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# Genetic Structure of a Soil Population of Nonsymbiotic *Rhizobium leguminosarum*

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**The genetic structure of a population of nonsymbiotic *Rhizobium leguminosarum* strains was determined by the electrophoretic mobilities of eight metabolic enzymes. Nonsymbiotic strains were isolated from the rhizosphere of bean plants and characterized by growth on differential media and at different temperatures, intrinsic antibiotic resistances, the lack of homology to a *nifH* probe, and their inability to form nodules on bean roots. All the isolates clustered with *R. leguminosarum* bv. phaseoli reference strains and did not encompass any other *Rhizobium* taxa. Their rRNA operon restriction fragment length polymorphisms and the nucleotide sequence of a fragment of the 16S rRNA gene were also found to be identical to those of *R. leguminosarum* bv. phaseoli reference strains. When complemented with an *R. leguminosarum* bv. phaseoli symbiotic plasmid (p42d), the nonsymbiotic isolates were able to fix nitrogen in symbiosis with bean roots at levels similar to those of the parental strain. The symbiotic isolates were found at a relative frequency of 1 in 40 nonsymbiotic *R. leguminosarum* strains.**

The bacteria of the family *Rhizobiaceae* are characterized by their ability to interact with higher plants; thus, *Agrobacterium* spp. are able to form tumors on plants and *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium* spp. can establish a symbiosis with the roots of legumes. These species have therefore been defined primarily on the basis of phenotypic characteristics such as host range, colony morphology, growth on selective media, and certain metabolic attributes (14). The only exception to this rule is *Agrobacterium radiobacter*, which does not elicit any kind of cortical hypertrophy in plants but shares many of the chromosomal characteristics of *Agrobacterium tumefaciens* (14).

In *Rhizobium* spp., most of the genes controlling nodulation, host range specificity, and nitrogen fixation are located on large transferable plasmids called sym plasmids. In *Rhizobium leguminosarum* these plasmids can be lost spontaneously at a high frequency and can also undergo frequent rearrangements, resulting in loss of the symbiotic capacity (14, 25).

The isolation of nonsymbiotic *Rhizobium* strains from soil has been described previously. Soberón-Chávez and Nájera (24) and Jarvis et al. (9) isolated strains from soil that are chromosomally similar to *R. leguminosarum*. Both groups reported that when complemented with a sym plasmid, these isolates were able to establish an effective symbiosis with the host plant as determined by the plasmid.

In the present study, we isolated nonsymbiotic bacteria by using growth on selective media, growth at specific temperatures, and intrinsic antibiotic resistances as selection criteria. The genetic relatedness of the nonsymbiotic isolates to different *Rhizobium* strains was determined by multilocus enzyme electrophoresis (MLEE) and corroborated by restriction fragment length polymorphism (RFLP) analysis of the rRNA operons. We sequenced a fragment of the 16S rRNA gene of three nonsymbiotic isolates and found that the sequences were identical to that of a symbiotic *R. leguminosarum* bv. phaseoli strain. We then transferred an *R. leguminosarum* bv. phaseoli sym plasmid to eight nonsymbiotic isolates belonging to different phylogenetic clusters. The transconjugants acquired the capacity to establish an effective symbiosis with bean plants.

## MATERIALS AND METHODS

**Bacterial strains.** Reference strains are listed in Table 1. Cells were grown in the following media: peptone yeast medium (PY) (18), yeast mannitol medium (YM), and minimal medium with lactose as the carbon source (MM-Lac; $10^{-7}$ g of $FeCl_3 \cdot ml^{-1}$, 2.3% [wt/vol] $K_2HPO_4$, 1% $MgSO_4 \cdot 7H_2O$, 1.5% $CaCl_2$, 1% $NH_4NO_3$, 0.2% lactose). When indicated, the following antibiotics were added: kanamycin (30 $\mu g \cdot ml^{-1}$), spectinomycin (100 $\mu g \cdot ml^{-1}$), nalidixic acid (100 $\mu g \cdot ml^{-1}$), and cycloheximide (150 $\mu g \cdot ml^{-1}$).

**Isolation of soil bacteria.** Six-week-old nodulated bean plants were harvested from a field in Tepoztlán, Morelos, Mexico. Beans have been cultivated at that site for the last 50 years in rotation with maize and without the addition of fertilizers. The root systems of 10 plants were placed in 50-ml sterile culture tubes containing 25 ml of a mixture of 10 mM $MgSO_4$ and 0.01% (vol/vol) Tween 40. Tubes were allowed to stand at room temperature for 30 min and then were vortexed for 10 min. The root system was then removed, and the suspension was left to sediment for 1 h. Aliquots (0.1 ml) were plated on PY containing nalidixic acid and cycloheximide and on MM-Lac containing nalidixic acid and cycloheximide. The plates were incubated at 30°C until colonies appeared (72 h). A total of 2,000 isolates from the MM-Lac plates and 500 from the PY plates were tested for their abilities to grow at 37°C in PY and MM-Lac and at 30°C in LB medium. Only 295 strains which grew exclusively at 30°C on PY and MM-Lac were preserved. These isolates were found at different frequencies on MM-Lac and PY (14 and 3%, respectively). They were further analyzed for their

