2.6-kb *Xho*I-*Eco*RI *fixA* fragment	15.0
pBN259	*Bradyrhizobium* sp. (*Parasponia*) *fixB* in pUC13	B. T. Nixon	Plasmid	3.5
pBN408	*Bradyrhizobium* sp. (*Parasponia*) *nifH fixB fixC* in pUC13	B. T. Nixon	1.1-kb *fixC Pst*I fragment	5.0
pBN176	*Bradyrhizobium* sp. (*Parasponia*) *ntrB* in pUC13	B. T. Nixon	0.9-kb *Eco*RI fragment	11.0
pBN386	*Bradyrhizobium* sp. (*Parasponia*) *ntrC* C-terminal 1 kb in SP6	B. T. Nixon	Plasmid	10.0, 3.5, 2.5
pPRC6	*Bradyrhizobium* sp. (*Parasponia*) *nodABCD* genes in pLAFR1	46	5.2-kb *Xho*I fragment	11.5
pBJ53A	*B. japonicum glnA* in pUC8	15	Plasmid	14.0
pBJ196A	*B. japonicum glnII* in pBR322	14	Plasmid	9.0

[a] Size of hybridizing NC92 *Eco*RI fragment(s) in kilobase pairs.

and groundnut (*Arachis hypogaea*). These particular host species were chosen because the latter two show different modes of *Bradyrhizobium* infection (root hair and crack entry infection, respectively), and siratro is a small-seeded legume that is routinely used for screening *Bradyrhizobium* strains in test tube assays. We report the identification of two unusual types of symbiotic mutants; in particular, a host-specific nodulation mutant and a novel class of symbiotic mutants which have host-specific defects in nitrogen fixation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Bradyrhizobium* sp. (*Arachis*) strain NC92 is an isolate from groundnut provided by G. Elkan (North Carolina State University). The bacterial strains and plasmids used in this study are listed in Table 1.

Bacterial media. LB (49), TY (50), YM (72), and Sherwood minimal medium (67) have been described. Nutrient agar was 5 g of peptone, 5 g of NaCl, 1.5 g of beef extract, and 1.5 g of yeast extract per liter. Minimal sucrose medium was 2% sucrose, 0.05 g of K_2HPO_4, 0.15 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.2 mg of Na_2MoO_4, 6.6 mg of $FeCl_3$, 0.5 g of sodium glutamate, 1 g of $CaCO_3$ per liter, and 0.025% bromothymol blue. Media were solidified with 1.5% agar (Difco Laboratories, Detroit, Mich.) as required. Congo red was added to YM plates to a final concentration of 25 μg/liter (72) to facilitate the detection of potential contaminants (39). LB medium was supplemented with kanamycin (20 μg/ml) or tetracycline (10 μg/ml) for the growth of *Escherichia coli* strains when appropriate. YM and minimal sucrose media were supplemented with Kanamycin (100 μg/ml), Sherwood medium was supplemented with tetracycline (150 μg/ml), and TY was supplemented with the tetracycline analog minocycline (10 μg/ml) (13) for the growth of *Bradyrhizobium* transconjugants when appropriate.

Isolation of symbiotic mutants. Bacterial matings between *E. coli* strain WA803 (64) and *Bradyrhizobium* sp. (*Arachis*) strain NC92 were carried out by coincubation of the two strains on nonselective TY plates at 28°C for 24 h. Km^r NC92 transconjugants were then selected by plating the resuspended mating mixture on minimal sucrose (kanamycin) plates and were purified three times through single colonies; 923 Km^r transconjugants were serially screened for their symbiotic phenotype on groundnut plants grown in pots in the greenhouse and on siratro plants grown individually in tubes in the growth chamber (two tubes per transconjugant). Putative symbiotic mutants were further tested in replicated trials conducted both at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and at Massachusetts General Hospital (MGH).

Preparation of inoculum. At ICRISAT, the inoculum was prepared by using 12 g of gamma-irradiated peat packets (70) obtained from Agricultural Laboratories Pty. Ltd., Australia. Log-phase cultures of Km^r NC92 transconjugants were grown in YM broth at 28°C (3 to 4 days of growth), and 10 ml of this broth was injected into a fresh peat packet; the broth and peat were thoroughly mixed and incubated for 10 days at room temperature. During this time the *Bradyrhizobium* population grows to 10^8 to 10^9 cells per g of peat. The peat packets were then stored at 4°C until use, when the peat was

diluted in sterile water to 10^6 cells per ml; 1 ml of inoculum was used per seed. This inoculum was used for all greenhouse and growth chamber trials conducted at ICRISAT. At MGH, frozen inoculum was prepared using a modification of the method of Bhuvaneswari et al. (9). Strains were grown to the midlog phase in TY broth (optical density at 620 nm of <0.4), 5×10^9 cells were pelleted and suspended in 5 ml of 1:10 phosphate-buffered saline (8 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of Na_2HPO_4, and 0.2 g of KCl per liter) plus 15% glycerol, and 0.5-ml samples were frozen at −70°C. These were subsequently diluted 10× or 100× in sterile water for use as inoculum with 10^8 or 10^7 cells per ml, respectively.

Plant culture and growth measurements. Groundnut cultivar Robut 33-1, pigeonpea cultivar ICP-1, and siratro were all obtained from ICRISAT. Additional pigeonpea, cultivar T-21, was obtained from Niftal, Paia, Hawaii, and additional siratro seed was from Wright Stephenson Co. (Australia) Pty. Ltd., New South Wales, Australia. Seeds were sterilized by immersion in concentrated sulfuric acid for 12 min (siratro) or 0.2% $HgCl_2$ for 3 min (groundnut and pigeonpea), followed by six rinses in sterile water.

