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Nitrogen-Fixing Nodules with *Ensifer adhaerens* Harboring *Rhizobium tropici* Symbiotic Plasmids

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***Ensifer adhaerens* is a soil bacterium that attaches to other bacteria and may cause lysis of these other bacteria. Based on the sequence of its small-subunit rRNA gene, *E. adhaerens* is related to *Sinorhizobium* spp. *E. adhaerens* ATCC 33499 did not nodulate *Phaseolus vulgaris* (bean) or *Leucaena leucocephala*, but with symbiotic plasmids from *Rhizobium tropici* CFN299 it formed nitrogen-fixing nodules on both hosts. The nodule isolates were identified as *E. adhaerens* isolates by growth on selective media.**

Rhizobia (*Sinorhizobium, Allorhizobium, Mesorhizobium, Bradyrhizobium, Rhizobium*, and *Azorhizobium*) form nitrogen-fixing nodules on the roots and stems of legumes. The genetic information for symbiosis is plasmid borne in *Rhizobium* and *Sinorhizobium*. Symbiotic plasmids may be eliminated, rendering the bacteria nonsymbiotic. Nonsymbiotic rhizobia exist naturally and can be more numerous than their symbiotic counterparts (16, 27).

Ensifer adhaerens strains are gram-negative soil bacteria that attach endwise to various living gram-positive and gram-negative bacteria and may cause lysis of these bacteria. *E. adhaerens* is a participant in a predatory chain involving other bacteria; however, it is an obligate predator only under nutrient limitation conditions. Its 16S rRNA gene sequence places *E. adhaerens* in the sinorhizobia (1).

Phaseolus vulgaris (bean), *Vigna*, and *Macroptilium* have been reported to be highly promiscuous hosts and are nodulated with a large range of rhizobia (18, 22, 25). We found that *E. adhaerens* ATCC 33499 did not form nodules on bean, *Leucaena leucocephala, Vigna mungo, Macroptilium atropurpureum*, or alfalfa when 10 plants of each species were grown in flasks with cotton, vermiculite, or agar as the support, as previously described (20). Thus, we wondered if *E. adhaerens* could become a bean and *Leucaena* nitrogen-fixing symbiont by acquiring symbiotic plasmids from a *Rhizobium* species. We chose *Rhizobium tropici* as the donor because *R. tropici* sym plasmids conferred on *Agrobacterium tumefaciens* the capacity to form nitrogen-fixing nodules on *P. vulgaris* and *L. leucocephala* (17).

R. tropici CFN299 and *E. adhaerens* ATCC 33499 are easily distinguishable by colony morphology on PY agar (5 g of peptone per liter, 3 g of yeast extract per liter, 0.7 g of calcium chloride per liter, 1.5% agar) plates; *E. adhaerens* produces larger amounts of slime and forms colonies faster than *R. tropici*. CFN299 does not grow in Luria broth (LB), while strain ATCC 33499 does. *E. adhaerens* ATCC 33499 is also resistant to 5 mg of gentamicin per liter, 100 mg of streptomycin per liter, 5 mg of chloramphenicol per liter, and 300 mg of erythromycin per liter, while *R. tropici* CFN299 is sensitive to all of these antibiotics.

No *nifH* genes were detected in *Ensifer* either by Southern blot hybridization or by PCR performed with *nifH* primers (6) (Table 1). Additionally, no *nod* gene products were obtained with *E. adhaerens* ATCC 33499 in a PCR with *nodC* primers 251F and 566R (28) or with *nodBC* primers (*nodB* 31 [TACC TGACSTTVGACGACGGTCC] and *nodC* RR [GAGACG GCGRCRRTGCTGGTTG]) that we have used to amplify *nodBC* or *nodC* gene sequences from *Sinorhizobium meliloti, Sinorhizobium medicae, Sinorhizobium arboris, Sinorhizobium terangae, Sinorhizobium kostiense, Sinorhizobium saheli, Sinorhizobium fredii, R. tropici*, and *Rhizobium etli*. The nucleotide sequences of the PCR products obtained with *R. etli* strains were determined and corresponded to the *nodBC* gene sequences (Claudia Silva, personal communication). No hybridization was obtained when the *S. meliloti nodC* PCR product was used as a probe in Southern blot hybridization with *E. adhaerens* ATCC 33499 total restricted DNA.

