Antonie van Leeuwenhoek **79:** 119–125, 2001.

Characterization of *Rhizobium loti* strains from the Salado River Basin

M.M. Fulchieri, M.J. Estrella* & A.A. Iglesias
*Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), Camino Circunvalación Laguna km 6, CC 164, Chascomús 7130, Buenos Aires, Argentina (*Author for correspondence; E-mail: estrella@intech.gov.ar)*

Received 10 March 2000; accepted 30 August 2000

Key words: Lotus spp., RFLP-PCR, *Rhizobium loti*, symbiotic interaction

Abstract

Thirty indigenous rhizobia strains, isolated from *Lotus tenuis* in the area of Chascomús and other regions of the Salado River Basin (Argentina), were characterized based on generation time, acid production, carbon utilization, protein profile, and molecular characterization by restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes amplified by the polymerase chain reaction (PCR). The results indicated that native rhizobia isolates from the Chascomús area are predominantly fast and intermediate-growers. The unclassified rhizobia examined by PCR-RFLP were found to be closely related to the reference strains of validly described *Rhizobium* species.

Abbreviations: MM – mineral medium; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; YEM – yeast extract mannitol

Introduction

The Salado River Basin is an extensive area in Buenos Aires province (Argentina). Soils in this area are highly saline, being poorly drained and periodically exposed to waterlogged conditions. The region is mainly used for beef and dairy cattle production with the principal feed for these purposes represented by native grassland and naturalized *Lotus tenuis* (Montes 1987). This species is a legume associated, in a symbiotic way, with *Rhizobium loti*, the nitrogen fixation process occurs hence in root nodules. *R. loti* strains commonly found in soils of the Salado River Basin are capable of forming nodules specifically with *L. tenuis* (Estrella et al. 1997). However, the characterization of the native *R. loti* strains has not been performed and data concerning the potential symbiotic capacity of these strains are not currently available.

R. loti-legume host associations show some characteristics which are different from those of other nitrogen fixation systems. Rhizobia that nodulate *Lotus* species include both fast-growing *R. loti* (Jarvis *et al.* 1982) and slow-growing *Bradyrhizobium* sp.(*Lotus*) (Jordan 1982). Strains of *R. loti* examined so far exhibit symbiotic promiscuity as they establish N_2-fixing associations not only with *Lotus* spp, but also with a variety of other legumes (Jarvis et al. 1982).

Bacterial classification can be based on phenotypic and/or genotypic characters. Phenotyping can be based on morphological, physiological, or biochemical aspects and, in the case of members of the family *Rhizobiaceae*, also on symbiotic compatibility with legume host plants. The different procedures render valuable data concerning the distinctive characteristics of the organisms. Phenotyping based on substrate utilization tests is commonly used for numerical taxonomic purposes (Dupuy et al. 1994; Gao et al. 1994) giving information of ecological significance when the substrates used occur in the organism's habitat.

Genotyping can be done by various methods, one of them being the variation in 16S rRNA genes estimated by PCR-RFLP. This method has been successfully used in determining genetic relationships between *Rhizobium* (Laguerre et al. 1994) and *Bradyrhizobium* strains (Vinuesa *et al*. 1998). The analysis of cellular proteins by SDS-PAGE fingerprinting is an intermediate method with respect to the phenetic-genetic dichotomy since the proteins analyzed represent both gene products and metabolic function (Moreira et al. 1993; Dupuy et al. 1994).

In this study, we characterized the native *R. loti* population of Chascomús, a representative area of the Salado River Basin region. A comparison was established between different isolates from the region and reference strains. The long term objective of this work is to obtain efficient plant-rhizobia pairs to optimize legume forage yields.

Materials and methods

Bacterial strains

The strains of *Rhizobium* described in this work (Table 1) were originally isolated from nodules of *L. tenuis* plants naturally growing in: (i) the outskirts of Chascomús city (strains 1-*INTECh*, 2-*INTECh,* 3-*INTECh,* 4-*INTECh* and 5-*INTECh*) and (ii) three different sites of the Salado River Basin area (strains 1-*PIRAN,* 2-*PIRAN* (from PIRAN); 1-*AYAC*, 2-*AYAC* (from AYACUCHO); and 1to 17*PILA* (from PILA)) . Reference strains (NZP, USDA) for fast and slow-growing *Rhizobium* and *Bradyrhizobium* were kindly provided by Dr Esperanza Martinez-Romero, Centro de Investigaciones sobre Fijación de Nitrógeno, UNAM, Cuernavaca, México. *R. loti* strains used as commercial inoculant for *Lotus* (*Lcom*1 and *Lcom*2) were also included. All bacteria were cultured in YEM or MM media (Vincent 1970) at 28 °C. Isolates were stored as 25% (v/v) glycerol stock cultures at −70 °C in YEM broth.

