***Mesorhizobium chacoense* sp. nov., a novel species that nodulates *Prosopis alba* in the Chaco Arido region (Argentina)**

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Low-molecular-weight RNA analysis was performed for the identification and classification of 20 Argentinian strains isolated from the root nodules of *Prosopis alba*. SDS-PAGE of total cellular proteins, determination of the DNA base composition, DNA–DNA reassociation experiments and physiological and biochemical tests were also carried out for these strains and the whole 16S rRNA gene was sequenced from one of the strains, strain LMG 19008^T. Results of the genotypic and phenotypic characterization showed that the strains isolated in this study belong to a group that clustered in the genus *Mesorhizobium*. The results of DNA–DNA hybridizations showed that this group is a novel species of this genus. The name *Mesorhizobium chacoense* sp. nov. is proposed for this species. The type strain is LMG 19008^T (= CECT 5336^T).

Keywords: taxonomy, *Mesorhizobium*, LMW RNA, 16S rRNA, *Prosopis*

INTRODUCTION

The taxonomy of the family *Rhizobiaceae* has been the subject of important advances in recent years. Six genera are able to nodulate diverse species of legumes: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Allorhizobium*. In the early years of *Rhizobium* taxonomy, species were, in general, described from legumes of agronomic importance, but a trend in recent years to investigate the symbionts of exotic plants has resulted in important contributions to rhizobial taxonomy. Thus, *Azorhizobium* was defined as a new genus based on strains able to nodulate the stems of *Sesbania rostrata* (Dreyfus *et al.*, 1988) and *Allorhizobium* was isolated from an aquatic legume, *Neptunia natans*, in Senegal (de Lajudie *et al.*, 1998b). Also, the analysis of rhizobial symbionts of leguminous trees of the genus

Acacia from Senegal resulted in the description of various species of *Sinorhizobium* (de Lajudie *et al.*, 1994) and *Mesorhizobium* (de Lajudie *et al.*, 1998a; Wang *et al.*, 1999). The species *Mesorhizobium plurifarium* contained strains isolated from nodules of *Acacia*, *Leucaena*, *Prosopis* and *Chamaecrista* in West Africa (Senegal), as well as East Africa (Sudan) and South America (Brazil). Nevertheless, a large number of legumes exists whose bacterial symbionts have not been studied systematically. Thus, in general, isolation of their bacterial symbionts is a promising strategy to increase our knowledge of the systematics and evolution of *Rhizobium*. Moreover, many of these rhizobia that remain as yet unexamined have established symbiotic relationships with legumes that are of great importance in the maintenance and economy of large and important ecosystems throughout the world. Therefore, knowledge and optimization in the management of these ecosystems may benefit from the study of these particular strains. Species of the genus *Prosopis* are important trees in many ecosystems throughout South America and some have been introduced into Africa, where they can be nodulated by indigenous rhizobia (de Lajudie *et al.*, 1998a;

Abbreviations: LMW RNA, low-molecular-weight RNA; PNP, *para*-nitro-phenyl substrates; SCE, staircase electrophoresis.

The GenBank accession number for the 16S rRNA sequence of *Mesorhizobium chacoense* LMG 19008^T is AJ278249.

Haukka *et al.*, 1996; McInroy *et al.*, 1999). Apart from the above references, little work has been described concerning the taxonomy of American rhizobial symbionts of *Prosopis*. In this work, rhizobial strains have been isolated from *Prosopis chilensis* plants growing in diverse geographical locations in central Argentina, including the Reserva Chancaní in the south of El Chaco Arido, an important ecosystem, covering several thousands of hectares in Argentina. Based on a polyphasic taxonomy approach, including staircase electrophoresis analysis (SCE) of low-molecular-weight (LMW) RNA, 16S rDNA sequencing, analysis of total cell protein, determination of base composition and DNA–DNA hybridization and a series of biochemical tests, the novel species *Mesorhizobium chacoense* is described.

METHODS

Bacterial strains. The reference strains and novel isolates used in this study are listed in Table 1. A total of 20 new rhizobial isolates was obtained from young *Prosopis alba*

plants growing in soil from two different locations. Nine isolates were from Luque, located some 200 km east of Cordoba Province in the Espinal region, and eleven isolates were from the Chancaní reserve, in the north-west of Cordoba Province in the Chaco Arido region (Argentina). Isolations were made according to Vincent (1970) using yeast mannitol agar [YMA, which contained (g l⁻¹): mannitol, 10; sodium glutamate, 0.5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.05; CaCl₂, 0.04; FeCl₃, 0.004; yeast extract, 1; agar, 20; pH 6.8] (Bergersen, 1961). The cultures used in further studies were purified from single colonies after 10 d incubation at 28 °C.