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TABLE 1. Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Nonsymbiotic isolates | | |
| CFN401 to CFN478 | | This study |
| *R. leguminosarum* bv. phaseoli type I | | |
| VS001 to VS010 | Wild type | V. Souza[a] |
| CFN42 | Wild type | CFN[b] |
| CFN3 | Wild type | CFN |
| CFN285 | Wild type | CFN |
| BRA8 | Wild type | CFN |
| VikingI | Wild type | E. Schmidt[c] |
| Nitragin-8251 | Wild type | Nitragin[d] |
| CFNX78 | Tn*5* insertion on p42d | S. Brom[b] |
| CFN2001 | CFN42 derivative cured of sym plasmid (p42d) | CFN |
| *R. leguminosarum* bv. phaseoli type II | | |
| CFN299 | Wild type | CFN |
| CIAT899 | Wild type | P. Graham[c] |
| C05-35 | Wild type | M. Tsai[e] |
| C05-II | Wild type | M. Tsai |
| UMR1063 | Wild type | P. Graham |
| AD822 | CIAT899 derivative cured of sym plasmid | Araceli Dávalos[b] |
| *R. leguminosarum* bv. viciae | | |
| USDA2337 | Wild type | USDA[f] |
| USDA2434 | Wild type | USDA |
| USDA2443 | Wild type | USDA |
| USDA2479 | Wild type | USDA |
| USDA2489 | Wild type | USDA |
| *R. leguminosarum* bv. trifolii | | |
| USDA2046 | Wild type | USDA |
| USDA2048 | Wild type | USDA |
| USDA2152 | Wild type | USDA |
| *R. meliloti* | | |
| SU47 | Wild type | J. Dénarié[g] |
| Rme2 | Wild type | CFN |
| Rme7 | Wild type | CFN |
| GR037 | Wild type | M. Megías[h] |
| GR4 | Wild type | M. Megías |
| *R. loti* | | |
| NZP2037 | Wild type | K. Marcker[i] |
| *S. fredii* | | |
| USDA191 | Wild type | USDA |
| USDA193 | Wild type | USDA |
| USDA214 | Wild type | USDA |
| HH003 | Wild type | M. Sadowsky[c] |
| HH103 | Wild type | M. Sadowsky |
| *A. tumefaciens* | | |
| C58 | Wild type | M. van Montagu[j] |
| A348 | Wild type | E. Nester[k] |
| *E. coli* | | |
| HB101 | | 1 |
| Plasmids | | |
| pCQ152 | *nifH* probe | 20 |
| pKK3535 | rRNA operon probe | 3 |
| pRK2013 | Mobilization helper | 23 |
| cos309 | 30-kb chromosomal fragment from CFN42 | 17 |

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abilities to produce mucous growth on YM plates and to produce yellow coloring in YM–0.0025% bromothymol blue (YM-BB) plates; 90% of the isolates produced mucous growth on YM, and only 27% produced a yellow coloring on YM-BB, as did control *R. leguminosarum* bv. phaseoli CFN42 and CFN285. We selected 85 isolates for further analysis.

**Preparation of lysates.** Isolates were grown overnight on an orbital shaker at 200 rpm in 30 ml of PY at 30°C. Cells were harvested by centrifugation at 7,500 × *g* for 10 min at 4°C. After suspension in 2 ml of 10 mM $MgSO_4$, the bacteria were sonicated twice for 30 s with a 30-s rest in an MSE sonifier with a microtip at 50% pulse; ice cooling was used during the procedure. The lysates were then stored at −70°C.

**Multilocus enzyme electrophoresis.** Starch gel electrophoresis and the selective staining of enzymes were done as described by Selander et al. (22). The following enzymes were assayed: alcohol dehydrogenase, NAD-malate dehydrogenase, isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase, xanthine dehydrogenase, leucine dehydrogenase (LDH1), lysine dehydrogenase, and indophenol oxidase. The electrophoretic buffer system for all enzymes was Tris citrate (pH 8) (22), except that for LDH2, the borate (pH 8.2) system was also used. Some species (all except *R. leguminosarum*) gave a much better reaction under these conditions. LDH electromorphs (ETs) were determined in both kinds of gels and were considered to be isozymes, not alleles, depending on the buffer system used.

Distinctive mobility variants (ETs) of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus, and the ET patterns were considered to be multilocus genotypes (Table 2).

From allele frequencies for ETs, the genetic diversity for an enzyme locus was calculated as

$$h = (1 - \Sigma x_i^2)\,[n/(n-1)]$$

where $x_i^2$ is the frequency of the $i^{th}$ allele and $n$ is the number of ETs. The mean genetic diversity per locus ($H$) is the arithmetic average of $h$ values for the nine loci (22). The genetic distance between each pair of ETs was estimated as the proportion of loci at which dissimilar alleles occurred (mismatches); clustering from a matrix of pairwise genetic distances was performed by the UPGMA method of Nei and Li (16).

**Filter blot hybridization.** DNA purification, digestion, and hybridization procedures were carried out as described by Flores et al. (7). The presence of *nifH* in the isolates was determined by hybridization of *Eco*RI digests of total DNA blotted on nylon membranes and probed with plasmid pCQ152, which carries a 0.3-kb *Sal*I fragment of the *R. leguminosarum* bv. phaseoli CFN42 *nifH*a gene (20).

Derivatives were traced and verified by their cos309 *Eco*RI RFLP; cos309 carries a 30-kb chromosomal fragment of strain CFN42 cloned in the *Eco*RI site of cosmid pLAFR1 (17).

The RFLPs of the rRNA operons were determined by hybridization of total DNA *Sau*3A digests probed with plasmid pKK3535 (3). This plasmid carries a 7.5-kb *Bam*HI fragment containing the *Escherichia coli rrnB* operon cloned in plasmid pBR322.

Relative levels of hybridization were estimated by using a modification of the Southern blot procedure as follows. DNA concentration was estimated spectrophotometrically at 260 nm. Total DNA digested with *Eco*RI was electrophoresed in 1% agarose gels, and DNA amounts in gels were further verified by transmittance scanning densitometry of acetate photographs. DNA was blotted on nylon filters and hybridized at 65°C with [$^{32}$P]DNA at high specific activity ($10^8$ cpm/μg). Autoradiography was carried out overnight at −70°C, and then filter lanes were cut and counted in a Beckman scintillation counter. The percentage of total homologous recombination was calculated for each strain tested.

