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Genetic Diversity and Host Range of Rhizobia Nodulating *Lotus tenuis* in Typical Soils of the Salado River Basin (Argentina)▽†

María Julia Estrella,[1,2] Socorro Muñoz,[1] María José Soto,[1] Oscar Ruiz,[2] and Juan Sanjuán[1]*

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidin, CSIC, Granada, Spain,[1] and Unidad de Biotecnología 3, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), UNSAM-CONICET, Chascomús, Buenos Aires, Argentina[2]

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A total of 103 root nodule isolates were used to estimate the diversity of bacteria nodulating *Lotus tenuis* in typical soils of the Salado River Basin. A high level of genetic diversity was revealed by repetitive extragenic palindromic PCR, and 77 isolates with unique genomic fingerprints were further differentiated into two clusters, clusters A and B, after 16S rRNA restriction fragment length polymorphism analysis. Cluster A strains appeared to be related to the genus *Mesorhizobium*, whereas cluster B was related to the genus *Rhizobium*. 16S rRNA sequence and phylogenetic analysis further supported the distribution of most of the symbiotic isolates in either *Rhizobium* or *Mesorhizobium*: the only exception was isolate BA135, whose 16S rRNA gene was closely related to the 16S rRNA gene of the genus *Aminobacter*. Most *Mesorhizobium*-like isolates were closely related to *Mesorhizobium amorphae*, *Mesorhizobium mediterraneum*, *Mesorhizobium tianshanense*, or the broad-host-range strain NZP2037, but surprisingly few isolates grouped with *Mesorhizobium loti* type strain NZP2213. *Rhizobium*-like strains were related to *Rhizobium gallicum*, *Rhizobium etli*, or *Rhizobium tropici*, for which *Phaseolus vulgaris* is a common host. However, no *nodC* or *nifH* genes could be amplified from the *L. tenuis* isolates, suggesting that they have rather divergent symbiosis genes. In contrast, *nodC* genes from the *Mesorhizobium* and *Aminobacter* strains were closely related to *nodC* genes from narrow-host-range *M. loti* strains. Likewise, *nifH* gene sequences were very highly conserved among the Argentinian isolates and reference *Lotus* rhizobia. The high levels of conservation of the *nodC* and *nifH* genes suggest that there was a common origin of the symbiosis genes in narrow-host-range *Lotus* symbionts, supporting the hypothesis that both intrageneric horizontal gene transfer and intergeneric horizontal gene transfer are important mechanisms for the spread of symbiotic capacity in the Salado River Basin.

The so-called Salado River Basin in Buenos Aires Province is the most important region devoted to beef and dairy cattle production in Argentina, and natural and cultivated grasslands are the main forage resource in this area (43). The growth and productivity of pastures in this region are limited by alternating cycles of extreme water excess and drought conditions. Soils in the Salado River Basin are heterogeneous and poorly drained and have low nutrient contents, high levels of sodic salts, and alkaline pHs (38, 57).

Legumes have the ability to establish mutualistic symbiotic relationships with soil bacteria collectively known as rhizobia, and these relationships allow the legumes to be independent of nitrogen levels in the soil. *Lotus tenuis* is a valuable forage legume native to the Mediterranean region which has become naturalized in the Salado River Basin during the last few decades and has shown good potential for adaptation to the soils there (32, 44). Thus, cultivation of *L. tenuis* could contribute to improvements in forage quality and production in the Salado River Basin.

It is generally accepted that *Lotus* species establish highly specific nitrogen-fixing symbioses with bacteria belonging to the genera *Mesorhizobium* and *Bradyrhizobium* (30, 31, 33). Based on the rhizobial partners, two groups can be distinguished in the genus *Lotus.* One of these groups includes *Lotus corniculatus* and *L. tenuis*, two species that form nitrogen-fixing nodules only with bacteria belonging to the genus *Mesorhizobium* (particularly *Mesorhizobium loti*). The second group comprises species like *Lotus subbiflorus* and *Lotus uliginosus*, which establish nitrogen-fixing nodules mainly in association with slow-growing *Bradyrhizobium*-like strains (4, 8, 28, 45). As a general rule, the rhizobia nodulating *Lotus* species have a narrow host range, but some *Mesorhizobium* strains (e.g., strain NZP2037) are thought to have a broad host range since they can form nitrogen-fixing nodules on most *Lotus* species. However, the symbiotic effectiveness of broad-host-range mesorhizobia is low compared with that of narrow-host-range strains (6, 50, 60).

Legume inoculants are usually based on selected highly efficient rhizobia and evaluated in particular environments. However, inoculant success is frequently limited by the presence of native soil rhizobia (14, 64). Inoculation often leads to improved productivity in soils with no previous history of the host legume where native rhizobial populations are small or nonexistent, whereas inoculant success is uncertain in soils where the native rhizobial populations are large. This is frequently due to the superior competitive ability of native strains, which occupy the majority of nodules under field conditions because of their large populations, their distribution through-

* Corresponding author. Mailing address: Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain. Phone: 34-958181600, ext. 259. Fax: 34-958129600. E-mail: juan.sanjuan@eez.csic.es.

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TABLE 1. Seventy-seven isolates from *L. tenuis* nodules, their habitats, and the chemical properties of the corresponding soils in the Salado River Basin

Isolates	Soil properties			
	Soil type or habitat	pH	Electrical conductivity (mS/cm)	Na^+ level (% of total)
BA148, BA146, BA128, BA125, BA124, BA120, BA116, BA144, BA139, BA135, BA115, BA150, BA151, BA152, BA138, BA140, BA121, BA136, BA143, BA149, BA131, BA134, BA113, BA123	Saline lowlands	9.7	7.93	48.3
BD53, BD61, BD46, BD68, BD65, BD66, BD74, BD51, BD67, BD70, BD72, BD56, BD44, BD60, BD50, BD63, BD57, BD58, BD41, BD48, BD43, BD45, BD40, BD47, BD49, BD59, BD55, BD54	Lowlands	5.66	0.51	9.6
ML108, ML106, ML110, ML90, ML79, ML104, ML105, ML91, ML103, ML100, ML93, ML83, ML92, ML77, ML101, ML87, ML98, ML95, ML97, ML85, ML81, ML96, ML88, ML102, ML84	Transitional plains	6.2	0.33	8.2

out the soil profile, or their better adaptation to the local soil environment (14, 64).

Inoculation of *L. tenuis* with selected rhizobia is expected to increase forage production in the Salado River Basin. However, knowledge about native rhizobia nodulating *Lotus* spp. in Argentinean soils is sparse. A preliminary study of rhizobia able to nodulate *L. tenuis* in the Salado River Basin suggested that there is considerable diversity (20). However, detailed information regarding taxonomic and physiological features of native rhizobial strains in this region is not available. The aim of the present work was to characterize bacteria able to nodulate *L. tenuis* that were obtained from three different soil environments typical of the Salado River Basin.

MATERIALS AND METHODS

Collection of rhizobia and culture conditions. Samples from three soil environments typical of the Salado River Basin (saline lowlands, nonsaline lowlands, and transitional plains) were composed of mixtures of subsamples taken at several sites located in Chascomús County in Buenos Aires Province. Soils samples were transported from the field to the laboratory, stored at 4°C for 2 days, and then used for recovery of rhizobia by using *L. tenuis* cultivar Pampa INTA as the trap plant. The isolates obtained are listed in Table 1. Yeast extract-mannitol medium was routinely used for rhizobial isolation, purification, and culture (71). All strains were stored at −80°C in the same medium with 20% (vol/vol) glycerol.