R. tropici CFN299 Tn*5-mob*-6 and CFN299 Tn*5-mob*-7 were obtained by mating CFN299 and S17-1(pSUP5011) and were selected on the basis of their ability to transfer to *Agrobacterium tumefaciens* GMI9023 the capacity to form nodules on bean as previously described (17). *R. tropici* CFN299 Tn*5-mob*-6 and CFN299 Tn*5-mob*-7 were able to form nitrogen-fixing nodules when they were tested individually with bean plants (Table 1). *R. tropici* CFN299 Tn*5-mob*-6 and CFN299 Tn*5-mob*-7 were shown to have Tn*5-mob* in the *nod-nif* plasmid by hybridization of Eckhardt gels with Tn*5* (data not shown).

E. adhaerens transconjugants obtained from matings on PY agar plates with *R. tropici* CFN299 Tn*5-mob*-6 and CFN299 Tn*5-mob*-7 were selected on LB containing 200 mg of neomycin per liter because *E. adhaerens* grows on LB containing 100 mg of neomycin per liter. Transconjugants grew in the presence of up to 800 mg of neomycin per liter, while the recipient *E. adhaerens* ATCC 33499 strain was sensitive to neomycin

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TABLE 1. Strains and characteristics

Strain[a]	Small-subunit rRNA patterns[b]	*R. tropici* plasmids[c]	*nifH* gene[d]	Acetylene reduction activity with *P. vulgaris* nodules (nmol of ethylene/h/plant)[e]	
				14 days postinoculation	18 days postinoculation
E. adhaerens transconjugants					
CFNEA40	ACEGIK	a, c[f]	+	102.7	ND[g]
CFNEA41	ACEGIK	a, b, c	+	104.8	ND
CFNEA50	ACEGIK	a, c	+	108.0	84.9
CFNEA51	ACEGIK	a, c	+	106.9	85.9
CFNEA52	ACEGIK	a, b, c[f]	+	103.8	84.9
CFNEA53	ACEGIK	a, b, c	+	115.3	96.4
CFNEA54	ACEGIK	b, c	+	113.2	94.3
CFNEA55	ACEGIK	b, c	+	126.9	104.8
CFNEA56	ACEGIK	b, c	+	114.3	94.3
E. adhaerens ATCC 33499	ACEGIK				
R. tropici strains					
CFN299	BDFHJL		+		
CFN299 Tn*5*-*mob*-6					166.8
CFN299 Tn*5*-*mob*-7					182.4

[a] Only transconjugant CFNEA41 was derived from CFN299 Tn*5*-*mob*-7; all other transconjugants were derived from CFN299 Tn*5*-*mob*-6. *R. etli* CFN42 was also used in this study.

[b] The different letters represent distinct patterns obtained with restriction enzymes *Hinf*I, *Msp*I, *Rsa*I, *Hha*I, *Sau*3A1, and *Dde*I; each letter position refers to a different restriction enzyme used for PCR-synthesized 16S rRNA genes (15) obtained with primers rD1 and fD1 (29).

[c] Eckhardt gel analysis was performed as modified (23) with liquid early-exponential-phase cultures in horizontal gels with sodium dodecyl sulfate in the agarose. The plasmid sizes were as follows: plasmid a, 185 kb; plasmid b, 220 kb; and plasmid c, 500 kb (*nod* and *nif* plasmid).

[d] The presence of *nifH* was determined by Southern blot hybridization or by PCR synthesis with *nif* primers as described previously (6).