**Nucleotide sequence of 16S rRNA genes.** The nucleotide sequence of the 16S rRNA genes was determined by directly sequencing double-stranded polymerase chain reaction products with Sequenase 2 (U.S. Biochemical Corp.). A region of 491 bp corresponding to nucleotides 872 through 1363 of the *A. tumefaciens* 16S rRNA gene was amplified by using the GenAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus) with a 28-mer (CCGCACAAGCGGTGGAG CATGTGGTTTA) and a 30-mer (CTTGTACACACCGC CCGTCACACCATGGGA) as primers. The reaction was carried out as specified by the manufacturer in 30 temperature cycles as follows: 30 s at 95°C for denaturation, 30 s at 55°C for primer annealing, and 3 min for polymerization at 72°C. The polymerase chain reaction products were purified by using QIAGEN Tip 20 minicolumns and the method suggested by the manufacturer. Direct sequencing with Sequenase was carried out by the method of Casanova et al. (5).

**Transfer of the *R. leguminosarum* bv. phaseoli p42d sym plasmid to nonsymbiotic isolates.** Spontaneous Sp$^r$ derivatives of the nonsymbiotic isolates were used as recipients in triparental matings with strain CFNX78 as the p42d donor and *E. coli* HB101(pRK2013) as the mobilization helper (2, 23). Transconjugants were selected by their ability to grow on PY containing spectinomycin, kanamycin, and nalidixic acid. The transconjugants were authenticated by their *nifH* and cos309 RFLPs.

**Bean nodulation and nitrogen fixation assays.** Bean (*Phaseolus vulgaris* L. cv. Negro Jamapa) nodulation assays and nitrogen fixation determination by the acetylene reduction assay were carried out as described previously (13).

## RESULTS

**Identification of nonsymbiotic *R. leguminosarum*.** Soil bacteria were isolated from the rhizosphere of bean plants and characterized by growth on differential media and at different temperatures. A total of 85 isolates having growth characteristics similar to those of a wild-type *Rhizobium leguminosarum* bv. phaseoli type I strain were chosen. The selected strains were examined by Southern hybridization for the ability to hybridize to a nitrogenase reductase-specific (*nifH*) probe (pCQ152). Only 2 of the 85 strains showed any homology to the *nifH* probe. These two strains proved to be *R. leguminosarum* bv. phaseoli, as they were able to nodulate and fix nitrogen in symbiosis with bean plants (data not shown). The other 83 strains did not nodulate beans.

**Genetic diversity of nonsymbiotic isolates.** Two types of *R. leguminosarum* bv. phaseoli strains have been previously defined among strains isolated from bean nodules (2, 13), corresponding essentially to two different symbiotic plasmids. Type I strains have multiple copies of nitrogenase *nifH* genes (12, 17) and a narrow nodulation host range (2). Type II strains have a single copy of the *nif* genes and nodulate *Leucaena* spp. (2, 12). To establish whether these isolates were properly nonsymbiotic *R. leguminosarum* strains, we