Nodulation tests. Surface-sterilized seeds of *P. chilensis*, *P. alba*, *Prosopis flexuosa* and *Acacia* sp. were heat treated (50 °C for 5 min) and germinated axenically in Petri dishes. Seedlings were transferred to pots with sterile vermiculite and watered with nitrogen-free nutrient solution (Rigaud & Puppo, 1975). Each plant was inoculated with 10 ml of a suspension of each strain containing 8 × 10⁸ cells ml⁻¹. The inoculated plants were placed for 30 d in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins m⁻² s⁻¹; 400–700 nm), programmed for a 16 h photoperiod day/night cycle, at a constant tem-

Table 1. Strains used in this study

Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; LMG, Collection of Bacteria of the Laboratory voor Microbiologie, Gent, Belgium; CECT, Spanish Type Culture Collection, Valencia, Spain.

Strain	LMG no.*	Host plant	Geographical origin†	Reference
<i>Mesorhizobium chacoense</i>				
Pr-5 ^T (= CECT 5336 ^T)	LMG 19008 ^{T1,2}	<i>Prosopis alba</i>	Chancaní	This study
Pr-11	LMG 19009 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-1	LMG 19010 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-2	LMG 19011 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-3	LMG 19012 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-4	LMG 19013 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-5	LMG 19014 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-6	LMG 19015 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-7	LMG 19016 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-8	LMG 19017 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pch-9	LMG 19018 ¹	<i>Prosopis alba</i>	Chancaní	This study
Pl-1	LMG 19019 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-3	LMG 19020 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-5	LMG 19021 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-6	LMG 19022 ^{1,2}	<i>Prosopis alba</i>	Luque	This study
Pl-7	LMG 19023 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-8	LMG 19024 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-10	LMG 19025 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-11	LMG 19026 ¹	<i>Prosopis alba</i>	Luque	This study
Pl-12	LMG 19027 ¹	<i>Prosopis alba</i>	Luque	This study
<i>Mesorhizobium loti</i> ATCC 33669 ^T	LMG 6125 ^{T1,2}	<i>Lotus tenuis</i>	New Zealand	Jarvis <i>et al.</i> (1982)
<i>Mesorhizobium loti</i> NZP 2014	LMG 6124 ²	<i>Lotus corniculatus</i>	New Zealand	Jarvis <i>et al.</i> (1982)
<i>Mesorhizobium ciceri</i> USDA 3383 ^T	LMG 14989 ^{T1,2}	<i>Cicer arietinum</i>	Spain	Nour <i>et al.</i> (1994)
<i>Mesorhizobium mediterraneum</i> UPM-CA142	LMG 14990 ²	<i>Cicer arietinum</i>	Spain	Nour <i>et al.</i> (1995)
<i>Mesorhizobium mediterraneum</i> USDA 3392 ^T	–	<i>Cicer arietinum</i>	Spain	Nour <i>et al.</i> (1995)
<i>Mesorhizobium tianshanense</i> USDA 3592 ^T	LMG 15767 ^{T1,2}	<i>Glycyrrhiza pallidiflora</i>	China	Chen <i>et al.</i> (1995)
<i>Mesorhizobium huakuii</i> USDA 4779 ^T	LMG 14107 ^{T1,2}	<i>Astragalus sinicus</i>	China	Chen <i>et al.</i> (1991)
<i>Mesorhizobium plurifarium</i> LMG 11892 ^T	LMG 11892 ^{T2}	<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998a)
<i>Mesorhizobium amorphae</i> ATCC 19665 ^T	LMG 18977 ^{T1,2}	<i>Amorpha fruticosa</i>	China	Wang <i>et al.</i> (1999)
<i>Sinorhizobium meliloti</i> USDA 1002 ^T	LMG 6133 ^T	<i>Medicago sativa</i>	USA	de Lajudie <i>et al.</i> (1994)
<i>Rhizobium leguminosarum</i> ATCC 10004 ^T	LMG 8817 ^T	<i>Pisum sativum</i>	USA	Skerman <i>et al.</i> (1980)
<i>Rhizobium galegae</i> ATCC 43677 ^T	LMG 6214 ^T	<i>Galega orientalis</i>	Finland	Lindström (1989)
<i>Azorhizobium caulinodans</i> ATCC 43989 ^T	LMG 6465 ^T	<i>Sesbania rostrata</i>	Senegal	Dreyfus <i>et al.</i> (1988)
<i>Bradyrhizobium japonicum</i> ATCC 10324 ^T	LMG 6138 ^T	<i>Glycine max</i>	Japan	Jordan (1982)
<i>Allorhizobium undicola</i>	LMG 11875 ^T	<i>Neptunia natans</i>	Senegal	de Lajudie <i>et al.</i> (1998b)
<i>Agrobacterium tumefaciens</i> ATCC 23308 ^T	LMG 187 ^T			Kerstens & De Ley (1984)
<i>Phyllobacterium myrsinacearum</i> ATCC 43590 ^T	LMG 6465 ^T	<i>Ardisia crispa</i>		Knösel (1984)

* Strains marked with '1' were included in LMW RNA analysis. Strains marked with '2' were used in DNA–DNA hybridization.