[e] Acetylene reduction activity was determined 14 and 18 days postinoculation. The data are averages for five plants from one experiment. The plant-to-plant variation was not more than 10% for each transconjugant. The levels of nitrogen fixation with the *E. adhaerens* transconjugants were around 40% of the levels with the parental CFN299 strains in the four other experiments. Plants were maintained in a growth chamber at 28°C.

[f] Plasmid cointegration or rearrangement occurred, which led to an approximately 700-kb plasmid or to an additional 300-kb plasmid.

[g] ND, not determined.

at concentrations greater than 100 mg per liter. Transconjugants CFNEA40 and CFNEA50 (from *R. tropici* CFN299 Tn*5*-*mob*-6) and CFNEA41 (from *R. tropici* CFN299 Tn*5*-*mob*-7) were selected.

Additionally, a mixture of *E. adhaerens* transconjugants derived from CFN299 Tn*5*-*mob*-6 and CFN299 Tn*5*-*mob*-7 was inoculated onto plants, and transconjugants CFNEA51 to CFNEA56 were selected from well-developed red nodules as follows. All bacteria isolated from bean nodules were recovered on PY medium, and 10 individual colonies per nodule were then tested for growth in LB containing 200 mg of neomycin per liter. All isolates on PY agar had the colony morphology of *E. adhaerens* ATCC 33499, not the colony morphology of *R. tropici* CFN299. All of the isolated colonies tested grew in LB containing 200 mg of neomycin per liter. One isolated colony from a nodule from each of six different plants was purified further by five serial steps involving colony isolation on LB containing 200 mg of neomycin per liter. The parental strain of transconjugants CFNEA51 to CFNEA56 was recognized on the basis of the size of the band hybridizing to a Tn*5* probe in each *Ensifer* transconjugant. The transconjugants were all derived from CFN299 Tn*5*-*mob*-6, perhaps indicating that the Tn*5*-*mob*-7 insertion had affected some loci involved in competition for nodule formation. The gene interrupted by Tn*5*-*mob*-7 will be described elsewhere.

E. adhaerens ATCC 33499 harbors two megaplasmids, as revealed by the Eckhardt procedure. *Ensifer* transconjugants acquired two or three plasmids from the *R. tropici* donor strain (Table 1; Fig. 1). *nif* genes were detected in the *Ensifer* transconjugants by using *nifH* primers in a PCR (Table 1), and the total DNA restriction fingerprints of all transconjugants were identical to the *E. adhaerens* ATCC 33499 fingerprint (data not

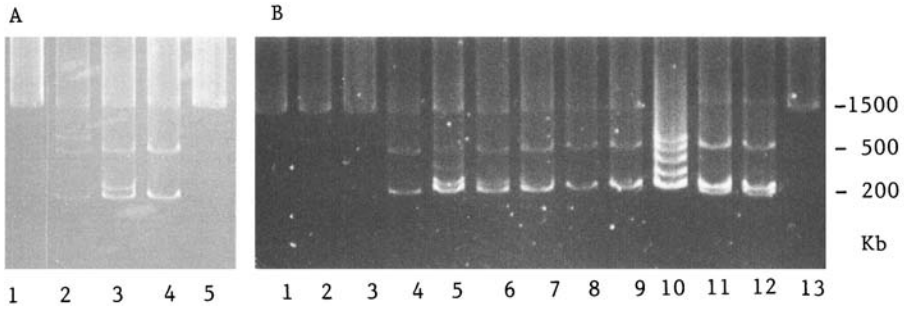

FIG. 1. Plasmids visualized by a modified Eckhardt procedure. (A) Lanes 1 and 5, *E. adhaerens* ATCC 33499; lane 2, CFNEA40; lane 3, CFNEA41; lane 4, CFNEA50. (B) Lanes 1 to 3 and 13, *E. adhaerens* ATCC 33499; lane 4, CFNEA51; lane 5, CFNEA52; lane 6, CFNEA53; lane 7, CFNEA54; lane 8, CFNEA55; lane 9, CFNEA56; lane 10, CFN42; lane 11, CFN299 Tn*5*-*mob*-6; lane 12, CFN299 Tn*5*-*mob*-7.