TABLE 2. Allele profiles at nine enzyme loci in 110 ETs

| ET | No. of alleles at indicated enzyme locus[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MDH | IDH | G6P | XDH | LDH1 | LYD | LDH2 | ADH | IPO |
| 1 | 5 | 5 | 2 | 2 | 5 | 5 | –[b] | 5 | 5 |
| 2 | 5 | 5 | 3 | 2 | 5 | 5 | – | 5 | 5 |
| 3 | 5 | 5 | 2 | 3 | 5 | 5 | – | 5 | 5 |
| 4 | 5 | 5 | 2 | 1 | 5 | 6 | – | 5 | 5 |
| 5 | 5 | 5 | 2 | 2 | 5 | 6 | – | 5 | 5 |
| 6 | 5 | 4 | 2 | 2 | 5 | 7 | – | 5 | 5 |
| 7 | 5 | 6 | 2 | 4 | 5 | 4 | – | 5 | 5 |
| 8 | 4 | 4 | 2 | 1 | 5 | 6 | – | 5 | 5 |
| 9 | 5 | 4 | 2 | 1 | 5 | 6 | – | 5 | 5 |
| 10 | 9 | 4 | 2 | 1 | 5 | 5 | – | 5 | 5 |
| 11 | 5 | 4 | 2 | 1 | 6 | 5 | – | 5 | 5 |
| 12 | 5 | 4 | 1 | 1 | 6 | 6 | – | 5 | 5 |
| 13 | 5 | 4 | 2 | 1 | 6 | 6 | – | 5 | 5 |
| 14 | 5 | 4 | 2 | 3 | 5 | 6 | – | 6 | 5 |
| 15 | 5 | 4 | 2 | 2 | 5 | 6 | – | 6 | 5 |
| 16 | 5 | 2 | 2 | 1 | 5 | 6 | – | 6 | 5 |
| 17 | 4 | 4 | 2 | 2 | 5 | 6 | – | 6 | 5 |
| 18 | 4 | 4 | 2 | 2 | 5 | 5 | – | 6 | 5 |
| 19 | 4 | 7 | 2 | 2 | 5 | 5 | – | 6 | 5 |
| 20 | 7 | 4 | 3 | 2 | 5 | 5 | – | 6 | 5 |
| 21 | 5 | 5 | 1 | 2 | 5 | 5 | – | 6 | 5 |
| 22 | 5 | 5 | 3 | 2 | 5 | 5 | – | 6 | 5 |
| 23 | 5 | 5 | 1 | 2 | 7 | 5 | – | 6 | 5 |
| 24 | 5 | 4 | 1 | 2 | 7 | 5 | – | 6 | 5 |
| 25 | 5 | 6 | 1 | 2 | 5 | 5 | – | 5 | 5 |
| 26 | 5 | 4 | 1 | 2 | 5 | 5 | – | 5 | 5 |
| 27 | 5 | 4 | 1 | 2 | 5 | 5 | – | 6 | 5 |
| 28 | 6 | 6 | 1 | 2 | 5 | 5 | – | 6 | 5 |
| 29 | 5 | 5 | 1 | 1 | 5 | 5 | – | 6 | 5 |
| 30 | 5 | 5 | 1 | 1 | 5 | 5 | – | 4 | 5 |
| 31 | 5 | 9 | 1 | 1 | 5 | 5 | – | 6 | 5 |
| 32 | 5 | 6 | 1 | 1 | 5 | 5 | – | 6 | 5 |
| 33 | 5 | 5 | 1 | 1 | 5 | 6 | – | 6 | 5 |
| 34 | 7 | 5 | 1 | 1 | 5 | 5 | – | 5 | 5 |
| 35 | 5 | 5 | 1 | 1 | 5 | 5 | – | 5 | 5 |
| 36 | 5 | 5 | 1 | 1 | 5 | 4 | – | 5 | 5 |
| 37 | 5 | 6 | 1 | 1 | 6 | 6 | – | 6 | 5 |
| 38 | 5 | 6 | 1 | 1 | 5 | 6 | – | 6 | 5 |
| 39 | 4 | 6 | 1 | 1 | 5 | 6 | – | 6 | 5 |
| 40 | 6 | 5 | 1 | 2 | 5 | 6 | – | 5 | 5 |
| 41 | 6 | 5 | 1 | 2 | 5 | 6 | – | 6 | 5 |
| 42 | 4 | 5 | 1 | 2 | 5 | 6 | – | 6 | 5 |
| 43 | 4 | 5 | 1 | 2 | 5 | 4 | – | 5 | 5 |
| 44 | 5 | 4 | 1 | 2 | 5 | 6 | – | 6 | 5 |
| 45 | 5 | 4 | 1 | 2 | 5 | 6 | – | 5 | 5 |
| 46 | 6 | 4 | 1 | 2 | 5 | 6 | – | 5 | 5 |
| 47 | 6 | 4 | 1 | 2 | 5 | 6 | – | 6 | 5 |
| 48 | 5 | 4 | 1 | 2 | 6 | 6 | – | 6 | 5 |
| 49 | 4 | 4 | 1 | 1 | 5 | 5 | – | 7 | 5 |
| 50 | 4 | 4 | 3 | 1 | 5 | 5 | – | 7 | 5 |
| 51 | 4 | 4 | 1 | 1 | 5 | 5 | – | 6 | 5 |
| 52 | 5 | 4 | 1 | 1 | 5 | 5 | – | 5 | 5 |
| 53 | 5 | 4 | 1 | 1 | 7 | 5 | – | 5 | 5 |
