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SHORT COMMUNICATION

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Diversity of rhizobia from nodules of the leguminous tree *Gliricidia sepium*, a natural host of *Rhizobium tropici*

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Abstract The *Rhizobium* species that nodulate the legume tree *Gliricidia sepium* were analyzed by phenotypic characteristics (including nodule formation in different hosts), PCR-RFLP patterns and sequences of 16S rRNA genes, multilocus enzyme electrophoresis, and plasmid patterns. Strains of *Rhizobium tropici* type A and B, *Sinorhizobium* spp., and *Rhizobium etli* bv. *phaseoli* were encountered in *G. sepium* nodules and their presence depended on the site sampled.

Keywords *Rhizobium* · Symbiosis · Legume trees · *Gliricidia sepium* · *Rhizobium tropici*

Introduction

Legume trees are valuable in reforestation programs, soil preservation and as green manure. Rhizobial symbionts of *Acacia* and *Leucaena* spp. have been extensively studied but there is a lack of knowledge on the *Rhizobium* species that nodulate other tree legumes such as *Gliricidia sepium*. *G. sepium* from the Papilionoideae subfamily has sometimes been called the "alfalfa of the tropics" because it may be used as forage by livestock (Ford 1987). *G. sepium* has a better efficiency of water usage than alfalfa and is native of the Americas (Hughes 1987).

By screening uncharacterized hosts, novel nodulating bacteria in the β-Proteobacteria were discovered (Moulin et al. 2001). Therefore, it was of interest to similarly analyze nodule bacteria from the vast amount of unexplored legumes. We previously characterized *Rhizobium tropici* and found that it is resistant to acidity, high temperatures, and heavy metals (Martínez-Romero et al. 1991). These properties highlight its advantages for use as an inoculant in the tropics. Today, several efficient strains of *Rhizobium tropici* are being used successfully in Brazil for *Phaseolus vulgaris* bean inoculation (Mostasso et al. 2002). In the laboratory, *Rhizobium tropici* nodulates and fixes nitrogen in *Gliricidia maculata* and in other host legumes (Hernández-Lucas et al. 1995). *Rhizobium tropici* has been catalogued as an opportunistic nodulator of bean (Martínez-Romero 1996), and its natural host is unknown (Martínez-Romero 1996; Graham and Vance 2000).

The aim of this work was to genetically characterize *Rhizobium* isolates from *G. sepium* with special interest in *Rhizobium tropici* strains.

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Materials and methods

Isolation of nodule bacteria and growth

Gliricidia sepium grows naturally in the two sampling sites Santa Rosa and Veracruz, Mexico. Santa Rosa is 900 m above sea level with an average temperature of 24 °C and clay/sandy soil (pH 6.3); Veracruz is at sea level with an average temperature of 25.2 °C and sandy soil (pH 7.9). Nodules were processed within 1–3 days of their recovery from 4-month-old plants, sterilized with sodium hypochlorite, and pressed onto plates of PY medium (casein peptone 5 g, yeast extract 3 g, $CaCl_2$ 0.6 g, agar 15 g per liter of H_2O) with or without nalidixic acid (Nal, 20 mg/l), or in minimal medium (González-Pasayo and Martínez-Romero 2000), or in YM medium (Vincent 1970). Nodule surface sterility tests were carried out; a single colony was purified from each nodule and tested for growth in PY medium without calcium, and with antibiotics at 28 °C or 37 °C. Growth was tested on Luria-Bertani (LB) medium plates. Other phenotypic tests were carried out as described by Wang et al. (1998).

PCR-RFLP analysis of 16S rRNA genes and sequences

Almost complete fragments of 16S rRNA genes were synthesized in PCR with *Taq* polymerase using fD1 and rD1 primers (Weisburg et al. 1991) and digested with the restriction enzymes *Hin*fI,

*Hha*I, *Msp*I, *Rsa*I, *Dde*I and *Sau3*A1. The patterns of the resulting fragments were compared to those obtained for the type strains of *Rhizobium*, *Sinorhizobium* or *Mesorhizobium* species. Partial 16S rRNA gene sequences were obtained from CFNEV21 (1029 bp, GenBank accession number AF480573), CFNER34 (754 bp, GenBank accession numbers AY079179 and AY079180) and CFNER90 (973 bp, GenBank accession number AF480572) as reported (Rogel et al. 2001). The sequences were aligned and analyzed with GCG and ClustalW. *nifH* gene PCR products were obtained from CFNER90 with nifH1 and nifH2 primers (Eardly et al. 1992) and sequenced (455 bp, GenBank accession number AY079181).