Isolation of genomic DNA. Total DNA was extracted from 3-ml cultures of the bacterial isolates grown in yeast extract-mannitol broth (71) at 30°C. Cultures were centrifuged at 13,500 × *g* for 3 min and washed in 0.5 ml of 0.1% (wt/vol) *N*-lauroylsarcosine in Tris-EDTA buffer (TE buffer) (10 mM Tris, 1 mM EDTA; pH 8). The pellets were suspended in 1 ml of 1 M NaCl and incubated for 1 h at 4°C in an orbital shaker. After this, the cell suspensions were centrifuged at 13,500 × *g* for 3 min, and the supernatants were removed. The pellets were suspended in 0.25 ml of 20% (wt/vol) sucrose in TE buffer prior to addition of 0.25 ml of a lysozyme solution (5 mg lysozyme in TE buffer) and 0.25 ml of RNase (1 mg/ml). After 30 min of incubation at 37°C, 0.1 ml pronase (10 mg/ml pronase in 5% *N*-lauroylsarcosine) was added, and the mixtures were incubated for 1 h at 37°C. A 0.07-ml aliquot of 3 M sodium acetate (pH 5.2) was added, and lysates were extracted with 0.4 ml of phenol–chloroform–isoamyl alcohol (25:24:1) saturated with 10 mM Tris-HCl (pH 8). The mixtures were vortexed and centrifuged at 13,500 × *g* for 3 min. Each aqueous phase was transferred to a clean tube, and 0.3 ml chloroform was added. The tubes were then vortexed and centrifuged at 13,500 × *g* for 3 min, the aqueous phase was transferred to clean tubes, and the DNA was precipitated with 0.7 ml of isopropanol. The precipitated DNA was washed with 0.5 ml of 70% ethanol, vacuum dried, and subsequently dissolved in 0.1 ml of MilliQ water by incubation for 30 min at 65°C.

Repetitive extragenic palindromic PCR (REP-PCR) genomic fingerprinting. Primers REP1R-1 (5′-IIIICGICGICATCIGGC-3′) and REP 2I (5′-ICGICTTA TCIGGCCTAC-3′) (70) were synthesized by the Service of Oligonucleotide Synthesis of the "Instituto de Parasitología y Biomedicina Lopez Neyra," CSIC, Spain. PCR amplification was carried out by using a 0.025-ml reaction mixture containing 100 ng of genomic DNA, 2 μM primer REP1R-1, 2 μM primer REP2-1, each deoxynucleoside triphosphate (Roche) at a concentration of 1.25 mM, 1× polymerase reaction buffer (Sigma, United States), 7 mM $MgCl_2$, and 4 U *Taq* DNA polymerase (Sigma, United States). Amplification was performed with a Px2 thermal cycler (Thermo Electron Corporation, United States) using the following temperature profile: initial denaturation at 95°C for 6 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and elongation at 65°C for 8 min; and a final extension for 16 min at 65°C (27). Amplified fragments were separated by electrophoresis in 1.5% agarose gels for 2 h at 85 V in 1× Tris-borate-EDTA buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA; pH 8.5). A 100-bp DNA ladder (Roche, Molecular Biochemicals, New England Biolabs) was used as the size marker. Gels were stained in an aqueous solution containing ethidium bromide (0.1%) and photographed using a UV transilluminator.

Computer-assisted analysis of the REP-PCR genomic fingerprints was performed by using the BioNumerics software program, version 4.0 (Applied Maths, Kortrijk, Belgium). The similarity between a pair of REP-PCR genomic fingerprints was calculated by using the product-moment correlation coefficient (*r* value) (51) applied to the whole densitometric curves for the gel tracks (52). Cluster analysis of the pairwise similarity values was performed by using the unweighted-pair group method using averages (UPGMA) algorithm (63).

PCR amplification and RFLP analysis of amplified 16S rRNA genes. Nearly full-length 16S rRNA genes were amplified from isolates using primers 41f (5-GCTCAAGATTGAACGCTGGCG-3) and 1488r (5-CGGTTACCTTGTTA CGACTTCACC-3) as previously described (27). The isolates were grouped according to their restriction fragment length polymorphism (RFLP) patterns into rRNA types, compared with 33 reference strains belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium* (Table 2), and analyzed further with the PAST software, version 1.30 (26). Levels of similarity for the 16S rRNA gene sequences of isolates and reference strains were estimated by using the proportion of shared restriction fragments. A dendrogram was constructed using the UPGMA (63).

PCR amplification of *nodC* and *nifH* genes. Forward primers nodCF, nodCFu, nodCF2, nodCF4, and nodCFn and reverse primer nodCI were used for amplification of *nodC* genes, as described by Laguerre and coworkers (35). Primers nifHI and nifHF were used for PCR amplification of about 780 bp of the *nifH* gene (35).

Sequencing of 16S rRNA, *nodC*, and *nifH* DNA fragments. PCR products were purified by electroelution using a dialysis membrane (Spectra/Por; Spectrum Laboratories, Inc.) and were sequenced by The Sequencing Service of the Instituto de Parasitología y Biomedicina "Lopez Neyra," CSIC, Spain. The 16S rRNA was sequenced using primers 41f and 1488r. In order to sequence the central portion of the amplified 16S rRNA genes, primer U-447 (5-GCGGTAATACG AAGG-3) was used. PCR products of *nodC* genes were sequenced using primers nodCF and nodCI. PCR products of the *nifH* gene were sequenced using primers nifHI and nifHF. The sequences obtained were compared with the sequences of reference strains deposited in the GenBank by using the BLASTN program (http://www.ncbi.nlm.nih.gov/blast).

PCR amplification and sequencing of *atpD* gene. Approximately 450 bp of the *atpD* gene was amplified by using forward primer atpD1 or atpD2 and reverse primer atpD3 (22). Purified DNA was added to a 50-μl PCR mixture containing each deoxynucleoside triphosphate at a concentration of 200 nM, 1.5 mM $MgCl_2$, 10 pmol each primer, 1 U *Taq* DNA polymerase, and 1× reaction buffer (Pro-