† Chancaní and Luque are two regions of Argentina.

perature of 25–27 °C and 50–60 % relative humidity. Root nodules appeared 2 months after inoculation.

RNA extraction and LMW RNA profile analysis. The LMW RNA of the strains studied was extracted as described by Höfle (1988). LMW RNA profiles were obtained using SCE in 14 % polyacrylamide gels under denaturing conditions in steps of 10 min, rising through a constant ramp with 50 V increases from 100 to 2300 V, as reported previously (Cruz-Sánchez *et al.*, 1997). The following commercial molecules, from Boehringer Mannheim and Sigma, were used as references: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nt) (Bidle & Fletcher, 1995), tRNA specific for tyrosine from *E. coli* (85 nt) and tRNA specific for valine from *E. coli* (77 nt) (Sprinzl *et al.*, 1985). Samples were prepared as reported elsewhere (Cruz-Sánchez *et al.*, 1997). After electrophoresis, the gels were silver-stained as described by Haas *et al.* (1994).

Determination of nucleotide sequences of the 16S rRNA genes and analysis of the sequence data. Amplification of the whole 16S rDNA was performed using the PCR primers FGPS 1509.153 (5'-AAGGAGGGGATCCAGCCGCA-3') and FGPS 4.281 [5'-ATGGA(GA)AG(TC)TTGATCCTG-GCTCA-3'] (Normand *et al.*, 1996) in a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer) under the following conditions: initial denaturation for 3 min at 94 °C; 35 cycles of denaturation (1 min at 94 °C), annealing (45 s at 60 °C) and extension (1 min at 72 °C); final extension for 7 min at 72 °C. The reaction volume was 50 µl and contained 5 µl cell suspension in 25 % glycerol, 5 µl buffer (10 µM Tris/HCl, pH 8.2; 1.5 mM MgCl₂; 50 mM KCl; 0.01 %, w/v, gelatin), 20 µM each dNTP (Pharmacia Biotech), 0.5 µM each primer and 2.5 U *Taq* DNA polymerase (Gibco-BRL). PCR amplification of DNA was checked by agarose gel electrophoresis (2 %, w/v) in Tris/borate/EDTA buffer. The PCR amplification products were visualized by ethidium bromide staining. Before sequencing, the amplification mixture was purified with a QIAquick kit (Qiagen).

Sequencing of the 16S rRNA gene (positions 58–1541 of the *E. coli* 16S rDNA sequence) was performed by Genome Express, Grenoble, France. Sequences of both strands were determined using the following five oligonucleotides: 5'-TGGCTCAGAACGAACGCTGGCGGC-3', 5'-AGCCT-TGCGGCCGTACTCCC3', 5'-CAGCAGCCGCGGTAA-3', 5'-AAGGAGGGGATCCAGCCGCA-3' and 5'-GCCT-GGGGAGCTCGGCCGCA-3', which correspond to positions 20–43, 518–532, 885–904, 1521–1540 and 880–899, respectively, of the *E. coli* small-subunit rDNA sequence (Normand *et al.*, 1996; Ritchie & Myrold, 1999).

The 16S rDNA of strains LMG 19008^T and LMG 19009 was amplified. For LMG 19009, only a 900 bp fragment was obtained. This fragment showed 100 % identity to the corresponding LMG 19008^T zone and PCR-RFLP analysis of their 16S rDNA using *Cfo*I and *Inf*I endonucleases gave identical patterns (data not shown). Consequently, only LMG 19008^T was used for further analysis. The sequence was compared with previously published sequences of members of the *Rhizobiaceae* and other closely related species (see Fig. 2) as determined by BLAST software (Altschul *et al.*, 1990). Sequences were aligned using the CLUSTAL X software (Thompson *et al.*, 1997). Alignment was refined manually using the SUNMASE algorithm (Faulkner & Jurka, 1988). The distances were calculated according to Kimura's two-parameter method (Kimura, 1980) and a dendrogram was obtained by the neighbour-joining algorithm (Saitou & Nei, 1987).