Greenhouse trials. Plants were grown in a 2:1 (vol/vol) mixture of sand and vermiculite in 6-in. (ca. 18-cm)-diameter pots. Two plants were grown per pot. Plants were watered with nitrogen-free nutrient solution, either Broughton solution (10) (ICRISAT) or Bergersen solution (29) (MGH). Greenhouse trials at MGH were conducted at 24°C with a 12-h day length. Higher temperatures were used at ICRISAT, where the ambient temperature could exceed 40°C, and no additional lighting was provided. At both ICRISAT and MGH the surface of the pots was covered with sterilized gravel after the plants had emerged to reduce splashing and possible cross-contamination among pots. At ICRISAT the pots were additionally fitted with watering tubes (P. T. C. Nambiar and P. J. Dart, Proceedings of the International Workshop on Groundnuts, ICRISAT, Patancheru, India, p. 110–124, 1980), whereas at MGH the pots were enclosed in open-ended plastic bags to further reduce this problem (35).

Plants were harvested after about 40 days of growth. The shoots were dried for 2 days at 60°C and weighed. The dried shoots were milled with a Ud Cyclone Sample Mill (Ud Corporation, Boulder, Colo.) and an approximately 15-mg subsample was analyzed for percentage combined nitrogen on a Carlo Erba automated nitrogen analyzer (ANA 1500; Carlo Erba Strumentazione, Milan, Italy). The relative amount of nitrogen fixed (*RN*) was calculated by using the formula RN = (mean total N_{mutant} − mean total $N_{639/807}$)/(mean total N_{NC92} − mean total $N_{639/807}$), where N is the total nitrogen fixed, and 639 and 807 are two NC92 derivatives that showed no detectable nitrogenase activity, as judged by the acetylene reduction assay, in symbiosis with any of the three host plants tested. For acetylene reduction assays, detached roots were incubated for 2 h in 10% acetylene in 125-ml Erlenmyer flasks sealed with a no. 49 Suba Seal (Gallenkamp, United Kingdom). After 2 h a 5-ml gas sample was removed and injected into a 10-ml Vacutainer (Becton Dickerson Laboratories, Oxnard, Calif.) for temporary storage. Gas samples were subsequently analyzed for acetylene reduction activity on a Sigma 3B gas chromatograph (Perkin-Elmer Corp., Nowalk, Conn.).

In all pot trials each treatment was replicated from three to five times. The pots were arranged in a randomized complete block or in a split plot design, and the results were analyzed statistically by using analysis of variance, by the Dunnett test for comparing a control mean to each other group mean (74), and by calculating the least significant difference (68).

Growth chamber trials. Siratro plants were also grown in seedling agar tubes (72), and pigeonpeas were grown in growth pouches (9). Growth pouches were watered with Broughton or Bergersen solution. Growth cabinets were maintained at 25°C with 16-h day length. Siratro acetylene reduction activity was measured on intact plants in tubes as described previously (49).

Isolation of bacteria from nodules. Nodules were immersed in 0.2% $HgCl_2$ for 60 s, rinsed six times in sterile distilled water, and squashed in 0.1 ml of sterile water. Nodule isolations were first streaked on nonselective YM plates and then streaked or replica plated on selective media as appropriate.

Complementation of mutant NC92 no. 21. Three-way matings were conducted as described previously (60) among NC92 no. 21, *E. coli* HB101 containing the helper plasmid pRK2013, and *E. coli* HB101 containing plasmid pRmSL26. Tc^r NC92 no. 21 transconjugants were selected on Sherwood minimal plates with 150 μg of tetracycline per ml.

Microscopy. Siratro nodules were fixed and prepared for light microscopy as described previously (33).

DNA biochemistry. *Bradyrhizobium* genomic DNA was prepared as follows. A 1.5-ml sample of a saturated culture grown in TY or YM at 30°C was pelleted in a 1.5-ml microcentrifuge tube, suspended in 600 μl of 0.5% sodium dodecyl sulfate–100 μg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated for 1 h at 37°C. The solution was then brought to approximately 0.7 M NaCl by the addition of 100 μl of 5 M NaCl before the addition of 80 μl of 10% hexadecyltrimethylammonium bromide in 0.7 M NaCl. The tubes were incubated for 10 min at 65°C and then extracted once with 0.7 ml of 24:1 (vol/vol) chloroform-isoamyl alcohol and once with 24:24:1 (vol/vol/vol) phenol-chloroform-isoamyl alcohol. The supernatant was precipitated with 0.6 volume of isopropanol, the chromosomal DNA was spun down, and the pellet was washed once with 70% ethanol, dried, and suspended in 100 μl of Tris-EDTA (pH 8.0). Samples (15 μl) of this DNA were digested with 10 U of *Eco*RI for use on Southern blots. Small- and large-scale preparations of plasmid DNA were carried out by using the alkaline lysis procedure described previously (45). Restriction endonucleases were purchased from Boehringer Mannheim and used as described by the manufacturer. Agarose gel electrophoresis was carried out as described previously (59), and the DNA in the gel was transferred to GeneScreen filters (New England Nuclear Corp., Boston, Mass.) according to the manufacturer's instructions, except that 20× SSC (3 M NaCl, 0.3 M sodium citrate) was used for the transfer buffer. Nick translations (45) and DNA hybridizations (46) were carried out as described previously. Where necessary, DNA fragments for use as hybridization probes were isolated from low-melting-temperature agarose gels (International Biotechnologies Inc.) by melting the agarose at 70°C in the presence of 0.4 M NaCl, cooling to 37°C, and then phenol extracting twice before ethanol precipitation.

