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 it naturally and can be more numerous than their symbiotic counterparts (16, 27).

*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* symb plasmids conferred *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 genes were obtained with *E. adhaerens* ATCC 33499 in a PCR with nodC primers 251F and 566R (28) or with nodBC primers (nodB 31 [TACC TGACSTTVGACGACGTCC] and nodC RR [GAGACG GCGRCRTGTGTTG]) that we have used to amplify nodBC or nodC gene sequences from *Sinorhizobium mellei,* *Sinorhizobium medicace,* *Sinorhizobium arboris,* *Sinorhizobium terrangae,* *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. mellei* nodC PCR product was used as a probe in Southern blot hybridization with *E. adhaerens* ATCC 33499 total restricted DNA.

*R. tropici* CFN299 Tn5-mob-6 and CFN299 Tn5-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* GM19203 the capacity to form nodules on bean as previously described (17). *R. tropici* CFN299 Tn5-mob-6 and CFN299 Tn5-mob-7 were able to form nitrogen-fixing nodules when they were tested individually with bean plants (Table 1). *R. tropici* CFN299 Tn5-mob-6 and CFN299 Tn5-mob-7 were shown to have Tn5-mob in the nod-nif plasmid by hybridization of Eckhardt gels with Tn5 (data not shown).

*E. adhaerens* transconjugants obtained from matings on PY agar plates with *R. tropici* CFN299 Tn5-mob-6 and CFN299 Tn5-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.
lated colony from a nodule from each of six different plants was grew in LB containing 200 mg of neomycin per liter. One iso-

R. tropici

goty of plasmid sizes were as follows: plasmid a, 185 kb; plasmid b, 220 kb; and plasmid c, 500 kb (Table 1).

All isolates on PY agar had the colony mor-

ered on PY medium, and 10 individual colonies per nodule

follows. All bacteria isolated from bean nodules were recov-

inoculated onto plants, and transconjugants CFNEA51 to

5-mob-6 and CFN299 Tn5-mob-7 were selected.

Additionally, a mixture of E. adhaerens transconjugants de-

rived from CFN299 Tn5-mob-6 and CFN299 Tn5-mob-7 was ino-

culated onto plants, and transconjugants CFNEA51 to

CFNEA56 were selected from well-developed red nodules as

follows. All bacteria isolated from bean nodules were recov-

ered on PY medium, and 10 individual colonies per nodule

were then tested for growth in LB containing 200 mg of neo-

mycin per liter. All isolates on PY agar had the colony mor-

phology of E. adhaerens ATCC 33499, not the colony mor-

phology of R. tropici CFN299. All of the isolated colonies tested

grew in LB containing 200 mg of neomycin per liter. One iso-

lated 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 recog-

nized on the basis of the size of the band hybridizing to a Tn5

probe in each transconjugant. The transconjugants were all derived from CFN299 Tn5-mob-6, perhaps indicating

that the Tn5-mob-7 insertion had affected some loci involved in

competition for nodule formation. The gene interrupted by

Tn5-mob-7 insertion had affected some loci involved in

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

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

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

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 Tn5-mob-6; lane 12, CFN299 Tn5-mob-7.

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 Tn5-mob-6; lane 12, CFN299 Tn5-mob-7.

TABLE 1. Strains and characteristics

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Small-subunit RNA patterns*</th>
<th>R. tropici plasmids*</th>
<th>nifH gene*</th>
<th>Acetylene reduction activity with P. vulgaris nodules (nmol of ethylene/h/plant)</th>
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* Only transconjugant CFNEA41 was derived from CFN299 Tn5-mob-7; all other transconjugants were derived from CFN299 Tn5-mob-6. R. etli CFN42 was also used in this study.

The different letters represent distinct patterns obtained with restriction enzymes HinfI, MspI, RsaI, HhaI, Sau3A1, and DdeI; each letter position refers to a different restriction enzyme used for PCR-synthesized 16S rRNA genes (15) obtained with primers rD1 and fD1 (29).

Acetylene reduction activity was determined with primers as described previously (6).

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

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.

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

ND, not determined.

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

KB, not determined.
Ribosomal fingerprints (15) were determined by 16S rRNA gene restriction enzyme digestion with HinfI, MspI, RsaI, HhaI, Sau3AI, and DdeI 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 P231q (TTAACCCTTTGGCGCTTTT), 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-
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 transconjugants CFN299 alone, and all of the nodules 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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REFERENCES