Multilocus enzyme electrophoresis

Bacterial isolates were grown in liquid PY medium. Extracts were prepared as described (Wang et al. 1998) and electrophoresed in starch or in starch (7.5%) with added agarose (1.5%) gels. The following metabolic enzymes were tested as described by Selander et al. (1986): hexokinase, glutamate, glucose 6-phosphate, malate, alanine and isocitrate dehydrogenases, phosphoglucomutase and malic enzyme.

Plasmid profiles and hybridization assays

A modified Eckhardt procedure (Hynes and McGregor 1990) was followed to visualize plasmids in agarose gels. DNA was extracted with genomic Prep (Amersham Pharmacia) following the manufacturer's instructions. Southern blot hybridization were performed as described previously (Wang et al. 1998).

Plant nodulation assays

G. sepium ecotype Morelos seeds were surface-sterilized and seedlings were placed in sterile vermiculite in black 20-cm-long cones in the greenhouse or in growth chambers. *Acacia farnesiana* seeds were treated with concentrated sulfuric acid. *Leucaena* and *Acacia* seedlings were grown in N-free Fahraeus medium (Fahraeus 1957) in jars with vermiculite. *Phaseolus vulgaris* beans were grown using a cotton support as described previously (Wang et al. 1998). Root seedlings were inoculated with 10^5 bacteria of each strain at the time of planting.

Results and discussion

Rhizobia isolates from *G. sepium* were obtained from 164 root nodules from four trees in Santa Rosa and from 40 nodules from 19 trees from Veracruz. Nodules in *G. sepium* are multilobed, indeterminate.

Santa Rosa isolates

Three main groups could be clearly distinguished among Santa Rosa isolates on the basis of morphological and phenotypic characteristics (see below). Several representatives from each group were analyzed by alloenzyme patterns (Fig. 1) and PCR-RFLP of 16S rDNA (Table 1). One group had 16S rDNA PCR-RFLP patterns similar to those of *Sinorhizobium terangae*. The second group, comprising 25% of the isolates, was assigned to *Rhizobium tropici* type B based on the sequence of the 16S rRNA genes of one representative (CFNER90), with 7 nucleotide differences in 863 bases (98.9% identity) compared to *Rhizobium tropici* type B strain CIAT899. This assignment was also made on the basis of the typical phenotypic characteristics that distinguish *Rhizobium tropici* type B strains, namely resistance to carbenicillin (1 g l^{-1}) and chloramphenicol (5 g l^{-1}), growth at 37 °C, and growth on LB and in PY without calcium; however, Santa Rosa type B *Rhizobium tropici* strains were gummier than the reference CIAT899 strain. A third group, encompassing 26% of the isolates, was found to be highly similar to *Rhizobium*