RESULTS

Transposon Tn*5* mutagenesis of *Bradyrhizobium* sp. (*Arachis*) strain NC92. Random Tn*5* mutagenesis was carried out by using the "suicide" plasmid pGS9 (64), and kanamycin-resistant NC92 transconjugants were obtained at a frequency of approximately 1 in 10^5 NC92 recipients. More

TABLE 2. Phenotypes of plants inoculated with putative *Bradyrhizobium* sp. strain NC92 symbiotic mutants[a]

Plant	Strain inoculated	Leaf color	Shoot wt (mg)	Total N[b] (mg)	Relative N fixed (%)	Ara
Groundnut	NC92	Dark	5,400	135.9	100	+
	284	Dark	3,800	83.0**	43	+
	302	Pale	3,500	54.7**	13	+
	639	Pale	3,600	46.0**	–	–
	727	Pale	3,800	60.3**	19	+
	748	Dark	5,000	124.4	88	+
	807	Pale	3,100	38.4**	–	–
	831	Dark	4,500	102.4**	64	+
	853	Dark	3,800	89.5**	50	+
	868	Dark	4,900	102.3**	64	+
	979	Dark	4,500	107.6**	70	+
Pigeonpea	NC92	Dark	583	15.9	100	+
	284	Pale	206	2.8**	6	+
	302	Pale	246	3.7**	13	+
	639	Pale	167	1.7**	–	–
	727	Dark	651	15.4	96	+
	748[c]	Pale	207	2.4**	4	–
	807	Pale	171	2.1**	–	–
	831	Medium	427	9.2*	52	+
	853	Medium	457	9.2*	52	+
	868	Dark	446	9.3*	53	+
	979	Medium	240	3.4**	11	+
Siratro	NC92	Dark	517	19.3	100	+
	284	Dark	327	11.0**	55	+
	302	Pale	53	0.8**	1	+
	639	Pale	42	0.6**	–	–
	727	Dark	437	14.4	74	+
	748	Pale	50	0.7**	1	–
	807	Pale	50	0.6**	–	–
	831	Dark	374	12.1**	62	+
	853	Dark	278	8.6**	43	+
	868	Dark	321	10.1**	51	+
	979	Dark	390	12.1**	62	+

[a] The data are presented as the mean of four replications. Values are given per pot, i.e., per two plants. N refers to combined nitrogen. The acetylene reduction activity (Ara) is defined as (+) when the average activity over all experiments was greater than 30% of the wild type and (−) when the average activity was less than 3% of the wild type. All numerical data were subjected to analysis of variance, and in each case treatment differences were found to be highly significant ($P < 0.01$). The standard errors of the mean were as follows for groundnut, pigeonpea, and siratro, respectively: ±300, ±66, and ±45 mg for shoot weight and ±6.1, ±1.8, and ±1.8 mg for total N.

[b] The data for total N were subjected to a two-tailed Dunnett test for comparing a control mean to each other group mean (74); * and ** indicate that the treatment mean differs from the wild-type control mean at a level of significance of 5 and 1%, respectively. The least significant differences for total N at a 1% level of significance are as follows: for groundnut, ± 21.4 mg; for pigeonpea and siratro, ±6.3 mg.

[c] There were no nodules on the roots of pigeonpea plants inoculated with strain 748.

than 900 of these Km^r NC92 transconjugants were individually screened for their symbiotic phenotypes on groundnut and on siratro. The screening on siratro was only partially successful in identifying Fix$^-$ mutants, because the restricted growth of the plants in small tubes resulted in all plants looking somewhat unhealthy. Nevertheless, one Fix$^-$ mutant (NC92 no. 748) and one Nod$^-$ mutant (NC92 no. 21) were identified. In contrast, groundnuts inoculated with NC92 symbiotic mutants could easily be recognized by their pale green, nitrogen-starved appearance after about 6 weeks of growth. Putative Fix$^-$ and Nod$^-$ NC92 mutants were further tested in replicated trials at ICRISAT and at MGH on three NC92 host plants, groundnut, pigeonpea, and siratro (at least two independent trials per mutant). Data from a representative trial conducted at MGH are given in Table 2. Trials conducted at ICRISAT gave similar results with regard to leaf color, acetylene reduction activity, and shoot weight, but total fixed nitrogen was not measured at ICRISAT. In all, two nodulation mutants and nine nitrogen fixation mutants were isolated. The phenotypes of these mutants are discussed in detail below and are summarized in Table 3.

Physical analysis of NC92 Tn*5* mutants. DNA hybridization was used to determine whether the NC92 Nod$^-$ and Fix$^-$ mutants contained Tn*5* sequences. *Eco*RI-digested genomic DNA from each mutant strain and from the wild type strain, NC92, was subjected to Southern blot analysis and probed with ^{32}P-labeled pGS9 or pACYC184 DNA. In 7 of the 11 mutants a single pGS9-hybridizing *Eco*RI fragment was observed, and no hybridization was detected to pACYC184. Since pACYC184 constitutes the region of the pGS9 vector that surrounds Tn*5* (64), this result indicates that these strains contain a single Tn*5* insertion and do not contain integrated vector sequences. The insertions were in different *Eco*RI fragments in each case. Two of the mutants, no. 21 and 748, showed two pGS9-hybridizing *Eco*RI fragments. These fragments also hybridized to pACYC184, demonstrating that these two mutants contain integrated vector sequences. The remaining two mutants, no. 831 and 979, showed no hybridizing bands with either probe, indicating that they must be spontaneous kanamycin-resistant mutants (data not shown).