Microplate DNA–DNA hybridization. Reference strains used in DNA–DNA hybridizations are listed in Table 1. Strains were grown on YMA or tryptone/yeast extract (TY) agar [containing (g l⁻¹): tryptone (Oxoid), 5; yeast extract (Oxoid), 0.75; KH₂PO₄, 0.454; Na₂HPO₄·12H₂O, 2.388; CaCl₂, 1; agar, 20; pH 6.8–7.0] at 28 °C for 2 d. Total DNA was prepared by using the method of Pitcher *et al.* (1989), in which volumes were scaled up 30-fold to allow the use of a larger quantity of cells for extraction. Hybridizations were performed using the microplate method as described by Ezaki *et al.* (1989).

DNA base composition. DNA of two representative strains, LMG 19008^T and LMG 19022, was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The resulting nucleoside mixtures were separated by HPLC using a Waters Symmetry Shield C8 column at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

Analysis of proteins by SDS-PAGE. Strains isolated from *Prosopis* in this study were grown at 28 °C for 48 h on TY agar. Strains LMG 15767 and LMG 14990, which did not grow sufficiently on TY, were grown on YMA. Cellular protein extracts were prepared and SDS-PAGE was performed as described previously (Pot *et al.*, 1994). Digitization, normalization and numerical analyses of the protein patterns were performed with the GELCOMP 4.0 software (Applied Maths) as described by Pot *et al.* (1994). The similarity between pairs of protein patterns is expressed by Pearson's product-moment correlation coefficient converted to percentages.

Plasmid profile analysis. The strains isolated were subjected to plasmid profile analysis according to Plazinski *et al.* (1985). Electrophoresis was modified as follows: electrophoresis was carried out at 30 V for 90 min, at 60 V for 60 min and at 40 V for 3 h. The plasmids of *Sinorhizobium meliloti* GR4, which has a cryptic plasmid of 140 MDa and another of 114 MDa (Toro & Olivares, 1986), were used as size markers. Plasmid DNA was capillary-transferred to a nylon membrane according to Southern (1975) and immobilized by baking at 80 °C for 2 h.

Oligonucleotide primers and preparation of DNA probe. Oligonucleotide primers were designed to amplify a fragment of the *nifH* gene that is conserved among members of the family *Rhizobiaceae*. These primers were: *unifH1*, 5'-CTATGCCGCCAACAACATCG-3'; and *unifH2*, 5'-TT-TGCATGGATCTTTTCAGCCA-3' (positions 635–654 and 888–910, respectively, of the *S. meliloti nifH* gene, accession no. J01781). These oligonucleotides amplify a 276 bp DNA fragment. Total DNA from *S. meliloti* GR4 was used as the template. PCRs were carried out in 25 µl volumes containing 1 ng total DNA, 25 pmol each primer, 0.1 mM dNTPs and 2 U *Taq* DNA polymerase. Amplifications were performed with a DNA thermo-robocycler (Stratagene). The program used in this work was as follows: 1 cycle at 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 30 s; and 1 cycle at 72 °C for 5 min. Amplified product was purified from 1 % agarose gel and used as template for PCR amplification-labelling with digoxigenin-11-dUTP (Boehringer).

Hybridization was carried out under high-stringency conditions according to the supplier's instructions. Washing was carried out twice (5 min each) in 2 × SSC, 0.1 % SDS at room temperature and twice (15 min each) in 0.1 × SSC,

Fig. 1. LMW RNA profiles of the strains isolated in this study and type strains of genus *Mesorhizobium*. Lanes: M, molecular mass marker; 1, *M. tianshanense* USDA 3592^T; 2, *M. mediterraneum* USDA 3392^T; 3, *M. ciceri* USDA 3383^T; 4, *M. amorphae* ACCC 19665^T; 5, *M. loti* ATCC 33669^T; 6, *M. plurifarum* LMG 11892^T; 7, *M. huakuii* USDA 4779^T; 8, *M. chacoense* LMG 19008^T; 9, LMG 19009; 10, LMG 19010; 11, LMG 19011; 12, LMG 19012; 13, LMG 19013; 14, LMG 19014; 15, LMG 19015; 16, LMG 19016; 17, LMG 19017; 18, LMG 19018; 19, LMG 19019; 20, LMG 19020; 21, LMG 19021; 22, LMG 19022; 23, LMG 19023; 24, LMG 19024; 25, LMG 19025; 26, LMG 19026; and 27, LMG 19027.

0.1% SDS at 68 °C. Detection of the hybridization signals was performed as specified by Boehringer Mannheim.

Morphological tests. Cell morphology was determined on Gram-stained cells and on living cells by phase-contrast microscopy.