TABLE 2. Reference strains and genes used in this study

Strain	Species	Host plant (origin)	16S rRNA accession no.	*nodC* accession no.	*nifH* accession no.	Reference
LMG6133	*Sinorhizobium meliloti*	*Medicago sativa*	X67222.2			74
IAM12611	*Sinorhizobium meliloti*	*Medicago sativa*	D14509.1			49
ATCC 35423	*Sinorhizobium fredii*	*Glycine max*	D14516.1			49
USDA 205	*Sinorhizobium fredii*	*Glycine max*	AY260147.1			58
NGR234	*Sinorhizobium fredii*	*Lotus purpureus*	AY260149.1			58
CIAT899	*Rhizobium tropici*	*Phaseolus vulgaris*	U89832.1			68
IFO15247	*Rhizobium tropici*	*Phaseolus vulgaris*	D11344.1			49
LMG9517	*Rhizobium tropici*	*Phaseolus vulgaris*	X67234.2			74
	Rhizobium leguminosarum bv. trifolii	*Trifolium repens*	U31074.1			7
USDA2671	*Rhizobium leguminosarum* bv. phaseoli	*Phaseolus vulgaris*	U29388.1			68
	Rhizobium leguminosarum bv. viciae	*Pisum sativum*	U29386.1			68
CFN 42	*Rhizobium etli*	*Phaseolus vulgaris*	U28916.1			68
SEMIA384	*Rhizobium etli*	*Phaseolus vulgaris*	AY904730.1			42
R 602	*Rhizobium gallicum*	*Phaseolus vulgaris*	AF008130.1			62
ICMP 12856	*Agrobacterium rhizogenes*		AY626393.1			76
DSM 30105	*Agrobacterium tumefaciens*		M11223.1			75
hpig4.1	*Rhizobium* sp.	*Mimosa pigra*	AY691401.1			5
H152	*Rhizobium giardinii*	*Phaseolus vulgaris*	U86344.1			1
DSM6450^T	*Aminobacter aminovorans*		AB167232.1			21
UPM-Ca7	*Mesorhizobium ciceri*	*Cicer arietinum*	U07934.1			47
A-1Bs	*Mesorhizobium tianshanense*	*Glycyrrhiza pallidiflora*	AF041447.1			73
ORS1096	*Mesorhizobium plurifarium*	*Acacia tortilis*	AJ295079.1			2
UPM-Ca36	*Mesorhizobium mediterraneum*	*Cicer arietinum*	L38825.1			47
LMG PR5	*Mesorhizobium chacoense*	*Prosopis alba*	AJ278249.1			69
ACCC 19665	*Mesorhizobium amorphae*	*Amorpha fruticosa*	AF041442.1			73
IFO 15243	*Mesorhizobium huakuii*	*Astragalus sinicus*	D13431.1			49
NZP2037	*Mesorhizobium loti*	*Lotus divaricatus*	Y14159.1			12
NZP 2213	*Mesorhizobium loti*	*Lotus tenuis*	D14514.1			37
USDA 110	*Bradyrhizobium japonicum*	*Glycine max*	D13430.1			49
1021	*Sinorhizobium meliloti*	*Medicago sativa*		M11268		16
USDA 257	*Sinorhizobium fredii*	*Glycine max*		M73699		34
NGR234	*Sinorhizobium fredii*	*Lotus purpureus*		U00090		19
IIACFN299	*Rhizobium tropici*	*Phaseolus vulgaris*		X98514		13
USDA2071	*Rhizobium leguminosarum* bv. trifolii	*Trifolium repens*		AF217271		35
H132	*Rhizobium leguminosarum* bv. phaseoli	*Phaseolus vulgaris*		AF217263		35
CFN 42	*Rhizobium etli*	*Phaseolus vulgaris*		U80928		35
FL27	*Rhizobium gallicum*	*Phaseolus vulgaris*		AF217270		35
SN33	*Mesorhizobium* sp.	*Oxytropis arctobia*		U53327		9
IC2091	*Mesorhizobium ciceri*	*Cicer arietinum*		AJ457929		39
IC60	*Mesorhizobium mediterraneum*	*Cicer arietinum*		AJ457928		39
ACCC 19665	*Mesorhizobium amorphae*	*Amorpha fruticosa*		AF217261		35
MAFF303099	*Mesorhizobium loti*	*Lotus corniculatus*		BA000012		33
LMG 6123 (= NZP2037)	*Mesorhizobium loti*	*Lotus divaricatus*		X52958.2		10
R7A	*Mesorhizobium loti*	*Lotus corniculatus*		AL672113		65
CCBAU 43063	*Bradyrhizobium elkanii*	*Macroptilium atropurpureum*		DQ010040		24
USDA 110	*Bradyrhizobium japonicum*	*Glycine max*		AF322013		24
8c-3	*Rhizobium etli*	*Phaseolus vulgaris*			DQ058415	18
CIAT899	*Rhizobium tropici*	*Phaseolus vulgaris*			M55225	15
Rch981	*Mesorhizobium ciceri*	*Cicer arietinum*			AY318755	39
SU329	*Rhizobium leguminosarum* bv. trifolii	*Trifolium repens*			K00490	61
Ss140	*Mesorhizobium plurifarium*	*Sesbania sericea*			AY688619	72
NGR234	*Sinorhizobium* sp.	*Lotus purpureus*			U00090	19
R7A	*Mesorhizobium loti*	*Lotus corniculatus*			AL672114	65
MAFF303099	*Mesorhizobium loti*	*Lotus corniculatus*			BA000012	33
RCAN13	*Mesorhizobium amorphae*	*Cicer arietinum*			DQ022841	54
FL27	*Rhizobium leguminosarum* bv. phaseoli	*Phaseolus vulgaris*			M55226	15
CCBAU 57015	*Rhizobium hainanense*	*Desmodium sinuatum*			AY934876	25
R602	*Rhizobium gallicum*	*Phaseolus vulgaris*			AF218126	35
CCBAU 65199	*Bradyrhizobium japonicum*	*Glycine max*			AY934872	25
CCBAU 23174	*Bradyrhizobium elkanii*	*Macroptilium atropurpureum*			AY934870	25

mega). The *atpD* gene was amplified as described previously by Gaunt et al. (22). The sequence obtained was compared with the sequences of reference strains deposited in the GenBank by using the BLASTN program (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analyses. Sequence alignment was performed with the ClustalW software from the EMBL server (http://www.ebi.ac.uk/). Aligned sequences were analyzed using the MEGA software, version 4.0 (67). Phylogenetic analyses of the 16S rRNA sequences were performed by the UPGMA (63). Phylogenetic

analyses of *nodC* and *nifH* sequences were performed by using the neighbor-joining method (56). The phylogenetic distances were computed by using the p-distance method and were calculated based on the proportion of different nucleotides (p-distance), which was obtained by dividing the number of nucleotide differences by the total number of nucleotides compared (46). Statistical support for tree nodes was evaluated by performing a bootstrap analysis (17).

DNA genomic hybridization. Total genomic DNAs were digested with endonuclease EcoRI, electrophoresed in 0.8% agarose gels, and then transferred to positively charged nylon membranes by the method of Southern (59). DNA hybridization probes were labeled with digoxigenin used according to the manufacturer's instructions (Roche, Barcelona, Spain). Hybridization and membrane washing were carried out under standard conditions. Membranes were prepared for chemiluminescence detection (Roche) and exposed to Kodak X-Omat film (Sigma).

Plant nodulation assays. Seeds of *L. tenuis* cv. Pampa INTA or *L. uliginosus* LE 627 (experimental line; INIA-La Estanzuela, Uruguay) were scarified and surface disinfected with concentrated sulfuric acid for 15 min. After acid treatment, the seeds were soaked for 2 min in a 1% aqueous sodium hypochlorite solution and then thoroughly washed with sterile water. The seeds were distributed on the surface of 1% water agar plates and incubated for 24 to 48 h at 25°C for germination in the dark. In order to evaluate the infectivity of isolates, 10 seedlings were transferred to square petri dishes containing Rigaud-Puppo agar (53). Seedlings were inoculated with individual strains by adding exponentially growing rhizobial cultures (10^8 cells per seedling). Uninoculated seedlings were used as negative controls. For nodulation assays with *L. tenuis* seedlings inoculated with *M. loti* NZP 2213 were used as positive controls, and for nodulation assays with *L. uliginosus* seedlings inoculated with *Bradyrhizobium loti* NZP 2309 were used as positive controls. In both assays, root nodules appeared 10 to 20 days after inoculation and were fully developed 2 weeks later.