These same Southern blots, or replicas, were reprobed with ^{32}P-labeled DNA of subclones of known heterologous symbiotic genes in an attempt to correlate the observed NC92 mutant phenotypes with Tn*5* (or vector) insertions in the vicinity of homologous NC92 DNA sequences. These would be detected by observing changes in the electrophoretic mobilities of the homologous NC92 restriction fragment(s). The hybridization probes used and the sizes of the hybridizing NC92 restriction fragments are listed in Table 1. Strain NC92 no. 21 was found to contain an insertion in DNA that is homologous to a 5.2-kilobase (kb) *Xho*I fragment which contains the common nodulation genes (*nodABCD*) of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501 (46). However, none of the other mutants contained insertions in NC92 restriction fragments that are homologous to *Bradyrhizobium nifH*, *nifD*, *nifK*, *fixA*, *fixB*, *fixC*, a *nifA*-like gene (B. K. Chelm, personal communication), *nifB*, *nifS*, *ntrB*, *ntrC*, *glnA*, or *glnII* (data not shown).

TABLE 3. Symbiotic phenotypes of NC92 mutants on three host plants

NC92 strain no.	Groundnut			Pigeonpea			Siratro		
	Nod	Fix	Ara	Nod	Fix	Ara	Nod	Fix	Ara
NC92	+	+	+	+	+	+	+	+	+
21	–			–			–		
639	+	–	–	+	–	–	+	–	–
807	+	–	–	+	–	–	+	–	–
302	+	–	+	+	–	+	+	–	+
727	+	–	+	+	+	+	+	+	+
284	+	±	+	+	–	+	+	±	+
979	+	±	+	+	–	+	+	±	+
748	+	+	+	–			+	–	–
831	+	±	+	+	±	+	+	±	+
853	+	±	+	+	±	+	+	±	+
868	+	±	+	+	±	+	+	±	+

Symbiotic phenotype of spontaneous kanamycin-resistant mutants. Since 2 of the 11 NC92 symbiotic mutants were found to be spontaneous kanamycin-resistant mutants (a frequency of 18%), the frequency of isolation of such spontaneous mutants was checked by plating serial dilutions of a log-phase culture of wild-type NC92 on minimal sucrose plates with and without the addition of kanamycin and was found to be 1 in 5×10^6. Four of these spontaneous Km^r mutants were checked for their symbiotic phenotype on pigeonpea; one was Fix^+, two were $Fix^\pm$, and one was Fix^- ARA^- (data not shown). Thus, there seems to be a link between spontaneous kanamycin resistance and symbiotic ineffectiveness in this strain.

Growth characteristics of NC92 Nod^- and Fix^- mutants. The NC92 kanamycin-resistant transconjugants were originally selected on minimal medium, thereby precluding the isolation of auxotrophic mutants. Nevertheless, two mutants do show altered growth properties. Mutant no. 868 takes 8 days to form a 1-mm-diameter colony on a plate when grown at 30°C (2 days longer than NC92 or the other Tn*5*-containing derivatives.) Mutant no. 284 is deficient in growth in liquid media; although it will form colonies in the usual 6 days on TY, YM, and minimal plates, it will not grow at all in TY broth and only very slowly in YM broth; no. 284 takes about 7 days to saturate 5 ml of YM broth after the inoculation of a single colony, compared with about 3 days for NC92 and the other Tn*5*-containing mutants.

Nitrogen fixation (Fix^-) mutants of NC92. The usual criterion used to identify Fix^- *Rhizobium* mutants is the acetylene reduction assay. Mutants which induce nodules but are unable to reduce acetylene in symbiotic association with their host plant are deemed to be Nod^+ Fix^- (49). However, as described below, several of the mutants isolated in this study formed symbiotic associations in which the host plant clearly received little or no symbiotically fixed nitrogen, yet acetylene reduction assays consistently gave positive results. Therefore, in this study, the criterion used to assign a Fix^- phenotype was the relative amount of nitrogen fixed. This was calculated as described in Materials and Methods by comparing the level of combined nitrogen present in plants nodulated by a particular mutant with that present in plants nodulated by the wild-type strain, NC92 (100% relative nitrogen fixation), and by the two mutants, no. 639 and 807, that showed no detectable acetylene reduction activity (0% relative nitrogen fixation). Uninoculated control plants were not used as the basis of any comparisons in the following discussions since, in contrast to previously inoculated pots, they became contaminated with other rhizobia (see below). In the following discussions we have designated the Fix phenotype of strains on the basis of their relative nitrogen fixation as follows: less than 25%, Fix^-; between 25% and 75%, $Fix^\pm$; and above 75% (or not statistically significantly different from NC92), Fix^+. Acetylene reduction activity, in contrast, is treated as a secondary property of Fix^- mutants (Ara phenotype). Since there can be a twofold variation in acetylene reduction activity from plant to plant, and since the data were not normalized to nodule weight, we have only interpreted these data qualitatively; we define positive acetylene reduction activity (Ara^+) as being an average over all experiments of greater than 30% of wild-type NC92 activity and negative activity (Ara^-) as being less than 3% of wild-type activity. Actual mean values for wild-type NC92 ranged from 4 to 23, 1 to 8, and 2 to 10 μmol of C_2H_2 per plant per h for groundnut, pigeonpea, and siratro, respectively. The data on shoot weight and combined nitrogen presented in Table 2 were gathered during a single-pot trial conducted at MGH. The qualitative acetylene reduction activities and the inferred symbiotic phenotypes described below and summarized in Table 3 are based on this trial and on replicative trials conducted at ICRISAT and at MGH.

Three mutants formed Fix^- symbioses with all three host plants tested. The nodules induced by two of these mutants, no. 639 and 807, also failed to reduce acetylene. However, we found that nodules induced by strain no. 302 showed levels of acetylene reduction activity comparable to those of nodules induced by wild-type NC92 on all three host plants (an average of 140, 50, and 78% of wild-type activity on groundnut, pigeonpea, and siratro, respectively), even though the relative amount of nitrogen fixed was less than 15% (Table 2).