Generation times and acid production

Doubling times were calculated from the exponential growth phase of cultures grown in YEM broth according to Martinez de Drets et al. (1974). Optical density (A= 620 nm) was measured every 2 h. Native isolates and reference strains were examined for acid or alkali production after growth for 2 d (fast-growing isolates) or 6 d (slow-growing isolates) according to Monza et al. (1992).

Carbon source utilization

For determination of carbon source utilization, cells were cultured in liquid YEM collected by centrifugation at 8000 *g*, at 4 °C for 5 min, washed twice with PBS buffer (20 mM sodium phosphate buffer pH 7, containing 150 mM NaCl and 3 mM KCl), and finally resuspended in liquid mm. Aliquots containing approximately 10^8 cells were used to inoculate 5 ml of liquid mm containing 5 mM KNO_3 and the appropriate carbon source at a final concentration of 5 mg/ml. The utilization of monosaccharides (D-glucose and D-galactose), polyols (glycerol and mannitol), disaccharides (raffinose and sucrose), carboxylic acids (succinate and citrate) and aromatic compounds (ferulic and coumaric acids) was determined as described by Arias et al. (1979). When aromatic compounds were used as the sole carbon source, stock solutions were prepared in ethanol and added to the MM at a final concentration of 20 mm.

SDS-PAGE of whole cell proteins

Rhizobia were grown on liquid YEM medium and harvested at stationary phase. Bacteria were recovered by centrifugation at 12,000 *g* for 5 min, and washed three times in Tris-HCl buffer (pH 7.0). Samples containing 10–20 mg of protein per ml were prepared as described by Wright et al. (1986). Gradient gel electrophoresis with SDS was performed according to Laemmli (1970) using 4.8% (w/v) acrylamide stacking gel and a resolving SDS-gel containing 10% (w/v) acrylamide .

Amplification of DNA by PCR

Template DNA was extracted from 4 ml stationary-phase cultures. The cells were pelleted, resuspended in 1 ml of Tris-EDTA, pH 8 (TE), and lysed in 2% (w/v) SDS. After adding 40 μg of RNase A per ml, the suspension was incubated at 37 °C for 10 min. The DNA was then precipitated by adding sodium acetate (to a final concentration of 250 mM) and isopropanol (to 70% v/v). The precipitate was collected on a hooked Pasteur pipette and resuspended in 400 μl of TE. Next, 20 μg of proteinase K was added and the solution was incubated at 50 °C for 3 h. The preparation extracted twice with equal volumes of phenol and three times with equal volumes of chloroform (Sambrook et al. 1989). The DNA in the supernatant was precipitated by adding a 0.1 volume of 3 M ammonium acetate and 2.5 volumes of absolute ethanol. The pellet was washed with ethanol (70% v/v), and dried and resuspended in TE. PCR amplification was carried out in a final volume of 50 μl by mixing template DNA (50 ug) with the reaction

Table 1. Generation time and carbon utilization of *Rhizobium* spp. and reference strains