Nucleotide sequence accession numbers. Sequences of the 16S rRNA genes obtained in this work have been deposited in the GenBank database under the following accession numbers: EU748908 (BD68), EU748909 (BD60), EU748910 (ML105), EU748911 (BA151), EU748912 (BA134), EU748913 (ML93), EU748914 (BA135), EU748915 (BD56), EU748916 (BA128), EU748917 (ML108), EU748918 (BD55), EU748919 (ML92), EU748920 (ML96), EU748921 (ML98), EU748922 (ML102), EU748923 (ML83), EU748924 (ML81), and EU748925 (ML85). Sequences of the *nodC* genes obtained in this work have been deposited in the GenBank database under the following accession numbers: EU748926 (ML93), EU748927 (BD56), EU748928 (ML108), EU748929 (BA135), EU748930 (BA128), EU748931 (BD68), EU748932 (BA151), EU748933 (ML105), and EU748934 (BA134). Sequences of the *nifH* genes obtained in this work have been deposited in the GenBank database under the following accession numbers: EU748935 (BA135), EU748936 (BD68), EU748937 (BA128), EU748938 (BA151), EU748939 (ML93), EU748940 (BA134), EU748941 (BD56), EU748942 (ML105), and EU748943 (ML108).

RESULTS

Analysis of REP-PCR genomic fingerprints. Purified genomic DNAs from 103 isolates were used as templates for PCR with REP primers to obtain DNA fingerprints. In the first step, REP fingerprints of isolates from each habitat were analyzed separately to determine similarities and to identify siblings (data not shown). As a general rule, each isolate had a unique and complex fingerprint, but some isolates with identical fingerprints were identified. In such cases, only one of the isolates was used for further analysis. A total of 77 fingerprints from all habitats were used to construct a dendrogram with the Bionumerics software (see Fig. S1 in the supplemental material). The REP-PCR analysis revealed a high level of genetic diversity among the isolates, and the degree of relatedness among strains isolated from different habitats ranged from 55 to 80% (see Fig. S1 in the supplemental material).

RFLP analysis of amplified 16S rRNA. The 16S rRNA genes of 77 nonredundant isolates were PCR amplified, which resulted in a single band at about 1,500 bp in all cases; this size corresponded to the expected size of the 16S rRNA genes of most members of the *Rhizobiaceae* (37). On the basis of previous work performed by Laguerre et al. (37), endonucleases HinfI and MspI were selected for 16S rRNA RFLP analysis in order to estimate the taxonomic positions of isolates by comparing their restriction profiles with the restriction profiles of 15 reference strains that represented different rhizobial species and genera (Table 2). This analysis resulted in 11 and 15 different restriction patterns for HinfI and MspI, respectively. Restriction patterns were arbitrarily identified by letters (Table 3) and used for classification of isolates into ribogroups. For the 77 isolates analyzed, 17 distinct ribogroups were distinguished, each comprising 1 to 19 isolates. A dendrogram constructed from the distance matrix as described in Materials and Methods confirmed the distribution of the isolates into two distinct clusters, clusters A and B, at a similarity level of 32%, and 17 subclusters that corresponded to the 17 ribogroups identified (Fig. 1). Only 7 of the 17 ribogroups identified matched any of the reference strains included in the analysis.

Isolates in cluster A, including isolates in ribogroups I, III, IV, VII, and X, comprised 61% of the strains analyzed and clustered with species of the genus *Mesorhizobium*, and the profiles of these ribogroups were identical to the profiles of reference strains *Mesorhizobium huakuii* IFO15243, *Mesorhizobium chacoense* LMGPR5, *M. loti* NZP2037, *Mesorhizobium tianshanense* A-1BS, and *Mesorhizobium mediterraneum* UMP-Ca36, respectively. Two cluster A isolates corresponded to ribogroup VI, which was not represented by any of the reference strains analyzed (Fig. 1 and Table 3). Cluster B comprised 39% of the strains analyzed, which appeared to be related to the genus *Rhizobium*. Some of the isolates in this cluster corresponded to ribogroups XIV and XX, whose profiles were identical to those of reference strains *Rhizobium etli* CFN42 and *Rhizobium tropici* IIB CIAT899, respectively. Other isolates in group B, corresponding to ribogroups XII, XIII, XV, XVII, XVIII, XIX, XXII, XXIII, and XXIV, did not match any of the reference strains used (Fig. 1 and Table 3).

When the environmental distribution of the isolates was examined, less taxonomic diversity was found in saline lowlands. Most the strains from this habitat were restricted to the genus *Mesorhizobium*; the only exception was isolate BA123, which grouped with reference strain *R. tropici* CIAT899. On the other hand, strains isolated from the nonsaline lowlands and transitional plains were found to be more diverse in terms of taxonomy, grouping with both the genus *Mesorhizobium* and the genus *Rhizobium* (Fig. 1 and Table 3).

Host range of *L. tenuis* isolates. The abilities of the 77 isolates to form nodules on the original host, *L. tenuis* cv. Pampa INTA, were examined. All 47 isolates in cluster A were able to nodulate and fix nitrogen on *L. tenuis* plants, as shown by the formation of pink nodules and the dark green color of the aerial parts of plants compared with noninoculated plants. In contrast, only isolates BD60, ML83, ML92, ML96, ML98, ML81, ML85, ML102, and BD55 in cluster B (related to the genus *Rhizobium*) were confirmed to nodulate *L. tenuis* and formed pink nodules similar to those formed by isolates in cluster A (Table 3). Nodulation of *L. tenuis* by the remaining isolates could not be verified under our experimental conditions, although it is worth noting that most ribogroups in 16S rRNA RFLP cluster B included both infective and noninfective isolates (Table 3).

On the other hand, several reports have suggested that most

TABLE 3. Restriction patterns of amplified 16S rRNA and 16S rRNA genotypes of rhizobial strains isolated from *L. tenuis* and reference strains

Isolates and/or reference strain[a]	Restriction pattern of amplified 16S rRNA digested with[b]:		RFLP ribogroup
	HinfI	MspI	
BA148, **BA146**, **BA128**, **BA125**, **BA124**, **BA120**, **BA116**, **BA144**, **ML108**, **ML106**, *Mesorhizobium huakuii* IFO15243	B	B	I
Mesorhizobium amorphae ACCC19665	B	D	II
BA139, **BA135**, *Mesorhizobium chacoense* LMGPR5	B	F	III
BD53, **BD61**, **BD46**, **BD68**, **BD65**, **BD66**, **BD74**, **BD51**, **BD67**, **BD70**, **BD72**, **ML110**, **ML90**, **ML79**, **ML104**, **ML105**, **ML91**, **ML103**, **ML100**, *M. loti* NZP2037	E	B	IV
Mesorhizobium plurifarium ORS 1096	E	D	V
BD56, **BD44**	E	A	VI
BA115, **BA150**, **BA151**, **BA152**, **BA138**, **BA140**, **BA121**, **BA136**, **BA143**, **BA149**, **BA131**, *Mesorhizobium tianshanense* A-1BS	C	C	VII
Mesorhizobium loti NZP2213	C	A	VIII
Mesorhizobium ciceri UPM-Ca7	A	A	IX
BA134, **BA113**, **ML93**, *Mesorhizobium mediterraneum* UMP-Ca36	A	C	X
Sinorhizobium fredii NGR234	A	J	XI
BD60	F	F	XII
BD50, BD63	D	E	XIII
ML83, **ML92**, ML77, ML101, ML87, **ML98**, *Rhizobium etli* CFN42	F	H	XIV
BD57, BD58	D	F	XV
Rhizobium leguminosarum bv. phaseoli USDA2671	D	H	XVI
ML95	F	N	XVII
ML97	F	K	XVIII
ML85, **ML81**	H	L	XIX
BD41, BD48, BD43, BD45, BD40, BD47, BD49, BA123, **ML96**, ML88, *Rhizobium tropici* IIB CIAT899	D	G	XX
Sinorhizobium meliloti LGM 6133	A	G	XXI
ML102, ML84	J	O	XXII
BD59	I	M	XXIII
BD55; BD54	F	E	XXIV
Bradyrhizobium japonicum USDA110	G	I	XXV

[a] Isolates that infect and do not infect *L. tenuis* are indicated by bold and light type, respectively.
[b] Different restriction patterns are indicated by different letters for each endonuclease used.

rhizobia that nodulate *L. tenuis* have a narrow host range for nodulation of *Lotus* spp. and cannot form nitrogen-fixing nodules on *L. uliginosus* (30, 45); the exceptions are strains, like *M. loti* NZP2037, that are considered broad-host-range strains since they can form nitrogen-fixing nodules on most, if not all, *Lotus* species.