Physiological and biochemical tests. The phenotypic characterization of strains isolated from *Prosopis* and type strains of the family *Rhizobiaceae* was based on pH changes for the different carbon sources (acid, basic or neutral), antibiotic resistance and extracellular enzyme production. The carbon sources were sucrose, galactose, lactose, L-arabinose, rhamnose, trehalose, maltose, adonitol, melibiose and raffinose. The basal medium comprised (l⁻¹): 0.2 g K₂HPO₄; 0.2 g MgSO₄; 1.0 g NH₄NO₃; 1 ml of a vitamin and trace element solution (Bergersen, 1961); and 0.05 g bromthymol blue. The pH was adjusted to 7.0 with KH₂PO₄. The discs with carbon sources (BBL; Becton Dickinson) were added under sterile conditions to 5 ml medium. The results obtained were based on the pH changes for the different carbon sources (acid, basic or neutral) and were recorded after 5 d for fast-growing species, 10 d for species of *Mesorhizobium* and 15 d for slow- and extra-slow-growing species. For testing antibiotic resistance, the following antibiotics were used: ampicillin, erythromycin, ciprofloxacin, penicillin, polymyxin, cloxacillin, oxytetracycline, gentamicin, cefuroxime and neomycin. The basal medium was YMB supplemented with 0.5% yeast extract. Each antibiotic disc was added under sterile conditions to 5 ml basal medium.

For biochemical characterization, ten extracellular glucosidases were tested. The chromogenic substrates used were *para*-nitrophenyl substrates (PNP): PNP α -D-arabino-

pyranoside, PNP β -D-arabinopyranoside, PNP α -D-fucopyranoside, PNP β -D-fucopyranoside, PNP α -D-galactopyranoside, PNP β -D-galactopyranoside, PNP α -D-xylopyranoside, PNP β -D-xylopyranoside, PNP α -D-maltopyranoside and PNP *N*-acetylglucosaminide. Substrates were used at a concentration of 0.4% in 50 mM phosphate buffer, pH 7. The enzymic reactions were carried out in multiwell plates by mixing 50 μ l substrate, prepared as specified above, and 50 μ l bacterial suspension in sterile water. The suspensions contained 6×10^9 c.f.u. ml⁻¹. To make up the suspensions, plate cultures with the minimal medium of Bergersen (1961) were incubated at 28 °C over 2 d for agrobacteria and phyllobacteria, 4 d for sinorhizobia, rhizobia and allorhizobia, 5 d for mesorhizobia and 8 d for bradyrhizobia and azorhizobia. The plates with the reaction mixture were incubated at 28 °C and were developed with a solution of 4% sodium carbonate, adding 100 μ l to each well. Abilities to grow at 37 and 40 °C and at pH 5 and 8 were determined on YMA medium.

RESULTS

Isolation and nodulation

All of the isolates grew slowly in YMA medium and were effective for symbiotic nitrogen fixation in the three species of *Prosopis* used in this study. Nitrogen fixation was confirmed as effective based on the nodulation and growth of plants for several months in nitrogen-free medium. None of strains was able to nodulate *Acacia* sp. All of the strains induced indeterminate nodules in *Prosopis*.

Fig. 2. Phylogenetic tree of rhizobia and some related bacteria in the α -Proteobacteria. The tree was constructed by the neighbour-joining method from 16S rRNA sequences. Bootstrap probability values greater than 50% are indicated at the branch-points. Bar, 0.01 substitutions per site. Sequences were derived from the type strains wherever possible.

Fig. 3. SDS-PAGE protein profiles of *Prosopis* isolates. Lanes M, molecular mass markers.

LMW RNA profiles

The LMW RNA profiles of strains isolated from *Prosopis* in this study are shown in Fig. 1. All strains had the same LMW RNA profile (Fig. 1, lanes 8–27), indicating that they belong to the same genus and species.

In the LMW RNA profiles, three zones can be distinguished: 5S rRNA, class 1 tRNA and class 2 tRNA. The 5S rRNA zone is characteristic of each bacterial genus and the tRNA zone is characteristic of each bacterial species. The 5S rRNA zone of the LMW RNA profile of all strains isolated from *Prosopis* in the Chaco Arido was similar to that of the genus *Mesorhizobium*. Therefore, all strains isolated in this study should be included in this genus. However, the tRNA profile of these strains differed from that of the currently accepted species of this genus (Fig. 1, lanes 1–7). This result indicates that the strains studied probably represent a novel species of the genus *Mesorhizobium*.