Three mutants, no. 831, 853, and 868, showed a partially effective phenotype on all three host plants; the relative amount of nitrogen fixed fell between 43 and 64% of that fixed by wild-type NC92. Plants of all three host species inoculated with these strains remained pale green for 5 to 10 days after NC92-inoculated plants became dark green. The plants did subsequently become dark green and showed wild-type levels of acetylene reduction activity at the end of the trial, although, as other results in this paper demonstrate, this does not necessarily indicate that dinitrogen gas is being fixed at wild-type levels.

Possible explanations for these partially effective phenotypes include delayed nodulation, delayed onset of nitrogen fixation, reduced efficiency of nitrogenase for dinitrogen reduction, or a lowered bacteroid concentration. These first two possibilities were investigated further by using siratro plants grown in seedling agar tubes so that the onset of nodulation could easily be monitored, and repeated, nondestructive acetylene reduction activity assays could be carried out. In two independent trials, with 10 tubes (1 plant per tube) per treatment, neither mutant no. 853 nor mutant no. 868 showed any significant delay in the onset of nodulation or of nitrogen fixation as determined by acetylene reduction activity, on siratro. In contrast, mutant no. 831 showed a 4- to 7-day delay in nodulation and a possible further delay of several days in the onset of nitrogen fixation.

The remaining three mutants show host specific nitrogen fixation phenotypes. Mutants no. 284 and 979 are Nod^+ Fix^- on pigeonpea, resulting in pale green plants with 6 and 11% relative amounts of fixed nitrogen, respectively (Table 2). In contrast, these two strains are partially effective (40 to 70% relative fixed nitrogen) on siratro and on groundnut. Acetylene reduction activity by both strains was detectable on all three hosts (an average of 31 to 185% of the wild type). Mutant no. 727 exhibits a different host-specific phenotype. It is Nod^+ Fix^+ on pigeonpea and siratro (on siratro its relative nitrogen fixation was only 74%, but this was not statistically different from the wild-type control) but clearly Nod^+ Fix^- on groundnut. However, mutant no. 727 does exhibit considerable acetylene reduction activity in symbiosis with groundnut (an average of 60% of wild-type levels.)

Nodulation (Nod^-) mutants of NC92. Two NC92 nodulation mutants were identified. Mutant no. 748 was initially identified as being Nod^+ Fix^- on siratro. We subsequently found that this mutant does not elicit any nodules on the roots of pigeonpea, whereas it elicits fully effective, nitrogen-fixing nodules on groundnut. These three distinct host-specific symbiotic phenotypes of mutant no. 748 have been confirmed in several experiments.

The discovery that a single mutant could exhibit three different host-specific symbiotic phenotypes was unex-

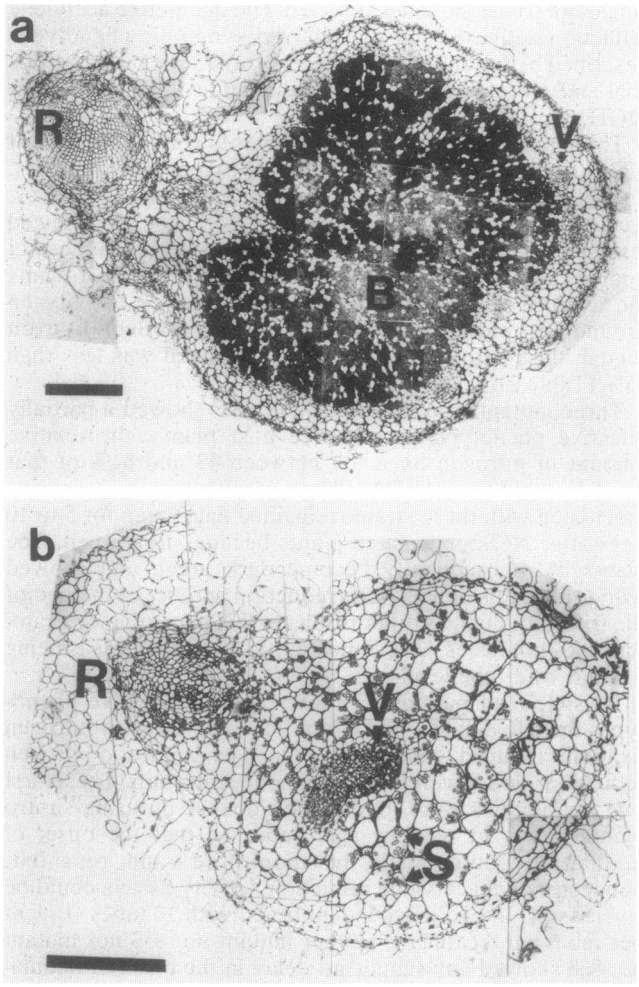

FIG. 1. Horizontal section through 3-week-old siratro root nodules induced (a) by the wild-type strain, NC92, and (b) by mutant no. 748. Both micrographs show the position of the attached root (R). (a) Vascular bundles (V) are peripheral, surrounding the central bacteroid-containing zone (B). (b) No bacterial infection is observed; the vasculature (V) is centrally located, and large, uninfected cortical cells contain prominent starch grains. Bar, 250 μm.

pected. We hypothesized that strain no. 748 might be defective in some function which is required for the root hair (pigeonpea and siratro [37, 57]) but not for the crack entry (groundnut [17]) mode of infection. Therefore, ineffective, mutant no. 748-induced siratro nodules were plastic embedded and sectioned for light microscopy to examine the nodule structure more closely. There were no darkly staining bacteroid packed cells, and these "empty" nodules had central (occasionally branched) vascular bundles, rather than the peripheral distribution of vascular bundles observed in wild-type siratro nodules (Fig. 1) (55). Occasional dark-staining bodies were observed in peripheral cortical cells, which could indicate limited bacterial invasion and would explain why it was possible to isolate some bacteria from these nodules. Mutant no. 748 does induce normal root hair curling and the formation of typical "shepherd's crooks" on siratro, a characteristic first step in the root hair infection pathway. This is consistent with no. 748 having a mutation in a nodulation function other than the common nodulation genes (*nodABCD*) (40).