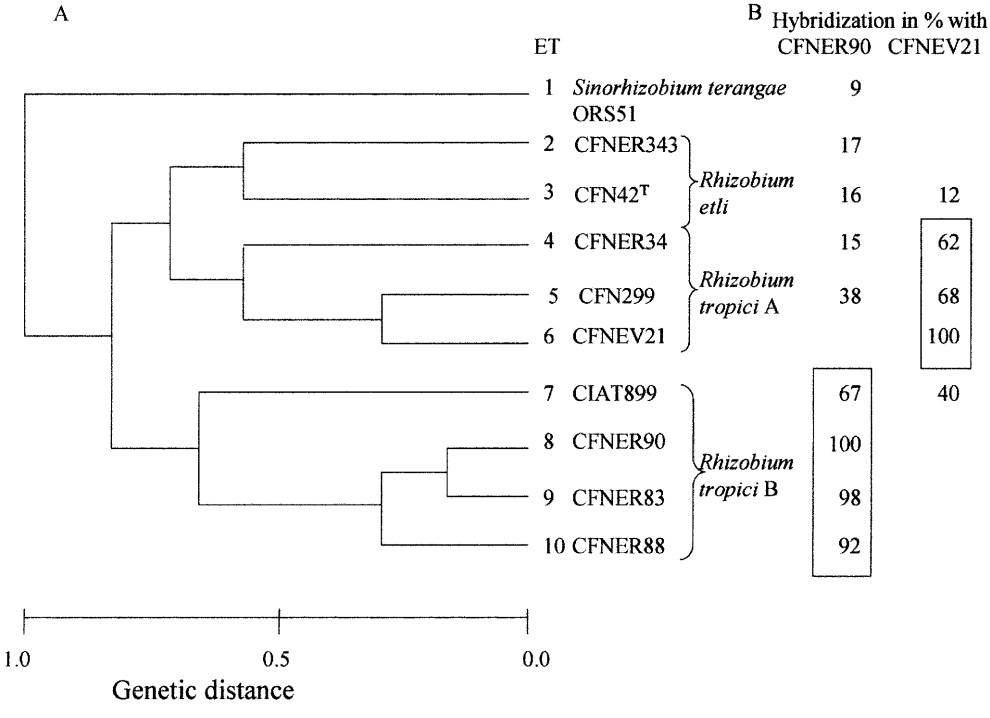

Fig. 1 **A** Genetic relatedness of isolates from *Gliricidia sepium*, including *Rhizobium tropici* and *Rhizobium etli* reference strains. The dendrogram was constructed from multilocus enzyme electrophoresis data for the metabolic enzymes referred to in Materials and methods. Other isolates corresponding to electrophoretic type 5 are CFNEV13, CFNEV16, CFNEV19, CFNEV24, CFNEV32, CFNEV34, CFNEV35, CFNEV37. **B** DNA-DNA hybridization as percent of the reference strains used as a probe. DNA from CFNER90 was also hybridized to DNA from the following species (% hybridization in parentheses): *Rhizobium leguminosarum* USDA2370 (15), *Rhizobium giardinii* H152 (9), *Rhizobium gallicum* R602 (12), *Rhizobium mongolense* USDA1844 (12), *Rhizobium galegae* USDA4128 (14)

Table 1 Patterns of 16S rDNA of selected isolates from *Gliricidia sepium* nodules and reference strains. The different letters represent the patterns of the PCR products obtained respectively with restriction enzymes (*Msp*I, *Hin*fI, *Hha*I, *Rsa*I, *Dde*I), as described by Wang et al. (1999) following the techniques of Laguerre et al. (1994)

	16S rDNA patterns
Sinorhizobium	
S. terangae ORS51 (reference strain)[a]	LBIAF
Rhizobium	
R. tropici CFN299 (reference strain)	EBFDL
R. tropici type A (from Veracruz)[b]	EBFDL
R. tropici type A (from Santa Rosa)[c]	EBFDL
R. tropici CIAT899 (reference strain)	FBDBE
R. tropici type B (from Santa Rosa)[d]	FBDBE
R. etli CFN42 (reference strain)	DBECE
R. etli CFNER343	DBECE

[a]de Lajudie et al. (1994)
[b]Isolates CFNEV3, CFNEV13, CFNEV16, CFNEV17, CFNEV19, CFNEV21, CFNEV24, CFNEV32, CFNEV34, CFNEV35, CFNEV37
[c]Isolates CFNER2, CFNER6, CFNER13, CFNER18, CFNER26, CFNER34, CFNER35, CFNER40, CFNER41, CFNER44, CFNER47, CFNER51
[d]Isolates CFNER76, CFNER83, CFNER85, CFNER88, CFNER90

tropici type A strains by morphological characteristics; the strains were not gummy, were sensitive to the above-mentioned antibiotics, and did not grow in LB or in PY without calcium. Furthermore, the 16S rRNA gene fragment of one representative (CFNER34) from this group was almost identical (99.8%) to that of CFN299 (1 base difference in 754 bases).

Only one of the isolates from Santa Rosa was found to group with *Rhizobium etli* strains according to the RFLP patterns of the 16S rDNA (Table 1). *nifH* gene reiterations characteristic of *Rhizobium etli* strains were encountered in CFNER343 (not shown); therefore this isolate was assigned to the biovar phaseoli. CFNER343 also had a large number of plasmids, similar to other *Rhizobium etli* strains (Fig. 2). Previously the *Rhizobium etli* bv. phaseoli strain CFN42 was found to nodulate *Gliricidia maculata* under laboratory conditions (Hernández-Lucas et al. 1995).

Veracruz isolates

The strains from *Gliricidia* nodules in Veracruz were all morphologically identical to each other and also identical in morphology and growth rate to *Rhizobium tropici* type A strain CFN299 in PY medium but less gummy than CFN299 in YM medium. They were also incapable of growing at 37 °C, on LB media or in the presence of carbenicillin and chloramphenicol, similar to CFN299. The isolates had identical 16S rDNA restriction patterns (Table 1) and the 16S rRNA gene sequence of one representative (CFNEV21) was almost identical (99.9%) to that of *Rhizobium tropici* CFN299 (1 base difference in 1,029 bases). Plasmid patterns found in some Veracruz isolates were similar to those in CFN299 but other, smaller plasmids were also detected (Fig. 2).