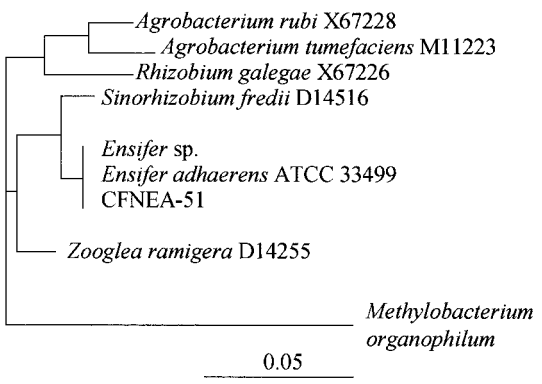

FIG. 2. Phylogenetic tree based on the 16S rRNA gene sequence.

shown). Ribosomal fingerprints (15) were determined by 16S rRNA gene restriction enzyme digestion with *Hinf*I, *Msp*I, *Rsa*I, *Hha*I, *Sau*3A1, and *Dde*I of PCR products generated with primers fD1 and rD1 (29) from all *E. adhaerens* transconjugants (Table 1), and the 16S rRNA gene sequence of *E. adhaerens* transconjugant CFNEA51 was determined (2). Almost the entire 16S rRNA gene was sequenced with an automated sequencer. The resulting sequence was hand aligned with selected comparison sequences, taking into consideration the secondary structure of the 16S rRNA molecule. The aligned sequences were then analyzed by the distance matrix method by using the FITCH option of the PHYLIP program (7). Distances were corrected by the method of Jukes and Cantor (14). Phylogenetic analysis of *E. adhaerens* transconjugant CFNEA51 (Fig. 2) indicated that the 16S rRNA gene sequence was identical to the original *E. adhaerens* ATCC 33499 sequence and the sequence of another strain of *E. adhaerens* included for comparison. As reported previously (1), the *Ensifer* strains were most closely related to *Sinorhizobium* spp.

Since it has been found that *E. adhaerens* sticks very tightly to other bacteria and that separation from these bacteria is difficult (1, 4), great effort was expended to purify *E. adhaerens* transconjugants prior to inoculation of plants. The procedure used to purify all *E. adhaerens* transconjugants included several steps consisting of dilution with Tween 40 and plating on LB containing 200 mg of neomycin per liter for single-colony isolation before the transconjugants were tested in plant nodulation assays to determine levels of nitrogen fixation.

E. adhaerens transconjugants (Table 1) were found to form nitrogen-fixing nodules in five independent experiments with bean (three to five plants per strain were tested in each experiment). CFNEA50 to CFNEA56 also formed nitrogen-fixing nodules on *L. leucocephala* plants, which were green (Fig. 3), while all uninoculated control plants lacked nodules and were yellow. *Leucaena* plant development indicated that nitrogen was transferred to the plants. The identities of the strains in all bean and *L. leucocephala* nodules were verified by growing colonies isolated from nodules on LB containing 200 mg of neomycin per liter, and isolates from the more than 800 nodules tested corresponded to the *Ensifer* transconjugants. Tests for nodule surface sterility were performed for all nodules as described previously (17).