Therefore, all isolates that were found to be infective on *L. tenuis* were tested using *L. uliginosus* LE627 in order to establish their host ranges for nodulation of *Lotus* species. Nodulation assays indicated that all the isolates were ineffective on *L. uliginosus*, as they formed no nodules or small red tumor-like structures that were unable to fix nitrogen (as shown by leaf chlorosis) similar to those on noninoculated plants.

Analysis of 16S rRNA gene sequences. An analysis of the sequences of 16S rRNA genes of representative isolates belonging to each ribogroup that infected *L. tenuis* (Table 3) was performed to confirm the phylogenetic position estimated using the RFLP analysis results. For this purpose, nearly full-length 16S rRNA sequences were obtained for 18 selected isolates that represented 12 16S rRNA RFLP groups (Table 3 and Fig. 1). Figure 2 shows a phylogenetic tree based on the similarity of the 16S rRNA sequences of the selected isolates and reference strains, which was constructed by using the p-distance model for estimating phylogenetic distances (46) and the UPGMA algorithm (63).

The classification of most isolates at the genus level obtained after analysis of 16S rRNA sequences (Fig. 2) was in good agreement with the classification obtained using the 16S rRNA RFLP data; the exceptions were isolates BA135 and BD59 (see below). However, 16S rRNA sequence analyses allowed better discrimination at the species level.

Thus, on the basis of 16S rRNA sequences, isolates BA128 and ML108 clustered with *Mesorhizobium amorphae* type strain ACCC 19665 (ribogroup II), sharing 99.5% sequence identity (five and six mismatches, respectively).

Isolates BD68 and ML105 exhibited the same ribotype (ribogroup IV) as strain *M. loti* NZP2037 (Fig. 1 and Table 3), and their 16S rRNA sequences shared 99.4% sequence identity with this reference strain. However, these two isolates appeared to be separated in the phylogenetic tree as isolate ML105 was in an undefined position between NZP2037 and *M. amorphae* ACCC 19665.

Isolate BD56 (ribogroup VI) was not related to any of the reference strains based on the 16S rRNA RFLP patterns, but its 16S rRNA gene sequence clustered in a branch together with the sequences of *Mesorhizobium ciceri* UPM Ca7 and *M. loti* NZP2213, with which it exhibited 99.2 and 98.9% sequence identity, respectively.

In the case of isolate BA151 (ribogroup VII), the 16S rRNA RFLP and sequence analyses provided similar results and grouped this isolate with *M. tianshanense* A-1BS (Fig. 1 and 2 and Table 3). Isolates ML93 and BA134, which grouped with

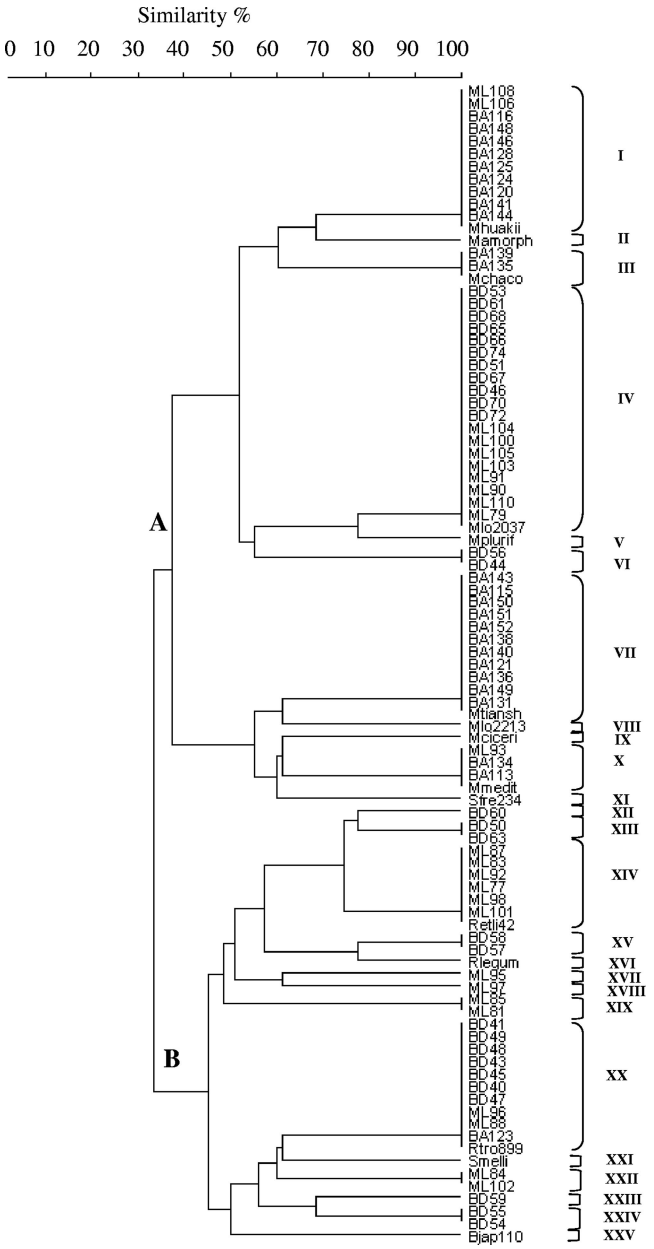

FIG. 1. 16S rRNA RFLP dendrogram showing genetic relationships among reference strains and rhizobial strains isolated from nodules of *L. tenuis* in typical soils of the Salado River Basin. The cluster analyses were performed using UPGMA. The endonucleases used were HinfI and MspI. Ribogroups I to XXVI (indicated on the right) are described in Table 3.