16S rDNA sequence analysis

Strain LMG 19008^T is proposed as the type strain of the group of rhizobia isolated from *Prosopis* in this study and the complete nucleotide sequence of its 16S rDNA was obtained and compared with those of other members of the α -*Proteobacteria* available in the databases. The results confirmed those obtained from

the LMW RNA analysis. The phylogenetic tree obtained with this method indicated that strain LMG 19008^T was related to *Mesorhizobium*, but formed a separate branch in this genus (Fig. 2). A search of the EMBL database revealed the new sequence to be most similar to the 16S rRNA sequences of species of *Mesorhizobium*. The similarity of the 16S rRNA sequence of *M. chacoense* sp. nov. to those of the type strains from all *Mesorhizobium* species was 98% or higher. Similarity to sequences of species from other closely related genera such as *Chelatobacter* or *Aminobacter* was lower than 98%. Based on these values, it is considered that the novel species proposed in the present work belongs to the genus *Mesorhizobium*.

Analysis of proteins by SDS-PAGE

Reproducibility was verified by preparing extracts in duplicate. Duplicate strains clustered above 93% (data not shown). Protein extracts of the newly isolated strains showed a striking similarity (Fig. 3) and clustered at 93% or higher (Fig. 4), indicating considerable homogeneity among the strains. Comparison of their SDS-PAGE profiles to those of other *Mesorhizobium* strains revealed that none of the protein profiles of these organisms was highly similar to those of the new group (Fig. 4).

DNA–DNA hybridizations and DNA base composition

The microplate method used in this study was generally in good agreement with the optical renaturation rate method (Goris *et al.*, 1998). In view of the very high similarity of the protein profiles of the isolates, confirmed by total identity in LMW RNA profiles, only two representative strains were used for DNA–DNA hybridizations. As expected, they showed a high degree of DNA relatedness (100%). With hybridization values of 2–17%, none of the known species of *Mesorhizobium* showed a significant degree of DNA relatedness to the new group. The G+C contents of genomic DNA of strains LMG 19008^T and LMG 19022, as determined by HPLC, were 61.71 and 61.55 mol%.

Plasmid profiles

Figure 5 shows the results of the plasmid profile analysis of strain LMG 19008^T (lane 1). All strains isolated from *Prosopis* in this study had plasmid profiles identical to that of the type strain (data not shown), with a unique plasmid of approximately 1000 MDa (data not shown). None of these plasmids hybridized with the probe of the *nifH* gene used in this study.

Physiological and biochemical characteristics

The results of physiological and biochemical tests are shown in Table 2. Different carbon sources were tested in a minimal medium with ammonium nitrate as the

Fig. 4. Dendrogram showing the relationships between the electrophoretic protein patterns from *Prosopis* isolates and reference strains of *Mesorhizobium* species. The mean correlation coefficient (r) was represented as a dendrogram constructed by the unweighted pair group method with arithmetic means. Scale bar, r value converted to a percentage.

Fig. 5. Plasmid profiles of *Prosopis* strains isolated in this study. Lanes: 1, *M. chacoense* LMG 19008T; 2, *S. meliloti* GR4.

nitrogen source. Four types of response can be expected in this medium: pH changes to acid; pH changes to basic; no change of pH, but growth detected; or no change of pH and no growth detected. The type strains of *Agrobacterium tumefaciens*, *Azorhizobium caulinodans* and *Bradyrhizobium japonicum* produced a basic pH in this medium, whereas most other strains produced an acid pH from the sugars used in this study. In general, the strains isolated from *Prosopis* in this study produced acid from these sugars, with the exception of raffinose.

Growth in the presence of ten antibiotics with different structural groups was also tested. Because some of the strains used in this study are slow growers, liquid medium (YMB) was used, as in the above experiment. The pattern of resistance to antibiotics allows the differentiation of the species of the family *Rhizobiaceae*. All strains isolated from *Prosopis* in this study showed the same pattern of resistance to the antibiotics.

Finally, several extracellular enzymic activities were detected using PNP substrates, which are easily detected from plate cultures. The results of extracellular

Table 2. Physiological and biochemical characteristics of rhizobia used in this study

The type strain of each species was used (see Table 1). +, Positive result; –, negative result or no growth (in the case of the carbon source utilization); A, acid; B, basic; N, neutral. Species are indicated as: 1, *A. undicola*; 2, *R. galegae*; 3, *R. leguminosarum*; 4, *S. meliloti*; 5, *M. ciceri*; 6, *M. mediterraneum*; 7, *M. loti*; 8, *M. huakuii*; 9, *M. tianshanense*; 10, *M. plurifarum*; 11, *M. amorphae*; 12, *M. chacoense*; 13, *B. japonicum*; 14, *A. caulinodans*; 15, *A. tumefaciens*; 16, *P. myrsinacearum*. Abbreviations: PNP α -Dara, PNP α -D-arabinopyranoside; PNP β -Dara, PNP β -D-arabinopyranoside; PNP α -Lfuco, PNP α -L-fucopyranoside; PNP β -Dfuco, PNP β -D-fucopyranoside; PNP α -Dgal, PNP α -D-galactopyranoside; PNP β -Dgal, PNP β -D-galactopyranoside; PNP α -Dxyl, PNP α -D-xylopyranoside; PNP β -Dxyl, PNP β -D-xylopyranoside; PNP α -Dmal, PNP α -D-maltopyranoside; PNP *N*-acglc, PNP *N*-acetylglucosaminide. All species were resistant to cloxacillin.