Rhizobium tropici characteristics

Both *Rhizobium tropici* type A and type B isolates from Veracruz and Santa Rosa were resistant to Nal (20 mg l^{-1}), grew at pH 4.5, and had a megaplasmid of similar size (Fig. 2). The large genetic distances detected by multilocus enzyme electrophoresis (MLEE) between the *Rhizobium tropici* B strains in Mexico and those from Brazil (Fig. 1) probably indicate that they diverged a long time ago. Since no bona fide *Rhizobium tropici* strains had been previously isolated in Mexico, a more complete characterization of a Santa Rosa isolate (CFNER90) and a Veracruz isolate (CFNEV21) was undertaken and the *nifH* gene hybridization patterns and total DNA-DNA hybridization levels were determined. Additionally, for CFNER90 a partial fragment of the *nifH* gene was sequenced. The *nifH* sequence of CFNER90 showed the highest similarity (96%) to a *Rhizobium tropici* type B strain BR6001 (Haukka et al. 1998) and to *Rhizobium tropici* reference type B strain CIAT899. *nifH* gene hybridization of CFNEV21, CFNER34, CFNER90, CIAT 899 and CFN299 revealed an identical band of around 8 kb with DNA digested with *Eco*R1 (not shown). Total DNA-DNA hybridization results are shown in Fig. 1.When used as a probe, DNA from CFNER90 hybridized 67% to the DNA of *Rhizobium tropici* strain CIAT899 and 92–98% to the DNAs of other *Rhizobium tropici* type B isolates from Santa Rosa. Total DNA of CFNEV21 (from Veracruz) hybridized 68% with DNA of CFN299, 100% to DNA of CFNEV34 (from Veracruz) and 40% to DNA of strain CIAT 899. It is worth mentioning that *Rhizobium tropici* type A and type B strains have DNA-DNA hybridization values of around 36% (Martínez-Romero et al. 1991). For this reason and due to their differences in megaplasmid sequences, it has been proposed that type A and type B strains are different species (Geniaux et al. 1995), but the existence of strains

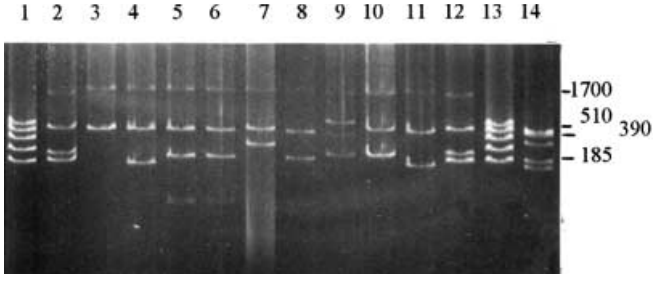

Fig. 2 Plasmid patterns revealed by a modified Eckhardt procedure (Hynes and McGregor 1990). *Lanes 1, 13 Rhizobium etli* reference strain CFN42; *lanes 2, 12 Rhizobium tropici* type A reference strain CFN299. *R. tropici* type A isolates from Veracruz: *lane 3* CFNEV19, *lane 4* CFNEV13, *lane 5* CFNEV24, *lane 6* CFNEV32, *lane 8* CFNEV21, *lane 11* CFNEV37; *lane 7* CFNER34 from Santa Rosa. *R. tropici* type B strains: *lane 9* CFNEVR90 from Santa Rosa, *lane 10* CIAT899.*Lane 14 R. etli* Santa Rosa isolate CFNER343. Molecular weight markers are in kb

with characteristics intermediate between type A and type B strains (Martínez-Romero 1996; Mostasso et al. 2002) argues against this proposal.

Nodules in *G. sepium* (ecotype Morelos) appeared 8 days after inoculation with the different strains tested in plant growth chambers. In the laboratory, *Rhizobium tropici* type A and B isolates from *G. sepium* were found to nodulate *Phaseolus vulgaris* (common bean) and formed an average of 15 nitrogen-fixing nodules at 14 days after inoculation, similar to the number of nodules formed by bean-borne *Rhizobium tropici* isolates. Type B isolates from *G. sepium* were additionally tested in *Acacia farnesiana* and *Leucaena leucocephala* and found to form nodules as well.

Rhizobium strains with different phenotypic characteristics depending on the geographic altitude have been isolated from *G. sepium* (Melchor-Marroquin et al. 1999) but the *Rhizobium* species involved were not defined. Nitrogen fixation by *G. sepium* was found to be influenced by the plant genotype, the bacterial strain (Awonaike and Hardarson 1992) and the environmental conditions (Sanginga et al. 1991). The elucidation of *G. sepium* symbionts will help to promote its inoculation and its optimal use as forage for livestock. Since *Rhizobium tropici* is frequently used as a model of plant-bacteria interactions, identification of a natural host for *Rhizobium tropici* will enrich the scope of research into its interactions with plants.

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