		Carbon source									
		Monosaccharide		Polyol		Disaccharide		Carboxylic acid		Aromatic compound	
Strains	Generation time (h)#	Glc	Gal	Gly	Man	Suc.	Raff	Citr	Succ	Fer	Cum.
NZP 2227	5.4 ± 0.2	+	+	+	+	+	+	–	+	+	–
NZP 2309	16.3 ± 0.2	+	+	+	+	–	–	+	+	+	+
1-*INTECh*	5.0 ± 0.1	+	+	+	+	+	+	–	+	+	–
2-*INTECh*	6.5 ± 0.1	+	+	+	+	+	+	–	+	+	+
3-*INTECh*	5.2 ± 0.2	+	+	+	+	+	+	–	+	+	–
4-*INTECh*	2.4 ± 0.3	+	+	+	+	+	+	–	+	+	–
5-*INTECh*	5.2 ± 0.2	+	+	+	+	+	+	–	+	+	–
1-*AYAC*	7.7 ± 0.3	+	+	+	+	–	–	+	+	+	+
2-*AYAC*	8.5 ± 0.2	+	+	+	+	–	–	+	+	+	+
1-*PIRAN*	7.0 ± 0.1	+	+	+	+	+	+	–	+	+	+
2-*PIRAN*	6.5 ± 0.2	+	+	+	+	+	+	–	+	+	+
1-*PILA*	5.0 ± 0.1	+	+	+	+	+	+	–	+	+	–
2-*PILA*	5.3 ± 0.1	+	+	+	+	+	+	–	+	+	–
3-*PILA*	5.2 ± 0.2	+	+	+	+	+	+	–	+	–	–
4-*PILA*	13.4 ± 0.3	+	+	+	+	–	–	+	+	+	+
5-*PILA*	10.7 ± 0.3	+	+	+	+	–	–	+	+	+	–
6-*PILA*	4.5 ± 0.2	+	+	+	+	+	+	–	+	+	+
7-*PILA*	14.5 ± 0.1	+	+	+	+	–	–	+	+	+	+
8-*PILA*	7.0 ± 0.3	+	+	+	+	+	+	–	+	+	–
9-*PILA*	nd	+	+	+	+	+	+	–	+	nd	nd
10-*PILA*	6.0 ± 0.2	+	+	+	+	+	+	–	+	+	+
11- *PILA*	4.9 ± 0.4	+	+	+	+	+	+	–	+	nd	nd
12- *PILA*	nd	+	+	+	+	–	+	–	+	+	nd
13- *PILA*	9.0 ± 0.1	nd	+	+	+	+	+	–	+	+	+
14- *PILA*	5.8 ± 0.2	+	+	+	+	+	+	–	+	+	–
15- *PILA*	5.6 ± 0.3	+	+	+	+	+	+	nd	+	+	–
16- *PILA*	6.6 ± 0.2	+	+	+	+	+	+	–	+	+	–
17- *PILA*	nd	+	+	+	+	+	+	–	nd	+	–
BALA	6.9 ± 0.1	+	+	+	+	+	+	nd	nd	+	–
BALB	6.5 ± 0.1	+	+	nd	+	+	+	nd	+	+	–
Lcom 1	11.5 ± 0.2	+	+	+	+	+	+	–	+	+	+
Lcom 2	10.2 ± 0.1	+	+	+	+	–	–	–	+	+	+

Abbreviations: Glc: glucose; Gal: galactose; Gly: glycerol; Man: mannitol; Suc: sucrose; Raff: raffinose; Cit: citrate; Succ: succinate; Fer: ferulic acid; Cu: coumaric acid. Growth (+); no growth (–); (nd) not determined.
Generation times were calculated from optical density measurements of cultures grown in YEM broth. Values are means of three replicate cultures ± standard deviation.

buffer (10 mM Tris-Cl, pH 9.0), 50 mM KCl, 1% Triton X-100, 1.5 mM $MgCl_2$), 20 μm (each) primers 27f, 5′-GAGATTTGATCCTGGCTCAG and 1495r, 5′-CTACGGCTACCTTGTTACG (derived from conserved regions of the 16S rRNA genes) and 2 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin). DNA amplification was done in a Minicycler MJ Research–Thermoblock (Watertown, Massachusetts) with the following temperature profile: an initial denaturation at 95 °C for 3 min; 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 56 °C) and extension (2 min at 72 °C) with a final extension at 72 °C for 10 min. Amplified DNA (5 μl aliquots of PCR product) was examined by 0.9% agarose gel electrophoresis.

Restriction fragment analysis

Aliquots (20 μl) of PCR products were digested with restriction endonucleases (Promega) with 2 U of enzyme per reaction as specified by the manufacturer. The following enzymes were used: *Hinf*I, *Sau3A*I, *Taq*I and *Hha*I. The digests were resolved by 2% agarose gel electrophoresis. A 100-bp ladder (GIBCO BRL, Eggenstein, Germany) was run at both sides and in the central lane of each gel. Gels were stained in an aqueous solution of ethidium bromide (1 μg/ml) and photographed under UV illumination with Polaroid type 667 positive/negative film. Sequence divergence between the 16S rDNA regions of pairs of strains were estimated from the proportion of shared restriction fragments by the method described by Nei & Li (1979). A dendrogram was constructed from the distance matrix by using the unweighted pair group method with arithmetic averages (UPGMA; Sneath & Sokal 1973).