Mutant no. 21 does not induce nodules on any of the three host plants tested. This result has been repeated in seedling agar tubes (siratro) and in growth pouch (pigeonpea) experiments at MGH as well as in a pot trial at ICRISAT (groundnut). In contrast to mutant no. 748, no. 21 does not induce root hair curling on siratro. These results suggested that mutant no. 21 might have a lesion in one of the so-called common nodulation genes (*nodABCD*), which are both structurally and functionally conserved in a number of *Rhizobium* and *Bradyrhizobium* species and appear to be essential for the induction of nodules on all *Rhizobium* host plants, including the nonlegume *Parasponia* (46a). Therefore we tried to genetically complement mutant no. 21 with plasmid pRmSL26, which carries the common nodulation genes cloned from *Rhizobium meliloti* (43). Plasmid pRmSL26 was introduced into mutant no. 21 by bacterial conjugation, and the resultant transconjugants were inoculated onto siratro. They were able to elicit nodules with high acetylene reduction activity on siratro, whereas no nodules formed on no. 21-inoculated plants. Minocycline-resistant colonies were isolated from these nodules (minocycline is a tetracycline analog which was found to have greater efficacy than tetracycline in selecting against wild-type NC92) and were able to elicit nodules on both siratro and pigeonpea. Southern blot analysis confirmed that these isolates did contain pRmSL26 sequences and were not revertants of the original mutant (data not shown). Complementation of mutant no. 21 has not been tested on groundnut.

Since mutant no. 748, which was originally identified as being Fix^- ARA^- on siratro, induced uninvaded siratro nodules, siratro nodules induced by the two non host-specific, Fix^- ARA^- mutants, no. 639 and 807, were also sectioned and examined under the light microscope. In both cases the nodules contained bacteria-filled cells, showing that these two mutants are not infection mutants on siratro. Mutant no. 639-induced nodules were indistinguishable from wild type-induced nodules; in contrast, mutant no. 807-induced nodules contained many fewer infected cells in the central, bacteroid-containing zone, and the uninfected cells showed the accumulation of large starch grains that is characteristic of ineffective nodules (data not shown).

Detection of potential cross-contaminants in the pot trials. The major drawback of testing symbiotic mutants in open pot trials is the possibility of cross-contamination by strains from adjacent pots. Indeed, nodules were observed on the secondary roots of uninoculated control plants. However, several arguments can be advanced to suggest that cross-contamination did not occur in inoculated pots. First, nodules did not form on the roots of plants inoculated with non-nondulating mutants of NC92 (no. 21, and no. 748 on pigeonpea), suggesting that an existing rhizobial population in the growth medium competes successfully with and prevents establishment of contaminating rhizobia. Second, in the trial conducted at MGH (Table 2), we isolated bacteria from nodules induced on one siratro and one pigeonpea plant for each treatment. All of the nodules from each treatment, including the later-formed nodules on the secondary roots, were pooled, and bacteria were isolated. These isolations were first streaked onto Congo red-containing YM plates and subsequently replica plated onto Congo red YM kanamycin plates. In all cases, all bacteria isolated were Km^r, indicating that no reversion of the mutants had occurred by excision of Tn*5*. However, there still remained the possibility of cross-contamination by the other Km^r strains. Therefore genomic DNA was prepared from nodule isolates from plants inoculated with mutants no. 284, 727, 831, and 979 as well as from

isolates from uninoculated plants. This DNA was digested with *Eco*RI, run on a 0.6% agarose gel, transferred to GeneScreen filters, and hybridized with ^{32}P-labeled pGS9 DNA. This analysis showed first that all nodules were formed by NC92 and its derivatives (as judged by *Eco*RI genomic fingerprints) and second that each isolate showed the pattern of pGS9 hybridization expected of the parent inoculum; that is, strains no. 284 and 727 showed a single hybridizing band of 18 and 9 kb, respectively, whereas no. 831 and 979, despite being Km^r, did not contain any pGS9-hybridizing bands at all. A single hybridizing band could be detected in each of the uninoculated controls. These lines of evidence, taken together, argue strongly against the possibility of cross-contamination of strains in pots with an inoculated *Bradyrhizobium* strain and therefore uphold the validity of the data presented in Table 2.

DISCUSSION

We used the suicide vector pGS9 to carry out random Tn*5* mutagenesis of a slow-growing *Bradyrhizobium* strain of the cowpea cross-inoculation group, and we successfully isolated nodulation and nitrogen fixation mutants at frequencies comparable to those obtained by other workers (16, 49). However, physical analysis of the mutants revealed two classes of mutants that do not contain simple Tn*5* insertions. Of the 11 NC92 mutants, two (no. 21 and 748) contain additional pGS9 vector sequences, and two (no. 831 and 979) are spontaneous kanamycin-resistant mutants. The remaining seven mutants do contain single Tn*5* insertions. However, since we were only able to correlate the phenotype of one of the mutants (no. 21) with an insertion in a known symbiotic locus (*nodABCD*), further analysis is required to demonstrate conclusively that the observed symbiotic phenotypes are caused by the Tn*5* insertions.