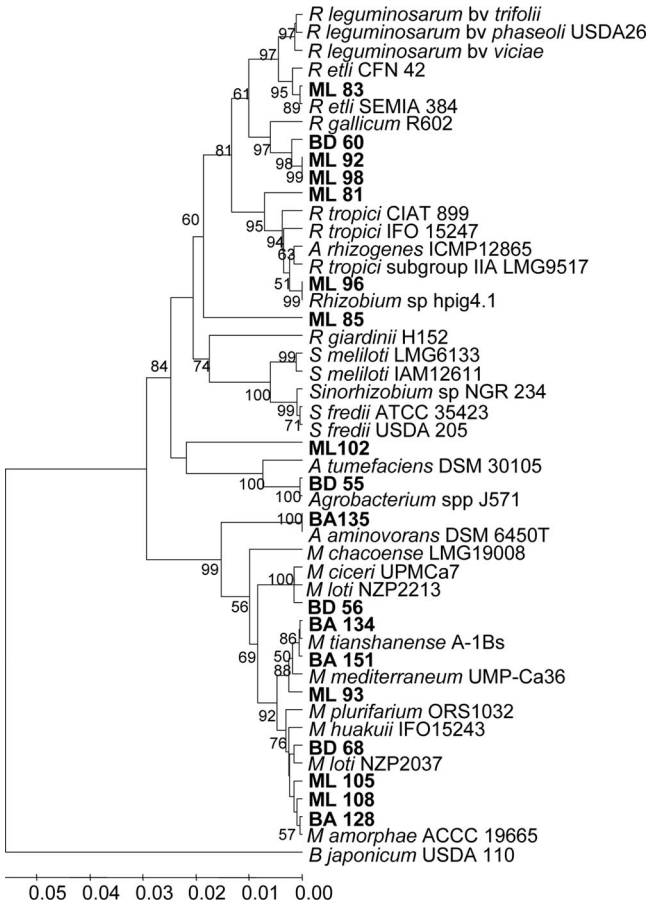

FIG. 2. 16S rRNA gene phylogeny of rhizobial strains isolated from nodules of *L. tenuis* grown in typical soils of the Salado River Basin. The tree was constructed from the nucleotide sequence data by using the UPGMA algorithm, and phylogenetic distances were calculated by the p-distance method. The numbers at branch points are the significant bootstrap values (expressed as percentages based on 1,000 replicates; only values greater than 50% are shown). The horizontal branch lines are proportional and indicate the p-distances.

M. mediterraneum UMP-Ca36 in RFLP ribogroup X, shared more than 99.0% 16S rRNA sequence identity with *M. mediterraneum* UMP-Ca36 and *M. tianshanense* A-1BS.

Although 16S rRNA RFLP analyses indicated that isolate BA135 (ribogroup III) grouped with *M. chacoense* LMG PR5 (Fig. 1 and Table 3), 16S rRNA sequencing revealed that BA135 had greater similarity to the nonsymbiotic bacterium *Aminobacter aminovorans* strain DSM6450^T (99.7% identity and four mismatches) than to *M. chacoense* LMG PR5 (97.6% identity and a difference of 30 nucleotides). In view of this result, the 16S rRNA sequence of strain *A. aminovorans* DSM6450^T was included in the alignment of 16S rRNA sequences for construction of the phylogenetic tree (Fig. 2).

Based on 16S rRNA sequences, isolates BD60 (ribogroup XII), ML77 (ribogroup XIV), BD63 (ribogroup XIII), and ML98 and ML92 (ribogroup XIV) clustered together in a branch separated from *Rhizobium gallicum* type strain R602; however, only BD60, ML98, and ML92 could be confirmed to be infective in *L. tenuis*, and their 16S rRNA sequences were included in the phylogenetic tree. (Fig. 2). The levels of 16S rRNA sequence identity between type strain R602 and the *L. tenuis* isolates varied from 96.8 to 97.1%.

Isolate ML83, which was one of the three infective isolates that represented RFLP ribogroup XIV (isolates ML98, ML92, and ML83), clustered with *R. etli* CFN42 on the basis of 16S rRNA sequence analysis. This isolate showed 98.9 and 99.4% sequence identity with *R. etli* CFN42 and SEMIA 384, respectively (Fig. 2).

Isolates ML88 and ML96 were placed in 16S rRNA RFLP ribogroup XX together with *R. tropici* IIB CIAT 899 (Table 3),

but 16S rRNA sequence analysis showed that both of these isolates were more closely related to *Rhizobium* sp. strain hpig 4.1 (three and four mismatches, respectively), which was isolated from *Mimosa* spp. in Costa Rica (5). However, only ML96 could be confirmed to be infective in *L. tenuis*.

Although isolates ML81 and ML85 both fell into RFLP ribogroup XIX, their 16S rRNA sequences were rather distinct (94.0% identity). ML81 branched out of the *R. tropici-Agrobacterium rhizogenes* cluster, whereas ML85 formed a differentiated branch within the *R. etli-R. leguminosarum-R. gallicum* cluster.

Isolates BD55 (ribogroup XXIV) and ML97 (noninfective, ribogroup XVIII) appeared to be closely related to the *Agrobacterium tumefaciens* cluster, showing 99.9 and 99.7% sequence identity, respectively, with *Agrobacterium* sp. strain J571, although they were more divergent from *A. tumefaciens* type strain DSM 30105 (Fig. 2). On the other hand, isolate ML102 was more closely related to *Rhizobium taeanense* PSB2-6 (a strain isolated from a leguminous sand dune plant; accession number DQ114473.1; not included in the tree), exhibiting higher sequence similarity to this strain (98.6% sequence identity) than to *A. tumefaciens* 358 (96.2% sequence identity) (Fig. 2).

Analysis of *nodC* and *nifH* sequences. PCR amplification of *nodC* and *nifH* gene fragments was carried out with isolates representing each of the 16S rRNA RFLP ribogroups identified for the *L. tenuis* isolates. A *nodC* gene fragment about 840 to 890 bp long could be amplified from all representative isolates belonging to ribogroups in 16S rRNA RFLP cluster A by using the nodCF-nodCI primer pair (35). In contrast, even though different combinations of primers described by Laguerre et al. (35) were used, a *nodC* gene fragment could not be amplified from any of the isolates in cluster B, regardless of their proven ability to nodulate *L. tenuis* under our experimental conditions (see above and Table 3). Genomic DNAs from nodulating and nonnodulating cluster B strains were hybridized with an *M. loti* R7A *nodC* gene probe, but no *nodC*-hybridizing bands were visualized for either strain, suggesting that the nodulation genes of the cluster B strains isolated from *L. tenuis* are very divergent from those of *M. loti*, which would at least partially explain our failure to amplify *nodC* from these isolates.

Likewise, an 800-bp *nifH* amplification product was obtained from all isolates in cluster A but from none of the infective representatives of cluster B. In contrast to the findings for *nodC*, when genomic DNAs from cluster B strains were hybridized with an *R. etli* CFN42 *nifH* probe, weak hybridization signals were observed for many of the isolates regardless of their infectivity, suggesting again that the symbiotic genes of these isolates may be rather divergent from those of other rhizobia (data not shown).

The sequences of *nodC* and *nifH* gene fragments were obtained and compared to corresponding rhizobial sequences reported previously. Phylogenetic analysis of *nodC* and *nifH* sequences was performed by using the neighbor-joining method (56), and the phylogenetic distances were calculated by using the p-distance method (46). As shown in Fig. 3, *nodC* sequences from *Lotus*-nodulating rhizobia clustered separately from *nodC* sequences from other legume symbiotic rhizobia. Furthermore, within the *Lotus* group, *nodC* phylogenies clearly differentiated broad-host-range strains (NZP2037) from narrow-host-range strains (R7A and MAFF303099), suggesting that *nodC* is a good indicator of host range for *Lotus* symbionts. All the Argentinian *nodC* sequences clustered in the narrow-host-range group of *Lotus* rhizobia, although they formed two subgroups diverging from the reference strains.

Phylogenetic analysis of *nifH* sequences also clustered the *Lotus* bacteria separate from other symbiotic rhizobia (Fig. 4). As observed with *nodC*, two separate subgroups were distinguished for the *Lotus* rhizobia, although in this case reference strains R7A and MAFF303099 appeared to be divergent and the Argentinian isolates fell into one of the subgroups regardless of the habitat from which they were isolated.