Results and discussion

Generation times and acid production

The generation times of 30 native isolates from nodulated *L. tenuis* are presented in Table 1. *R. loti* NZP2227 and *B. loti* NZP2309 were used as reference strains for fast and slow-growing rhizobia, respectively. As shown in Table 1, the reference strains *R. loti* NZP2227 and the isolates 1-*INTECh,* 3-*INTECh,* 4-*INTECh*, 5-*INTECh*, 1-*PILA*, 2-*PILA*, 3-*PILA,* 6-*PILA*, 10-*PILA*, 11-*PILA,* 14-*PILA* and 15-*PILA* exhibited fast growth rates with generation times ranging from 2.4 to 6.0 h. Strains 4-*PILA,* 5-*PILA* and 7-*PILA,* and the commercial *R. loti* showed generation times ranging from 10.2 to 14.5 h, which classified them as slow-growing rhizobia, similary to the reference strain *B. loti* NZP2309 (16.3 h). The remaining isolates exhibited intermediate growth rates (6.5–9 h).

The isolates with fast and intermediate growth rates produced an acid reaction in YEM (data not shown), which is consistent with the previous results indicating that fast-growing *R. loti* strains are able to acidify the growth medium (Monza et al. 1992). All other isolates, as well as the slow-growing reference strain *B. loti* NZP2309, did not render any significant change in pH of the medium (data not shown).

Carbon source utilization

No differences in the utilization of monosaccharides were observed among isolates (Table 1). All grew well with either D-glucose or D-galactose as the sole carbon source. Similarly, all rhizobia tested were able to grow on MM with either glycerol or mannitol. It has been shown that members of fast and slow-growing species of *Rhizobiaceae* can utilize a variety of disaccharides, the utilization of this carbon source being a possible criterion to distinguish between rhizobia and bradyrhizobia (Stower 1985). In good agreement with this, a clear difference was observed in relation to the utilization of sucrose and raffinose, since the fast-growing isolates utilized these compounds, whereas the slow and intermediate-growing isolates 1-*AYAC*, 2-*AYAC*, 4-*PILA*, 5-*PILA*, 7-*PILA,* and *Lcom* 2 did not (Table 1).

Amongst the carboxylic acids, succinate allowed the growth of both slow and fast-growing isolates whereas citrate supported the growth of the slow-growing isolates and of some of those exhibiting intermediate growth rates. It is interesting to note that the slow-growers, strains 4-*PILA*, 6-*PILA,* 7-*PILA,* *Lcom*1, and *Lcom*2; as well as the intermediate-growers strains 2-*INTECh,* 1-*AYAC,* 2-*AYAC,* 1-*PIRAN,* 2-*PIRAN,* 10-*PILA* and 13-*PILA* utilized coumaric acid, similary to the reference strain *B. loti* NZP 2309. This result is consistent with other reports indicating that the ability to use aromatic compounds is widespread among members of the slow-growing species of the family *Rhizobiaceae* (Irisarri et al. 1996).

Protein profiles

Electrophoretic protein profiles of whole cells of native isolates 1-*INTECh,* 2-*INTECh*, 3-*INTECh,* 4-*INTECh,* 5-*INTECh*, 1-*AYAC* and 1-*PIRAN* and of references strains *R. loti* NZP 2227 and*B. loti* NZP 2309, obtained by using one-dimensional SDS-PAGE, are shown in Figure 1. The banding patterns appeared to be very similar in the majority of the isolates studied, specially in the fast-growing strains 1-*INTECh* and 3-*INTECh,* and the reference *R. loti* NZP 2227. High similarities among native strains of different origins were found, and proteins of molecular masses < 79 kDa appeared to be quite conserved between fast and intermediate-growing rhizobia. These observations are consistent with those of Roberts et al. (1980) in showing that similarities between banding patterns may be due to proteins involved in *Lotus* specificity.