The frequency of occurrence of spontaneous kanamycin-resistant mutants among the NC92 symbiotic mutants was surprising. We therefore examined the phenomenon further and found that there is a high background of isolation of spontaneous mutants (1 mutant in 10^5 to 10^6 kanamycin-susceptible cells, comparable to the frequency of isolation of kanamycin-resistant colonies after pGS9 mutagenesis) and a strong correlation between acquired kanamycin resistance and symbiotic defectiveness in strain NC92. Correlations between spontaneous antibiotic resistance and reduced symbiotic effectiveness have previously been reported (52, 53), although Pankhurst (53) found that spontaneous kanamycin resistance reduced the symbiotic effectiveness of *Rhizobium* but not *Bradyrhizobium* strains. It is possible that the acquired resistance results from alterations in cell wall permeability and that these changes also disrupt bacterial communication with the plant partner. Alternatively, the observed symbiotic phenotypes could result from transposition of an endogenous insertion element, as was found in *R. meliloti* (61), although this hypothesis offers no explanation for the correlated acquisition of kanamycin resistance. Such spontaneous kanamycin-resistant mutants could be avoided if it were possible to select for the presence of Tn*5* by coselection with kanamycin and streptomycin, thus exploiting the additional Tn*5*-encoded streptomycin resistance gene which is expressed in *Rhizobium* species (22, 56, 58, 65). Unfortunately, the endogenous streptomycin resistance of strain NC92 is too high (resistant to >500 μg/ml) to make this approach feasible, and this may be a common problem with other *Bradyrhizobium* cowpea group strains (38).

The symbiotic mutants which we have isolated exhibit several novel phenotypes. Among the Fix$^-$ mutants, only two mutants (no. 639 and 807) exhibit a conventional Fix$^-$ phenotype; the inability of these two mutants to supply their plant hosts with fixed nitrogen is attributable to the absence of active nitrogenase enzyme, as demonstrated by the failure of nodules elicited by these mutants to reduce acetylene. Light microscopic examination of no. 639- and 807-elicited siratro nodules did not reveal any obvious defects in bacterial invasion. It was therefore particularly surprising that even these two mutants did not appear to contain insertions in the nitrogenase structural genes, *nifHDK*, or in any other known symbiotic genes, and that we did not isolate any such mutants.

Three mutants (no. 831, 853, 868) show partially effective phenotypes on all three host plants. One of these (no. 831, which is one of the spontaneous kanamycin-resistant mutants) exhibits a small nodulation delay in siratro. If nodulation were also delayed on the other two host plants, this could perhaps account for the reduced effectiveness of this strain. The reason for the reduced effectiveness of the other two strains is not known.

Another mutant (no. 302) is clearly Fix$^-$ on all three host plants and yet exhibits considerable acetylene reduction activity, demonstrating the presence of nitrogenase enzyme with at least partial activity. The only well-characterized precedent for such a phenotype lies with the free-living, nitrogen-fixing species *Klebsiella pneumoniae*; *K. pneumoniae* strains with a mutation at the *nifV* locus fail to process the nitrogenase iron-molybdenum cofactor correctly, producing an enzyme which is able to reduce acetylene but not nitrogen (31, 48). A consequence of this incomplete processing is that the acetylene reduction activity is not susceptible to competitive inhibition by dinitrogen, unlike the wild-type enzyme. Dinitrogen inhibition of acetylene reduction activity has not been examined in the present mutants. Moreover, no *nifV*-like gene has yet been identified in any *Rhizobium* or *Bradyrhizobium* species, and so the possibility that mutant no. 302 is defective in a *nifV*-like gene could not be ruled out by DNA hybridization. There are also some reports of *B. japonicum* strains with similar phenotypes; soybean (*Glycine max*) plants inoculated with these strains are yellow and nitrogen starved, but the nodules exhibit considerable acetylene reduction activity. One such strain contains an engineered deletion in a gene that is homologous to the *K. pneumoniae nifS* gene, a gene encoding a factor involved in molybdenum cofactor processing (32a; T. Adams and B. K. Chelm, personal communication). However, Southern blot analysis ruled out the possibility that any of the NC92 mutants contained insertions in an NC92 *nifS*-homologous gene. Another ineffective *B. japonicum* mutant which retains acetylene reduction activity has been reported (75). This mutant lacks glutamate-oxaloacetate amino transferase and may be defective in transferring fixed nitrogen to the plant. Thus, possible explanations for this phenotype (Fix$^-$ Ara$^+$) include defects in enzyme processing or in molecular transport.

The remaining three NC92 Fix$^-$ mutants are Fix$^-$ on only a subset of the host plants tested. These mutants are no. 284 and 979, which are Fix$^-$ on pigeonpea but Fix$^\pm$ on siratro and groundnut, and no. 727, which is Fix$^-$ on groundnut but Fix$^+$ on siratro and pigeonpea. In each case it does not seem possible to attribute the Fix$^-$ or Fix$^\pm$ phenotype directly to reduced nitrogenase activity, since each mutant exhibited an average of at least 31% of wild-type NC92 acetylene reduction activity, even on the host plants that received less than

25% relative fixed nitrogen. It is curious that mutants no. 284 and 979 both show such similar phenotypes when no. 979 lacks any Tn*5* insertion at all. The one difference between no. 284 and 979 is that only no. 284 shows impairment of growth in liquid media.

Such bacterial host specific fixation mutants, which we term Hsf mutants by analogy to Hsn (host specific nodulation) mutants (40), have not previously been reported. This may simply be because *Rhizobium* mutants are not usually screened for symbiotic effectiveness on more than one host plant. The phenomenon of host-specific fixation is well known among natural isolates of both *Rhizobium* and *Bradyrhizobium* species (27), and Appelbaum et al. were recently able to transfer cultivar-specific nitrogen fixation ability between strains of *Rhizobium fredii*, the fast-growing symbiont of soybean, by transferring the indigenous symbiotic plasmids (1). Moreover, plant mutants that exhibit altered strain specificity for the formation of effective nodules have been isolated (12). We have not tested the NC92 mutants on sufficient cultivars to determine whether the Hsf phenotypes lie at the host cultivar or species level.