| 54 | 4 | 4 | 1 | 1 | 7 | 5 | – | 5 | 5 |
| 55 | 8 | 4 | 1 | 1 | 6 | 5 | – | 5 | 5 |
| 56 | 5 | 4 | 1 | 1 | 6 | 5 | – | 7 | 5 |
| 57 | 5 | 5 | 1 | 1 | 6 | 5 | – | 7 | 5 |
| 58 | 5 | 7 | 2 | 1 | 6 | 5 | – | 7 | 5 |
| 59 | 5 | 4 | 1 | 3 | 6 | 5 | – | 6 | 5 |
| 60 | 5 | 4 | 3 | 1 | 6 | 5 | – | 6 | 5 |
| 61 | 5 | 4 | 1 | 1 | 6 | 5 | – | 6 | 5 |
| 62 | 5 | 4 | 1 | 1 | 7 | 6 | – | 7 | 5 |
| 63 | 5 | 5 | 1 | 3 | 7 | 6 | – | 6 | 5 |
| 64 | 5 | 5 | 1 | 2 | 7 | 6 | – | 7 | 5 |
| 65 | 5 | 5 | 1 | 2 | 6 | 6 | – | 7 | 5 |
| 66 | 4 | 5 | 1 | 1 | 5 | 6 | – | 7 | 5 |
| 67 | 4 | 5 | 1 | 3 | 6 | 6 | – | 7 | 5 |
| 68 | 4 | 5 | 1 | 3 | 5 | 6 | – | 5 | 5 |
| 69 | 5 | 4 | 2 | 2 | 6 | 5 | – | 8 | 5 |
| 70 | 5 | 4 | 3 | 2 | 6 | 5 | – | 5 | 5 |
| 71 | 5 | 5 | 2 | 2 | 6 | 5 | – | 6 | 5 |
| 72 | 4 | 5 | 1.5 | 2 | 6 | 5 | – | 5 | 5 |
| 73 | 4 | 5 | 2 | 2 | 6 | 5 | – | 5 | 5 |
| 74 | 6 | 5 | 1 | 2 | 6 | 5 | – | 5 | 5 |
| 75 | 4 | 5 | 1 | 2 | 6 | 5 | – | 4 | 5 |
| 76 | 4 | 5 | 1 | 2 | 6 | 5 | – | 6 | 5 |
| 77 | 4 | 5 | 1 | 2 | 5 | 5 | – | 6 | 5 |
| 78 | 8 | 1 | 1 | 2 | 6 | 4 | – | 5 | 5 |
| 79 | 5 | 5 | 1 | 2 | 6 | 4 | – | 5 | 5 |
| 80 | 4 | 4 | 2 | 3 | 6 | 5 | – | 5 | 5 |
| 81 | 6 | 5 | 2 | 1 | 6 | 5 | – | 5 | 5 |
| 82 | 6 | 5 | 1 | 1 | 6 | 6 | – | 5 | 5 |
| 83 | 6 | 6 | 1 | 1 | 6 | 6 | – | 5 | 5 |
| 84 | 4 | 5 | 2 | 1 | 7 | 4 | – | 6 | 5 |
| 85 | 5 | 5 | 2 | 1 | 5 | 4 | – | 4 | 5 |
| 86 | 4 | 6 | 7 | 4 | – | 6 | 5 | 7 | 5 |
| 87 | 3 | 6 | 6 | 4 | – | 6 | 5 | 7 | 5 |
| 88 | 3 | 6 | 7 | 4 | – | 6 | 5 | 8 | 5 |
| 89 | 3 | 6 | 7 | 4 | – | 6 | 6 | 7 | 5 |
| 90 | 4 | 7 | 7 | 4 | – | 5 | 5 | 7 | 5 |
| 91 | 7 | 7.5 | 1.5 | 4 | 7 | 4 | – | 7 | 5 |
| 92 | 9 | 7.5 | 2 | 3 | 7 | 6 | – | 7 | 5 |
| 93 | 8 | 7 | 1 | 4 | 7 | 5 | – | 7 | 5 |
| 94 | 7 | 7 | 1.5 | 4 | 7.5 | 6 | – | 6 | 5 |
| 95 | 7 | 7 | 1 | 3 | 7 | 4 | – | 6 | 5 |
| 96 | 7 | 7 | 2 | 4 | 6.5 | 4 | – | 6 | 5 |
| 97 | 7 | 6 | 2 | 3 | 6.5 | 4 | – | 5 | 5 |
| 98 | 7 | 6 | 4 | 3 | 6.5 | 4 | – | 4 | 5 |
| 99 | 6 | 3 | 2 | 3 | – | 6 | 6 | 8 | 6 |
| 100 | 7 | 2 | 2 | 3 | – | 6 | 5 | 9 | 6 |
| 101 | 7 | 2 | 3 | 3 | – | 7 | 5 | 8 | 7 |
| 102 | 8 | 3 | 3 | 4 | – | 7 | 5 | 8 | 7 |
| 103 | 8 | 2 | 3 | 3 | – | 6 | 6 | 9 | 7 |
| 104 | 3 | 2 | 3 | 3 | – | 6 | 6 | 8 | 7 |
| 105 | 3 | 3 | 4 | 3 | – | 6 | 5 | 5 | 7 |
| 106 | 4 | 2 | 4 | 3 | – | 6 | 5 | 9 | 7 |
| 107 | 4 | 2 | 4 | 2 | – | 6 | 5 | 6 | 7 |
| 108 | 4 | 2 | 4 | 3 | – | 6 | 5 | 6 | 7 |
| 109 | 7 | 6 | 7 | 5 | – | 7 | 7 | 5 | 6 |
| 110 | 7 | 6 | 7 | 5 | – | 7 | 7 | 6 | 6 |