Twenty nodules recovered from different nitrogen-fixing plants inoculated with CFNEA51 and CFNEA53 were surface sterilized and individually macerated, and 1 drop of each preparation was placed on PY agar and analyzed for resistance to antibiotics as described above. The remainder of the nodule extract was used for PCR with *nifH* primers (6) or with *R. tropici* citrate synthase gene (11) primers P231p (AAGAA GCCCATTTGCTTCC) and P2318 (TTAACCCTTTGGCGC TTTTT), which yielded a 624-bp product. While PCR products were obtained with *nifH* primers from nodules formed by either *R. tropici* or *E. adhaerens* transconjugants, PCR products were obtained with citrate synthase gene primers from *R. trop-*

FIG. 3. *Leucaena* plants (45 days old) inoculated with *R. tropici* CFN299 (plant 1), *E. adhaerens* transconjugant CFNEA51 (plant 2), and *E. adhaerens* ATCC 33499 (plant 3). Plant 4 was an uninoculated control plant.

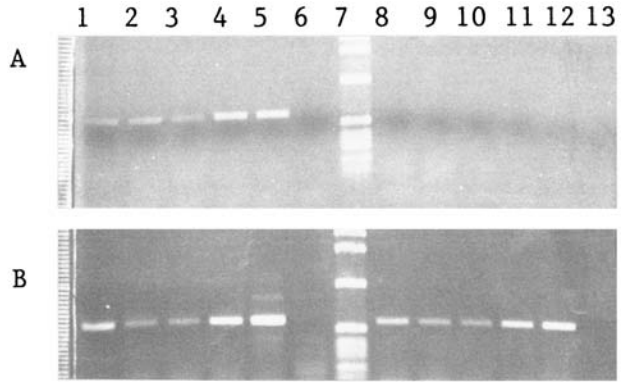

FIG. 4. PCR products obtained directly from nodule extracts. Each surface-sterilized nodule was squeezed in 0.1 ml of sterilized water and extracted twice with phenol-chloroform-isoamyl alcohol and once with chloroform; the resulting preparation was precipitated with ethanol and resuspended in 15 μl of H_2O, and 5-μl aliquots were used either with *R. tropici* citrate synthase gene primers (see text) (A) or with *nifH* gene primers (6) (B). Lanes 1 to 4, nodules formed by *R. tropici* strain CFN299; lane 5, *R. tropici* CFN299 DNA; lane 6, no-DNA control; lane 7, 1-kb DNA ladder marker; lanes 8 to 12, nodules from *E. adhaerens* transconjugants (lanes 8 and 9, CFNEA51; lane 10, CFNEA53; lanes 11 and 12, CFNEA51), lane 13, *E. adhaerens* ATCC 33499 DNA.

ici nodules but not from *E. adhaerens* CFNEA51 or CFNEA53 nodule extracts or *E. adhaerens* ATCC 33499 purified DNA (Fig. 4). Citrate synthase gene PCR products were digested with *Msp*I which yielded the expected digestion fragments. These results support the result that *R. tropici* CFN299 parental strains were not present as contaminants in *E. adhaerens* transconjugant nodules.

E. adhaerens ATCC 33499 was a suitable recipient for rhizobial symbiotic plasmids. Sym plasmid stability was assessed by using isolated colonies of CFNEA50 and CFNEA55 after growth for 100 generations in PY liquid medium without antibiotics. All 600 colonies tested (300 colonies of each transconjugant) were resistant to neomycin (200 mg/liter), suggesting that they harbored the *R. tropici* CFN299 symbiotic plasmid, but two of six *E. adhaerens* transconjugants lost the symbiotic plasmid after 2 months of storage at 4°C, indicating that there was some instability.

There was a short delay (1 or 2 days) in the onset of bean nodulation with *Ensifer* transconjugants CFNEA52 to CFNEA56 compared to the *R. tropici* mutant CFN299 Tn*5-mob*-6. At 16 days postinoculation, the average number of nodules elicited by transconjugants CFNEA52 to CFNEA56 was 80% of the number of nodules obtained with donor strain CFN299 Tn*5-mob*-6. The nodules formed by the transconjugants were red and large. There were no nodules on any of the control (uninoculated) bean plants.