It is possible that host-specific fixation is similar to host-specific nodulation in that a specific negative interaction occurs at an early stage in the nodulation pathway, but that the barrier lies in nodule invasion rather than nodule induction. However, light microscopic examination of ineffective siratro nodules induced by a *B. japonicum* strain, USDA 122, which forms a fully effective symbiosis with soybean, revealed that these siratro nodules are indeed invaded by strain USDA 122 (K. Wilson, unpublished results). Thus, the host-specific failure to fix nitrogen in this case must be due to some postinfection breakdown in the interaction between the plant and the bacterium. This is almost certainly also true for the NC92 mutants, since each exhibits significant nodule acetylene reduction activity (in contrast to the siratro nodules induced by USDA 122). Such Hsf defects could lie in specific signaling between the symbiotic partners or in some aspect of nodule physiology. For example, the pathway of infection (17) and the structure of the bacteroids (66) are both very unusual in groundnut, and may require unique capabilities in the bacterium which are absent in mutant no. 727. Another possibility is that different host plants may supply different carbon substrates to the bacteroids, and these NC92 mutants might be deficient in the utilization of host-specific carbon sources. It has also been shown that a reduction in the levels of exported, fixed nitrogen can lead to a failure by the plant to induce the enzymes necessary for nitrogen assimilation and to early senescence of the nodules (2, 3), and this effect might be greater in certain host species than in others. We hope that these three bacterial Hsf mutants may enable us to examine these and other possibilities and to specifically identify some of these additional steps which exist between the initial induction of a root nodule and the final establishment and maintenance of a fully effective, nitrogen-fixing symbiosis.

The identification of such a high proportion of Fix^- Ara^+ mutants in strain NC92 may be a consequence of the different modes of regulation of *nif* genes between fast- and slow-growing rhizobia. It has not been possible to demonstrate ex planta acetylene reduction activity in fast-growing *Rhizobium* strains, whereas such ex planta activity is well documented in *Bradyrhizobium* strains (42, 47, 54). Therefore it could be speculated that fast-growing *Rhizobium* species might exhibit more stringent regulatory mechanisms which prevent the expression of nitrogenase when any of a number of symbiotic genes are mutated, whereas analogous defects in the symbiotic genes of *Bradyrhizobium* strains would not shut down the induction of nitrogenase.

In addition to these nine Fix mutants we describe the isolation of two Nod^- mutants. Mutant no. 21 fails to elicit nodules on all three host plants. Genetic complementation and DNA hybridization studies indicate that it carries a mutation in the common nodulation genes. The fact that mutant no. 21 fails to elicit nodules on groundnut, a crack entry host, as well as on pigeonpea and siratro, which are both infected via the root hairs, confirms the findings of others (7, 46a, 51) that common *nod* genes are required for both modes of infection, and therefore that they are not simply genes for the induction of root hair curling or infection thread formation.

The second nodulation mutant, no. 748, is a host-specific nodulation mutant. It is now known that both fast- and slow-growing rhizobia which exhibit a broad host range carry separate recognition factors for at least some of their hosts; certain genes have been identified by the transfer of partial host range to other *Rhizobium* species (5, 6, 11), and a mutant *Bradyrhizobium* sp. (*Parasponia*) strain has been constructed that fails to elicit nodules on the nonlegume host *Parasponia* sp. but is still able to elicit Fix^+ nodules on two legume hosts, siratro and cowpea (*Vigna unguiculata*) (46a). Mutant no. 748 is particularly interesting because it exhibits a different mutant phenotype on each of three host plants. On pigeonpea it fails to induce any nodules at all, suggesting that it may lack a specific "pigeonpea factor." It does induce nodules on siratro, but the nodules are devoid of bacteria and have abnormal, centralized vasculature. *Rhizobium* mutants which induce such empty nodules have recently been identified in the fast-growing *Rhizobium* species (24, 26, 71), and mutant no. 748 demonstrates that the processes of nodule induction and nodule invasion are also separable in the slow-growing *Bradyrhizobium* species. The structure of these empty siratro nodules resembles that of *Phaseolus vulgaris* (common bean) nodules induced by noninfective mutants of *Rhizobium phaseoli* (71) and may therefore be characteristic of uninvaded, determinate nodules. On groundnut, the crack entry host, no. 748 shows no defect in nodulation or nitrogen fixation. Thus, actual infection by mutant no. 748 may be limited to crack entry hosts, making no. 748 a root hair infection mutant, although it is clear that infection by no. 748 should be tested on other hosts, particularly on the crack-entry *Stylosanthes* species (18), before any definite conclusions can be drawn. This hypothesis does not fully explain the difference in the interaction of no. 748 with the two root hair infection hosts, siratro and pigeonpea. One possibility is that siratro and pigeonpea have very different sensitivities for nodulation factors, as demonstrated by Bauer et al. (8) for soybean and cowpea, such that the absence of a certain factor affects the two hosts differentially. It is also not yet certain whether these different phenotypes are attributable to a single or to multiple mutations in mutant no. 748. However, this mutant does clearly demonstrate that different host plants do have different qualitative or quantitative requirements for nodulation factors.

In this report we have demonstrated the utility of random transposon mutagenesis followed by careful screening in the isolation of novel symbiotic *Bradyrhizobium* mutants. Several of these mutants do not have a direct phenotypic counterpart among known symbiotic mutants of the fast-growing *Rhizobium* species, and only one (no. 21) was shown to carry an insertion in a DNA sequence that shares homology with a known *Rhizobium* symbiotic gene. Further

analysis of these mutants may help to elucidate the genetic basis for some of the symbiotic differences between fast- and slow-growing *Rhizobium* species.

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