[a] Abbreviations: MDH, NAD-malate dehydrogenase; IDH, isocitrate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; XDH, xanthine dehydrogenase; LDH1, leucine dehydrogenase in Tris citrate (pH 8); LYD, lysine dehydrogenase; LDH2, leucine dehydrogenase in the borate (pH 8.2) system; ADH, alcohol dehydrogenase; IPO, indophenol oxidase.

[b] –, No detectable activity in this buffer system.

analyzed the genetic diversity of our sample together with bona fide *R. leguminosarum* bv. phaseoli type I strains. Several strains were isolates from the same field collected at the same time from nodules found on the roots of the bean plants, and others were reference strains from our collection (Table 1). To establish whether the isolates belonged exclusively to *R. leguminosarum* bv. phaseoli type I strains or whether they could be other *Rhizobium* species, we incorporated isolates of other biovars or species: *R. leguminosarum* bv. phaseoli type II (12), *R. leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, *R. meliloti*, *R. loti*, *Sinorhizobium fredii*, and *A. tumefaciens*.

Cluster analysis of the ETs showed four defined divisions, I (ETs 1 to 85), II (ETs 86 to 98), III (ETs 99 to 108), and IV

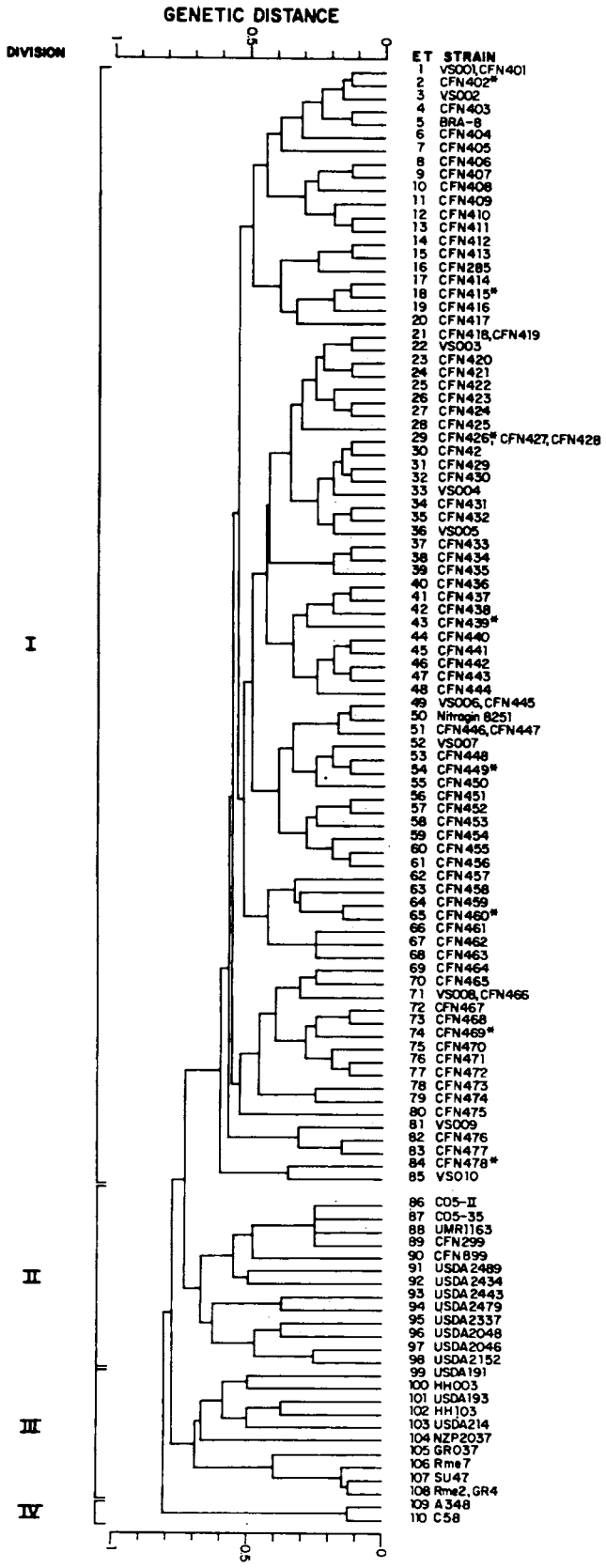


FIG. 1. Genetic relatedness among 110 ETs of *Rhizobium* spp. and nonsymbiotic isolates based on electrophoretically detectable allelic variation at nine enzyme loci (Table 2). Strains used in rRNA operon RFLP analysis, sym plasmid transfer, and DNA relative hybridization are marked with an asterisk. I, II, III, and IV correspond to the divisions as defined in the text.

TABLE 3. Genetic diversity at nine enzyme loci among ETs

| Enzyme locus[a] | Characteristics of: | | | |
|---|---|---|---|---|
| | 110 ETs[b] | | 38 ETs[c] | |
| | No. of alleles | Genetic diversity (*h*) | No. of alleles | Genetic diversity (*h*) |
| MDH | 7 | 0.723 | 6 | 0.592 |
| IDH | 9 | 0.762 | 7 | 0.558 |
| G6P | 6 | 0.663 | 4 | 0.525 |
| XDH | 5 | 0.728 | 4 | 0.589 |
| LDH1 | 5 | 0.739 | 3 | 0.556 |
| LYD | 4 | 0.633 | 4 | 0.574 |
| LDH2 | 3 | 0.978 | 0 | – |
| ADH | 6 | 0.704 | 5 | 0.643 |
| IPO | 4 | 0.272 | 1 | 0 |
| Mean | 5.4 | 0.689 | 4.5[d] | 0.504[d] |

[a] Abbreviations are as in Table 2, footnote *a*.
[b] Represented by the total sample of 121 isolates examined.
[c] Represented by division I, the *R. leguminosarum* bv. phaseoli type I cluster.
[d] Means were calculated without taking LDH2 into account.

(ETs 109 and 110) (Fig. 1). All the nonsymbiotic isolates were enclosed in division I with a genetic distance of 0.6. This division also included all the *R. leguminosarum* bv. phaseoli type I strains. Cluster I was separated from all the other divisions at a genetic distance of at least 0.73. The outermost group, division IV, at a distance of 0.81, was formed by the *A. tumefaciens* strains; division III, at 0.78, was composed, on one extremity, of the *R. meliloti* strains forming a tight cluster at 0.4, and on the other, the single *R. loti* strain separated by 0.67 from the *S. fredii* strains in a 0.59 cluster. Division II exhibited the most diverse composition: one cluster formed by the three *R. leguminosarum* bv. trifolii strains and three of the *R. leguminosarum* bv. viciae strains at a genetic distance of 0.63 divided by the 0.67 of a second cluster made up of the remaining *R. leguminosarum* bv. viciae strains and the *R. leguminosarum* bv. phaseoli type II strains.

In this analysis, all enzymes assayed were polymorphic, with allele frequencies (*h*) ranging from 0.272 to 0.978 for indophenol oxidase and LDH2, respectively (Table 3), and a mean genetic diversity for the whole collection of $H = 0.689$, including all species. In estimations of the genetic diversity of division I only (Fig. 1), the *Rhizobium leguminosarum* bv. phaseoli cluster, indophenol oxidase became monomorphic, as reported by Eardly et al. for *R. meliloti* (6), and LDH2 was not measurable as it was not present in this cluster (Table 3); the mean genetic diversity was $H = 0.504$, which is slightly lower than the 0.615 value found by Piñero et al. for a collection of *R. leguminosarum* bv. phaseoli type I strains (19).

**Ribosomal hybridization RFLPs.** Eight nonsymbiotic isolates (strains CFN402, CFN415, CFN426, CFN439, CFN

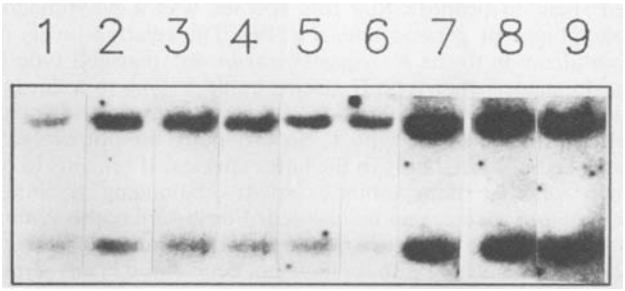


FIG. 2. Hybridization patterns of *Sau*3A-digested total DNA of eight nonsymbiotic isolates and six *R. leguminosarum* bv. phaseoli reference strains probed with plasmid pKK3535. Lanes: 1, CFN402; 2, CFN415; 3, CFN426; 4, CFN439; 5, CFN449; 6, CFN460; 7, CFN469; 8, CFN478; 9, CFNX78.

```
          10         20         30         40         50
          |          |          |          |          |
  1 CTGCAGAGAT GCAGGGGTTC TTCGGGACCG GCACACAGGT GCTGCATGGC
 51 TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC
101 AACCCTCGCC CTTAGTTGCC AGCATTTGGT TGGGCACTCT AAGGGGACTG
151 CCGGTGATAA GCCGAGAGGA AGGTGGGGAT GACGTCAAGT CCTCATGGCC
201 CTTACGGGCT GGGCTACACA CGTGCTACAA TGGTGGTGAC AGTGGGCAGC
251 GAGCACGCGA GTGTGAGCTA ATCTCCAAAA GCCATCTCAG TTCGGATTGC
301 ACTCTGCAAC TCGAGTGCAT GAAGTTGGAA TCGCTAGTAA TCGCGGATCA
351 GCATGC
```

FIG. 3. Nucleotide sequence of an *R. leguminosarum* bv. phaseoli 16S rRNA gene fragment.