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Carbon source utilization:																
Sucrose	A	A	A	A	A	A	A	A	A	B	A	A	–	B	B	A
Galactose	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	A
Lactose	A	A	A	A	A	A	A	A	A	A	A	A	–	B	B	–
L-Arabinose	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	A
Rhamnose	A	A	A	A	A	A	A	A	A	A	A	A	N	B	B	A
Trehalose	A	A	A	A	A	A	A	A	A	A	A	A	–	B	B	A
Maltose	A	A	A	A	A	A	A	A	A	A	A	A	–	A	B	A
Adonitol	–	A	A	A	A	A	A	A	B	A	A	A	B	B	B	A
Melibiose	–	A	A	A	N	A	A	A	A	A	A	A	A	B	B	A
Raffinose	–	A	A	A	N	B	A	A	–	B	A	N	–	B	B	A
Antibiotic resistance:																
Ampicillin	–	–	+	+	–	–	–	–	–	–	+	–	+	+	+	+
Erythromycin	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ciprofloxacin	–	–	–	+	–	–	–	–	–	+	–	+	+	+	–	–
Penicillin	–	–	+	+	–	–	–	–	–	–	+	–	+	+	+	+
Polymyxin	–	–	–	–	+	–	+	+	–	+	–	–	+	+	–	+
Oxytetracycline	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	+
Gentamicin	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
Cefuroxime	–	–	+	+	–	–	–	–	–	–	–	–	+	+	+	–
Neomycin	+	+	–	–	+	–	+	+	–	+	+	–	+	+	+	+
Enzyme activity against:																
PNP α -Dara	+	+	+	+	–	–	–	–	–	–	+	–	–	–	+	–
PNP β -Dara	–	–	+	+	+	–	+	–	–	–	+	–	–	–	+	–
PNP α -Lfuco	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
PNP β -Dfuco	+	+	+	+	–	–	–	–	–	–	+	–	+	–	+	–
PNP α -Dgal	+	–	+	+	+	–	–	+	–	+	+	–	–	–	+	–
PNP β -Dgal	+	+	+	+	–	–	–	–	–	–	+	–	–	–	+	–
PNP α -Dxyl	–	–	+	–	–	+	–	–	+	–	+	–	–	–	+	–
PNP β -Dxyl	–	+	+	+	+	+	+	–	+	–	+	+	–	–	+	–
PNP α -Dmal	–	–	+	+	–	+	–	–	–	–	+	+	–	–	+	–
PNP <i>N</i> -acglc	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+

enzyme production show that the species of the family *Rhizobiaceae* have a different pattern of glycosidase production. All strains isolated from *Prosopis* in this study showed the same pattern of extracellular enzymes.

DISCUSSION

The application of new molecular techniques to the classification and description of new species and genera has, in the last few decades, led to a large increase in the size of the family *Rhizobiaceae*, for example through the description of several new genera (*Mesorhizobium*, *Allorhizobium*). One of them, the genus

Mesorhizobium (Jarvis *et al.*, 1997), includes a group of species related to *Mesorhizobium loti* and obtained from a diverse range of host legumes including *Lupinus*, *Ornithopus*, *Lotus*, *Anthyllis*, *Caragena*, *As-tragalus*, *Ononis*, *Genista*, *Mimosa*, *Cicer* and *Leucaena*. At the time of description of the genus, it included the species *Mesorhizobium ciceri* (Nour *et al.*, 1994), *Mesorhizobium huakuii* (Chen *et al.*, 1991), *M. loti* (Jarvis *et al.*, 1982), *Mesorhizobium mediterraneum* (Nour *et al.*, 1995) and *Mesorhizobium tianshanense* (Chen *et al.*, 1995). Later, two new species were described: *M. plurifarum*, for a group of isolates from *Acacia*, *Leucaena*, *Prosopis* and *Chamaecrista* (de Lajudie *et al.*, 1998a), and *Mesorhizobium amorphae*,

corresponding to a group of isolates from *Amorpha fruticosa* (Wang *et al.*, 1999).