In competition experiments, when a 1:1 or 1:1,000 ratio of *R. tropici* CFN299 to *E. adhaerens* transconjugant CFNEA53 was used to inoculate bean plants (a total of 10^6 bacteria were inoculated per plant), no nodules were formed by the *E. adhaerens* transconjugants since only *R. tropici* CFN299 was recovered from inside nodules. The nodule isolates did not form colonies on LB containing 200 mg of neomycin per liter. PCR analysis of some of the nodules revealed only CFN299 with the *R. tropici ccsA* gene primers. In the experiments in which we deliberatedly mixed *R. tropici* CFN299 with *E. adhaerens* transconjugants, we were able to easily distinguish nodules formed by *R. tropici* CFN299. This finding supports the notion that in the experiments described above the *E. adhaerens* transconjugants, and not a contaminating parental strain, really formed the nodules.

To test if *E. adhaerens* ATCC 33499 could adhere to and be introduced into nodules together with *R. tropici* CFN299, mixtures of the two bacteria in various proportions were inoculated onto roots of bean seedlings. *R. tropici* was found to be the sole occupant of the bean nodules. It has been reported that *E. adhaerens* does not attack *A. tumefaciens, Rhizobium leguminosarum,* or *S. meliloti* (9, 31). When mixtures of *E. adhaerens* and *R. tropici* were coinoculated onto bean plants, the numbers of nodules obtained were similar to the numbers of nodules obtained with *R. tropici* CFN299 alone, and all of the nodules were formed by CFN299. A similar result was obtained with an inoculum prepared from a coculture of *R. tropici* CFN299 and *E. adhaerens* grown in PY medium for 24 h. The ability of *E. adhaerens* to attack other bacteria has been reported to be dependent on the growth conditions (3).

Genes involved in uptake of bean root exudates have been located on plasmid c (which carries the *nod-nif* genes) and on plasmid a (180 kb) of *R. tropici* CFN299, and these uptake genes have a role in symbiosis (26). Plasmid b was found to contain symbiotic determinants that conferred a symbiotic advantage to *A. tumefaciens* harboring only plasmid c (17). We found that plasmids a and b were cotransferred from *R. tropici* CFN299, along with the *nod-nif* plasmid, into *A. tumefaciens* (17) or into *Ensifer* (this study). The *A. tumefaciens* transconjugant containing all three plasmids nodulated better and fixed more nitrogen than transconjugants containing only plasmid c (17). Nevertheless, in competition experiments with *R. tropici* CFN299, *A. tumefaciens* harboring *R. tropici* plasmids a, b, and c was not recovered from the nodules, indicating that this transconjugant was not as competitive for nodule formation as the wild-type *R. tropici* strain (20). Similarly, in this study we found that *Ensifer* transconjugant CFN299 containing *R. tropici* plasmids b and c was not as competitive as *R. tropici* wild-type strain CFN299.

Several reports have addressed the role of plasmids in rhizobia with regard to symbiosis and metabolism (24; reviewed in references 8 and 21). Catabolic genes (23) and genes for lipopolysaccharide (5) or exopolysaccharide (10, 13) biosynthesis are plasmid borne. Rhizobial plasmids have been transferred between different strains and species in the laboratory (reviewed in reference 19). *S. meliloti* transconjugants that acquired the *R. leguminosarum nod-nif* plasmids either formed ineffective nodules (non-nitrogen-fixing nodules) or no nodules on pea or vetch (12, 30). In these examples, *S. meliloti* contained its normal complement of symbiotic megaplasmids, and functional incompatibility of plasmids may have occurred.

Levels of DNA-DNA homology greater than 30% between *E. adhaerens* ATCC 33499 and all *Sinorhizobium* species (unpublished data) also support the hypothesis that these bacteria are closely related. Taken together, our data suggest that *E. adhaerens* might be a misclassified bacterium, seemingly a nonsymbiotic bacterium, but comprehensive polyphasic taxonomic characterization of *E. adhaerens* is required to clarify the taxonomic position of this organism. Additionally, it would be

interesting to search for predatory activities in *Sinorhizobium* species.

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