449, CFN460, CFN469, and CFN478) belonging to the main groups found on the genetic distance tree in division I were selected for RFLP analysis of the rRNA operons. Total DNA *Sau*3A digests were blotted and hybridized with plasmid pKK3535. The hybridization patterns of the nonsymbiotic isolates were the same as that of *R. leguminosarum* bv. phaseoli type I strains (Fig. 2).

**Nucleotide sequence of the 16S rRNA gene.** Double-stranded polymerase chain reaction products of a fragment of the 16S rRNA gene from three nonsymbiotic isolates (CFN426, CFN439, and CFN460) and from the wild-type strain CFN42 were sequenced as described in Materials and Methods. The sequenced region encompasses a zone of variation among species (15). All proved to be identical in the 356-bp sequence (Fig. 3).

**DNA homology among nonsymbiotic isolates.** To estimate DNA sequence similarity coefficients, we measured the total DNA homologous hybridization percentage of the eight nonsymbiotic isolates probed with $^{32}$P-labeled total DNA from strains CFN2001 and AD822, two derivatives cured of their symbiotic plasmids belonging to *R. leguminosarum* bv. phaseoli types I and II, respectively. All the *R. leguminosarum* bv. phaseoli type I strains, as well as the nonsymbiotic isolates, have similar relative percentages of hybridization (≤59%) to strain CFN2001 (Table 4). The two *R. leguminosarum* bv. *viciae* and *trifolii* strains, USDA2337 and USDA2048, have, respectively, 48 and 49% homology to strain CFN2001. The *R. leguminosarum* bv. phaseoli type II strain CIAT899 and *R. meliloti* SU47 both have low levels of homology to strain CFN2001 (28 and 26%, respectively). All strains have low levels of homology to *R. leguminosarum* bv. phaseoli type II strain AD822, except for its parent strain, CIAT899 (Table 4).

**Symbiotic effectiveness of nonsymbiotic derivatives carrying a sym plasmid.** The symbiotic plasmid of *R. leguminosarum* bv. phaseoli type I strain CFN42 (p42d) was transferred to spontaneous Sp$^r$ derivatives of the eight nonsymbiotic isolates in triparental matings with strain CFNX78 as the donor and *E. coli* HB101(pRK2013) as the mobilization helper.

Bean plants, four per strain, were inoculated with the transconjugants, and 2 weeks after inoculation nitrogen fixation levels were determined by the acetylene reduction assay. Under our conditions, all plants were nodulated and the levels of nitrogen fixation were comparable to those of the parental wild-type strain CFNX78 (data not shown).

To verify that the nodules had indeed been formed by the transconjugants, three nodules from each plant were surface sterilized and crushed on PY plates devoid of any antibiotic. Twenty isolated colonies from each nodule were tested for their resistances to kanamycin, spectinomycin, and nalidixic acid. All colonies tested were Sp$^r$, Km$^r$, and Nal$^r$. Strains were further authenticated by their *nifH* and cos309 *Eco*RI RFLPs (Fig. 4).

## DISCUSSION

**Genetic relatedness of the nonsymbiotic isolates.** We report here the isolation from soil of a population of nonsymbiotic *R. leguminosarum* strains. These strains were selected by their ability to grow in different media (PY, YM, and MM-Lac) supplemented with nalidixic acid at 30°C and their inability to grow at 37°C or in LB medium. These are traits known to be common to all *R. leguminosarum* bv. viciae, trifolii, and phaseoli type I strains. This screening eliminates strains belonging to different *Rhizobium* species; for instance the *R. leguminosarum* bv. phaseoli type II strains are capable of growth at 37°C and in LB (11), some Brazilian type I isolates can also grow at 37°C but not in LB, and not all *R. meliloti* strains are Nal$^r$.

The genetic structure of this collection, as determined by the MLEE data, shows that the nonsymbiotic strains are clustered with the *R. leguminosarum* bv. phaseoli type I strains. The DNA sequence similarity coefficients of the nonsymbiotic strains were determined by hybridizing, under

TABLE 4. Relative levels of DNA-DNA hybridization at 65°C[a]

| Source of probe | % Homology to probe source for strain[b]: | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CFN 402 | CFN 415 | CFN 426 | CFN 439 | CFN 449 | CFN 460 | CFN 469 | CFN 478 | NIT-8251 | CFN 285 | BRA 8 | CFN 42 | USDA 2048 | USDA 2337 | CIAT 899 | SU 47 |
| CFN2001 | 65 | 63 | 84 | 73 | 69 | 65 | 60 | 59 | 77 | 63 | 62 | 100 | 49 | 48 | 28 | 26 |
| AD822 | 15 | 17 | 18 | 14 | 15 | 17 | 18 | 20 | 16 | 17 | 20 | 18 | 25 | 23 | 100 | 15 |

[a] See text for details.
[b] Strain phenotypes and species are described in Table 1.