Some legumes, such as *Prosopis*, are indigenous to the American continent and have been cultivated in other geographical locations since the fifteenth century. The adaptation of these legumes to new habitats can include the nodulation of their roots by different genera and species of rhizobia in each soil. Thus, it is of great interest to study the populations that nodulate *Prosopis* in large ecosystems in South America, because the symbionts of *Prosopis* in America have not been extensively studied.

Stable LMW RNAs are molecules whose interest for taxonomic purposes lies in their presence in all cells from the beginning of evolution, in the fact that they always play the same role (protein synthesis, an indispensable function for cell growth) and in their ubiquitous distribution in living beings. SCE allows an optimal separation of these molecules (Cruz-Sánchez *et al.*, 1997). This technique has already been applied to genera and species of the family *Rhizobiaceae*, obtaining different profiles for each bacterial species assayed (Velázquez *et al.*, 1998a, b). Therefore, LMW RNA profiles allow rapid analysis of populations of bacteria and permit rapid identification of species and genera from the family *Rhizobiaceae* and the detection of new ones.

Analysis of the LMW RNA profiles of the strains isolated in this study showed that: (i) all strains isolated from *Prosopis* in this study displayed the same LMW RNA profile and, therefore, should be included in the same genus and species; (ii) the 5S rRNA zone of their profile indicated that these strains should be included in the genus *Mesorhizobium*; and (iii) the tRNA profile of strains studied indicated that these strains should be included in a novel species of this genus.

Analysis of the 16S rRNA sequence of strain LMG 19008^T confirmed the results obtained from the LMW RNA profiles. As can be seen in Fig. 2, this strain grouped with the other species of *Mesorhizobium*, although this grouping was not strongly supported in the bootstrap analysis. Strain LMG 19008^T formed a new and separate line (Fig. 2) in the *Mesorhizobium* group, with sequence similarities of 98.0–98.4% to the other *Mesorhizobium* species. The degree of similarity to 16S rRNA sequences of other closely related genera such as *Chelatobacter* or *Aminobacter* was lower than 98%. Results obtained by SDS-PAGE of whole-cell protein extracts also confirmed those obtained by LMW RNA analysis. The results showed high similarity among the patterns of strains isolated from *Prosopis* in this study (Fig. 3) and low similarity between the patterns of these strains and those of other species of *Mesorhizobium* (Fig. 4). Results obtained for DNA–DNA hybridization showed that the two representative strains, LMG 19008^T and LMG 19022, were very closely related and their homology to the

others species of *Mesorhizobium* was low. Thus, it is concluded that the strains isolated in this study constitute a novel species of the genus *Mesorhizobium*.

All strains of *Prosopis* isolated in this study had the same patterns of resistance to antibiotics and of exoglucosidase production, but variations could be observed in the assimilation of carbon sources (Table 2). Comparison of phenotypic results for the *Prosopis* strains, strains representing other *Mesorhizobium* species and some related species showed that the novel isolates could be differentiated from all other taxa studied.

Results of all the above techniques, 16S rDNA sequencing, LMW RNA profiling, SDS-PAGE protein analysis, DNA–DNA hybridization and phenotypic analysis, thus corroborate and complement each other very well and lead to a similar conclusion. A novel species, *Mesorhizobium chacoense* sp. nov., is therefore proposed for the *Prosopis* isolates from the Chaco Arido (Argentina).

Description of *Mesorhizobium chacoense* sp. nov.

Mesorhizobium chacoense (cha.co.en'se. N.L. neut. adj. *chacoense* from El Chaco, Argentina, where the type strain was isolated).

Gram-negative, aerobic, motile, non-spore-forming rods. Colonies on YMA are circular, convex, white, opaque and usually 1–3 mm in diameter within 7 d at 28 °C. Maximum temperature for growth is 37 °C. The generation times are 10–24 h in YM broth. Strains of this species produce acid from sucrose, galactose, lactose, L-arabinose, rhamnose, trehalose, maltose, adonitol and melibiose in media with ammonium nitrate as nitrogen source. These strains are resistant to erythromycin, ciprofloxacin and cloxacillin. All strains produce β -xylosidases, α -maltosidases and *N*-acetylglucosaminidases. This species shows the 5S rRNA zone in LMW RNA profiles typical of the genus *Mesorhizobium*. All strains have the same tRNA profile. The plasmid present in the strains did not carry the *nifH* gene. The type strain, LMG 19008^T (= CECT 5336^T), has the characteristics described for this species. The G + C content of genomic DNA of strains LMG 19008^T and LMG 19022, as determined by HPLC, is 62 mol%.

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