DISCUSSION

Analysis of REP-PCR fingerprints revealed a high level of genetic diversity among the *L. tenuis* nodule isolates, considering that 77 unique and complex genomic fingerprints were obtained. A comparison of REP-PCR profiles for different habitats revealed levels of similarity among isolates that ranged from 80% to less than 60%. Similarly, other authors found considerable genetic diversity among rhizobia nodulating *L. corniculatus* by using other methodological approaches, like DNA-DNA hybridization (11, 29, 30) or numerical taxonomy (48).

Seventy-seven isolates representing unique REP-PCR genomic fingerprints were further differentiated into two clusters, clusters A and B, after 16S rRNA gene RFLP analysis. A majority of the isolates (61%, cluster A) appeared to be related to the genus *Mesorhizobium* and were distributed in six ribogroups; the sixth ribogroup was not related to any of our reference strains. In contrast, cluster B, comprising 39% of the isolates, included 11 16S rRNA RFLP ribogroups related to species in the genus *Rhizobium*. When reinoculated onto the original host, *L. tenuis*, all of the cluster A isolates formed nitrogen-fixing nodules, in contrast to the cluster B isolates (related to *Rhizobium*), only nine of which were able to nodulate *L. tenuis* roots under our experimental conditions. It is possible that the noninfective isolates may have arisen through loss of symbiotic genes during the process of isolation and subsequent culture (36), an explanation supported by the fact that most ribogroups in cluster B included both nodulating and nonnodulating isolates. Furthermore, only *Rhizobium*-like bacteria were isolated from the nodules formed after reinoculation onto *L. tenuis* roots. Nevertheless, other explanations, such as the possibility that certain plant-bacterium interactions can occur only under particular soil conditions not met by our experimental protocol, should not be ruled out.

The distribution of most of the symbiotic isolates among the genera *Rhizobium* and *Mesorhizobium* is further supported by the results of the analyses of nearly full-length PCR-amplified 16S rRNA genes; the only exception is isolate BA135, whose 16S rRNA sequence was closely related to the *A. aminovorans* sequence. The phylogenetic relationship of strain BA135 with *A. aminovorans* is further supported by its *atpD* gene sequence, which also shows the highest level of similarity with the *atpD* gene sequence of strain *A. aminovorans* DSM 10368 (98% identity; accession number EU748944). The genus *Aminobacter* is phylogenetically related to *Mesorhizobium* but has never been reported to nodulate legumes (41). Thus, this is the

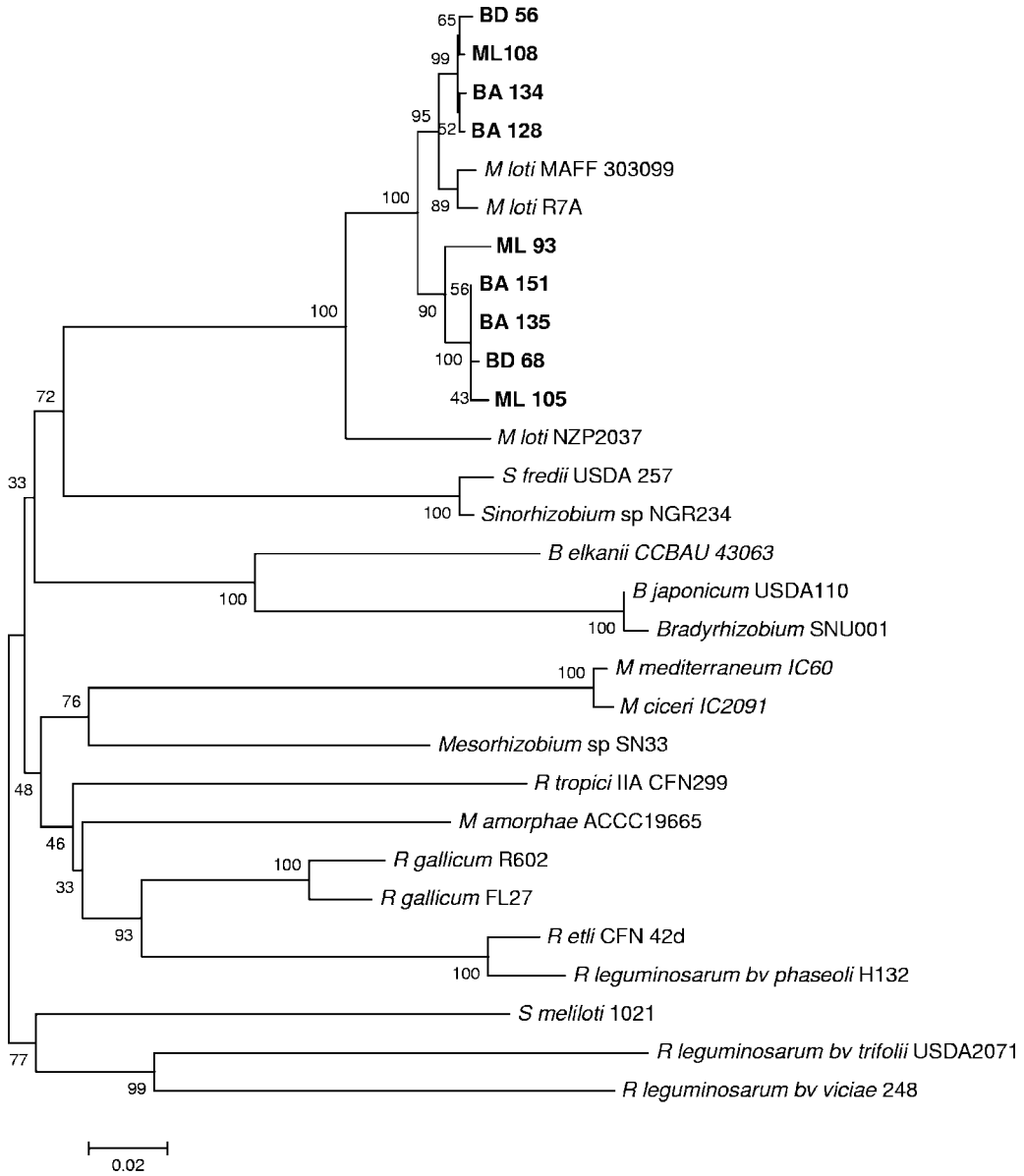

FIG. 3. Phylogenetic analysis of *nodC* genes from isolates nodulating *L. tenuis*. Aligned sequences of *nodC* genes that were 800 bp long (positions 286 −1123 of the *nodC* nucleotide sequence of *M. loti* MAFF 303099) were used. The tree was constructed by using the neighbor-joining method. The percentages of replicate trees in which the isolates and reference strains clustered together in the bootstrap test (1,000 replicates) are indicated at branch points (only values greater than 50% are shown). The tree is drawn to scale, and the units for the branch lengths are the same as the units for the phylogenetic distances used to infer the phylogenetic tree.

first report of a member of this genus with symbiotic capacity. The genus *Aminobacter* includes several pesticide- and herbicide-degrading bacteria isolated from agricultural soils that are able to grow on CH_3Cl, CH_3Br, CH_3I, and methylated amines as sole carbon and energy sources (41). Given the high proportion of species with bioremediation activity in this genus, the isolation and further characterization of new symbiotic isolates belonging to this genus in soils of the Salado River Basin could have important practical environmental implications.