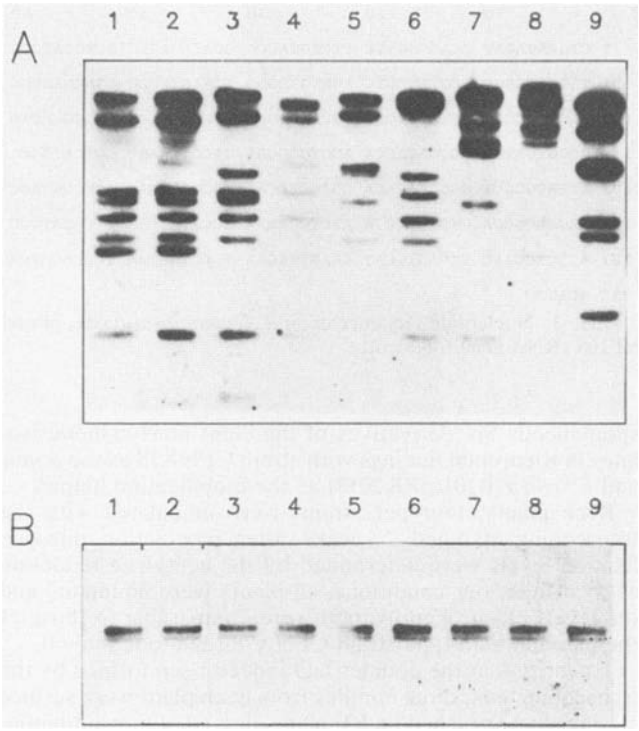


FIG. 4. RFLPs of *Eco*RI-digested total DNAs of eight transconjugants of nonsymbiotic derivatives carrying the p42d sym plasmid and *R. leguminosarum* bv. phaseoli CFNX78. (A) Hybridization pattern with cos309 as the probe. (B) Hybridization pattern with pCQ152 as the probe. Lanes: 1, CFN402; 2, CFN415; 3, CFN426; 4, CFN439; 5, CFN449; 6, CFN460; 7, CFN469; 8, CFN478; 9, CFNX78.

high-stringency conditions, blotted total DNA with labeled total DNAs from strains cured of their symbiotic plasmids. These strains were used as probes to minimize the signals due exclusively to the sym plasmids, which would artificially enhance the hybridization levels of symbiotic strains compared with the nonsymbiotic isolates. The relative levels of homology also group the nonsymbiotic isolates with the reference *R. leguminosarum* bv. phaseoli type I strains. These data are in accordance with previous studies of other genera of bacteria, in which it has been shown that the estimates of genetic relatedness of strains obtained by MLEE and by DNA-DNA hybridization are strongly correlated (21). Furthermore, the nonsymbiotic isolates and the *R. leguminosarum* bv. phaseoli type I strains have identical ribosomal hybridization patterns and identical nucleotide sequences in a fragment of the 16S rRNA gene. These data, taken in conjunction, strongly indicate that the nonsymbiotic isolates are indeed *R. leguminosarum*, even though they are incapable of establishing a symbiosis with the plant.

**Taxonomy of *Rhizobium* species.** The different divisions found in our analysis reflect the taxonomy as defined in the current *Rhizobium* systematics, apart from the fact that the *R. leguminosarum* bv. phaseoli type I strains seem to form a division detached from *R. leguminosarum* bv. viciae and trifolii and from *R. leguminosarum* bv. phaseoli type II (12). This is supported by the observation that the most diverse group in the *R. leguminosarum* species is that of the biovar phaseoli (19). Piñero et al. found a 0.691 level of mean genetic diversity for *R. leguminosarum* bv. phaseoli, which led them to propose that this species was a polyphyletic assemblage of genetic lineages (19). The relative levels of hybridization to the *R. leguminosarum* bv. phaseoli type II strain suggest that type II strains could belong to a lineage independent from that of *R. leguminosarum* bv. viciae, trifolii, and phaseoli type I. So far, nonsymbiotic isolates have been isolated only in the latter species. It remains to be seen whether nonsymbiotic isolates belonging to other *Rhizobium* species can be isolated. For instance, the symbiotic information resides in nontransmissible megaplasmids in *R. meliloti*, and so far these have not been cured in any strain belonging to this species.

**Ecological relevance of the nonsymbiotic *R. leguminosarum* strains.** *Rhizobium* species are common inhabitants of soils. A measure of their adaptation is the persistence of strains in soil in the absence of their host plants.

When complemented with a sym plasmid, nonsymbiotic strains are indistinguishable from the symbiotic strains because they have the same metabolic characteristics and are capable of fixing nitrogen in symbiosis with beans (data not shown). Although the nitrogenase activities and nodule numbers are comparable to those obtained with a control strain under our laboratory conditions, we do not know whether these strains have the same competitive abilities as the natural symbiotic strains for nodulation in the soil. The generation of new symbiotic strains with new adaptive traits thus would also depend on the potential for lateral transfer of genes. Interstrain plasmid transfer seems to be a rather common phenomenon in *Rhizobium* species, although its frequency in soil seems to be much lower than that determined under laboratory conditions (4, 8). The natural occurrence of interstrain plasmid transfer is substantiated by inconsistencies found by grouping strains under plasmid genotypes defined by RFLPs and under chromosomal genotypes defined by either RFLPs or MLEE (10, 21, 26).

In our screening we found a ratio of 1 symbiotic strain to 40 nonsymbiotic strains. The true proportion may very well be higher because we cannot reject the possibility that the symbiotic strains escaped from the nodules in the roots from which we isolated our strains. This ratio could vary as a function of the time and method of sampling and the prevailing conditions in the soil.

Because the genus *Rhizobium* is defined by an ability to establish a symbiosis with the roots of legumes, nonsymbiotic isolates, although chromosomally identical, are excluded. As other authors have already pointed out, a biologically meaningful classification of the genus *Rhizobium* should be based on allelic variation in chromosomal genes rather than on plasmid-encoded phenotypes (6, 19). The inclusion of nonsymbiotic strains in the phylogeny of *Rhizobium* species provides a new framework for the understanding of the evolutionary history of the bacterium-plant interaction. To further understand the ecology of *Rhizobium* species it will be necessary to take into account the existence of these apparently abundant nonsymbiotic *Rhizobium* species and their participation in the generation of new symbiotic strains with different adaptive traits.

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