All other isolates that infect *L. tenuis* were related to previously described rhizobial species, particularly members of the genera *Mesorhizobium* or *Rhizobium*, as suggested by their 16S rRNA gene sequences. Unexpectedly, an overwhelming majority of these isolates were related to species other than *M. loti*, which is the type species for *L. tenuis* and *L. corniculatus* symbionts (20, 30, 55, 66). Thus, only isolates BD56 and BD44 were phylogenetically closely related to *M. loti* type strain NZP2213, whereas the remaining isolates in 16S rRNA RFLP cluster A were more closely related to other *Mesorhizobium* species, like *M. amorphae*, *M. mediterraneum*, or *M. tianshanense*. A significant proportion of isolates were closely re-

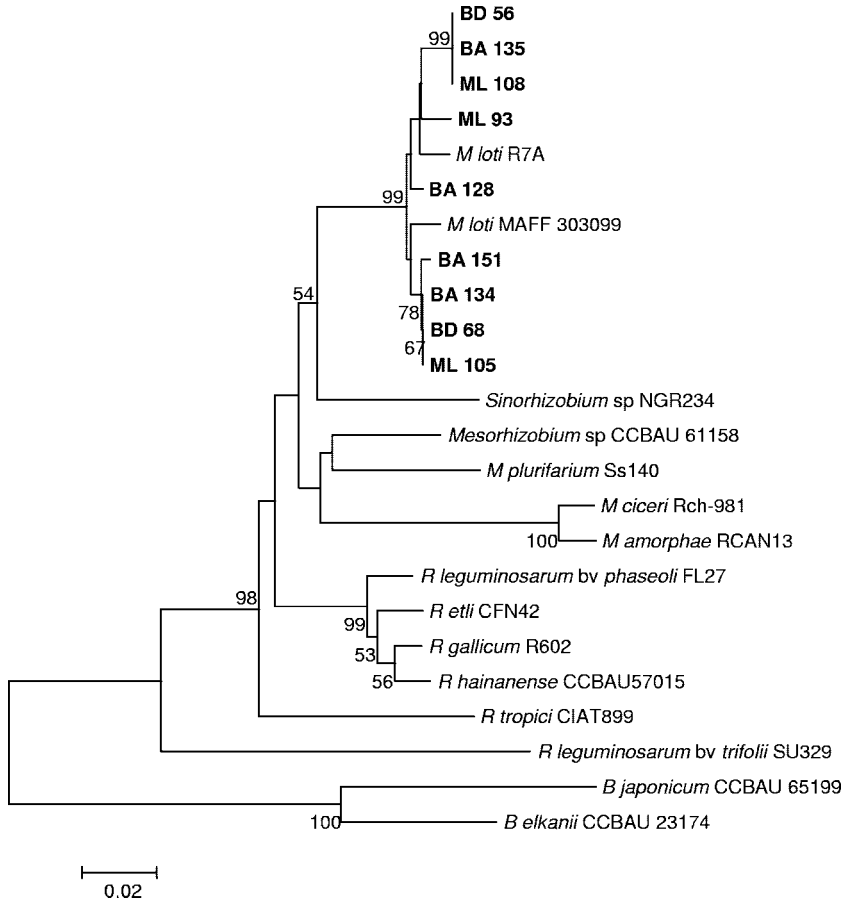

FIG. 4. Phylogenetic analysis of *nifH* genes from isolates nodulating *L. tenuis.* Aligned sequences of *nifH* genes that were 500 bp long (positions 317 to 815 of the *nifH* nucleotide sequence from *M. loti* MAFF303099) were used. The tree was constructed by using the neighbor-joining method. The percentages of replicate trees in which the isolates and reference strains clustered together in the bootstrap test (1,000 replicates) are indicated at branch points (only values greater than 50% are shown). The tree is drawn to scale, and the units for the branch lengths are the same as the units for the phylogenetic distances used to infer the phylogenetic tree.

lated to broad-host-range strain NZP2037; however, according to 16S rRNA phylogenies, this strain is more closely related to other *Mesorhizobium* spp. than to the *M. loti* type strain.

Another surprising result was the fact that a significant number of *L. tenuis* symbionts seem to be closely related to *Rhizobium* species, like *R. gallicum*, *R. etli*, or *R. tropici*. Although *R. etli* has previously been shown to nodulate *Lotus japonicus* (3), this is the first time that *Lotus* was identified as the primary host of a *Rhizobium* sp. strain. Intriguingly, the *L. tenuis* symbionts were closely related to rhizobial species that have been shown to be common symbionts of *Phaseolus vulgaris* (25, 40). Unfortunately, despite various attempts we were unable to amplify *nodC* or *nifH* gene sequences from *Rhizobium* symbionts of *L. tenuis*, which prevented us from obtaining further insight into the origin of their symbiotic capacities. However, the fact that no *nodC* gene sequences could be amplified when various primer sets were used indicates that the nodulation genes of these *L. tenuis* symbionts may be different from those of other rhizobia. This conclusion is supported by the negative results obtained when genomic hybridization with symbiotic gene probes was performed. In particular, no *nodC*-hybridizing DNA fragments were found in either nodulating or nonnodulating *Rhizobium* isolates, which could even suggest the rare but previously proven presence of nodulation in the absence of Nod factors, as recently reported for certain photosynthetic bradyrhizobia (23).

In contrast, *nodC* and *nifH* gene fragments from all of the *Mesorhizobium* and *Aminobacter*-like isolates could be amplified. The *nodC* genes were all very similar and closely related to *nodC* genes from narrow-host-range *M. loti* strains, such as R7A and MAFF303099. This perfectly correlates with the narrow host range of the Argentinian isolates inferred from their inability to nodulate *L. uliginosus*. Indeed, *nodC* gene phylogenies clearly differentiated between narrow- and broad host-range *Lotus* symbionts, suggesting that *nodC* is a valid marker for this purpose.

nifH gene sequences in the Argentinian and reference *Lotus* bacteria were very highly conserved and formed a phylogenetic cluster differentiated from other rhizobial *nifH* gene sequences. The fact that similar symbiotic genes are shared by distinguishable 16S rRNA species and genera suggests that genes for symbiosis with *L. tenuis* had a common origin and that horizontal gene transfer has played an important role in the spread of symbiotic capacity among *Mesorhizobium* species

and even to the closely related genus *Aminobacter*. Although intrageneric lateral transfer of symbiotic determinants has been shown previously for *Mesorhizobium* (66), our results with isolate BD135 are the first evidence that there has been symbiotic gene exchange between *Mesorhizobium* and *Aminobacter*.

Our work indicates that the high level of genetic diversity of *L. tenuis* symbionts in the Salado River Basin is not evenly distributed in all habitats screened in this work. A comparison of isolates from three typical habitats that characterized the soils in the Salado River Basin revealed that the more stressful environments, the saline lowlands, contain mainly mesorhizobia, in contrast to habitats such as the nonsaline lowlands and transitional plains, where genotypically more diverse symbionts were found, including bacteria belonging to the genera *Mesorhizobium*, *Rhizobium*, and *Aminobacter*. Until now, *M. loti* has been reported to be the type symbiont for *Lotus* spp., such as *L. japonicus*, *L. tenuis*, or *L. corniculatus* (55). There have been a few reports of genetic diversity of *Lotus* symbionts, and it should be noted that much of the previous work on *Lotus* rhizobia was done in only a few countries, like New Zealand or Uruguay (27, 45, 66). Our work suggests that in environments like the Salado River Basin, *M. loti* strains represent a minority of the symbionts of *L. tenuis*. This indicates that *Lotus*-nodulating rhizobia should be characterized in other countries and environments, and it would not be surprising if species other than *M. loti* or even genera other than *Mesorhizobium* were the dominant *Lotus* symbiotic bacteria.

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