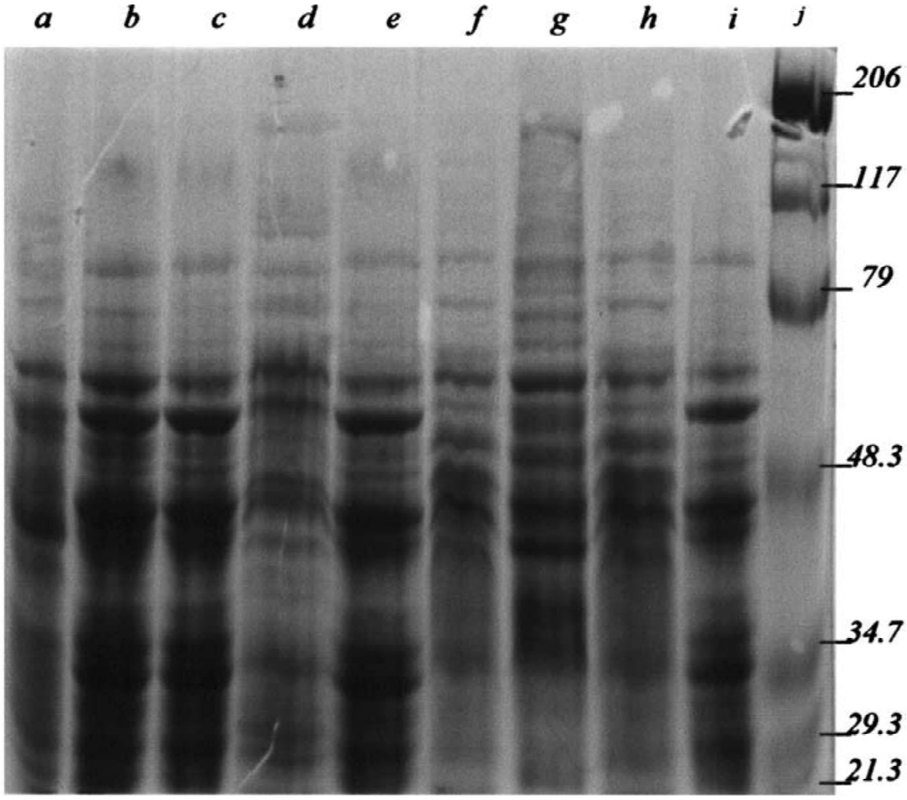

Figure 1. Protein patterns (SDS-PAGE) of whole cells of rhizobia nodulating *Lotus tenuis*. Lane a: *B. loti* NZP 2309, lane b: *R. loti* NZP 2227, lanes c–i: native isolates: c: 1-*INTECh*, d: 2-*INTECh*, e: 3-*INTECh*, f: 4-*INTECh*, g: 5-*INTECh*, h:1-*AYAC*, i:1-*PIRAN,* lane j: Molecular weight markers (kDa).

16S rDNA restriction patterns

Nearly full-length 16S rDNAs of twenty-six strains isolated from *L. tenuis* growing in the area of the Salado River Basin region and five reference strains (Table 1) were amplified by PCR using the universal primers, 27f and 1495r. All of the strains produced a single band of about 1,500 bp (data not shown) corresponding to that expected for the 16S rRNA gene (Weisburg et al. 1991). The PCR products were digested using enzymes *Sau3*AI, *Taq*I, *Hinf*I and *Hha*I. The individual RFLP patterns are shown in Figure 2. The combined *Sau3A*I, *Taq*I, *Hinf*I and *Hha*I restriction patterns of the amplified 16S rDNAs were examined by cluster analysis using the unweighted pair group method (UPGMA). The 16S rDNA genotypes of the unclassified *Rhizobium* strains were compared with those of recognized species. The analysis revealed fifteen well-defined groups (indicated with numbers 1 to 15 in Figure 2) out of the 26 isolates indicating the existence of high genetic diversity within the collection.

The groups were separated into three well defined main clusters (A, B and C, see Figure 2) at a linkage level of 77%. Cluster A, which comprised groups 1 (isolate 4-*PILA*), 2 (isolate 5-*PILA*), 3 (isolate 7-*PILA*), and the reference strain *B. loti* NZP 2309 was defined at a similarity of 87%. Figure 2 shows that the quantitatively most important cluster (B) consisted of groups 4 to 13 and reference strains NZP 2213, USDA 1002 and NZP 2227; these organisms were defined at a 82% similarity. The restriction patterns of strains 3-*INTECh*, *PIRAN,* 2-*PILA,* 12-*PILA* and 1-*PILA* were identical to that of the type strain *R. loti* NZP 2213, and hence were included in the same group (number 4). Strains 4-*INTECh* and 10-*PILA* (assigned to groups 5 and 6) were 94 and 93% similar to *R. loti* NZP 2213, respectively. Strains 18-*PILA* and 15-*PILA* (both included in group 7) shared a 92% similarity with *R. loti* NZP 2213. The patterns of strains *BALA*, *AYAC*, *L. com* 1, *BALB,* and *L. com* 2 were identical to that of *R. meliloti* USDA1002, and were assigned to group 8. Strain 5-*INTECh*, which shared a 94%

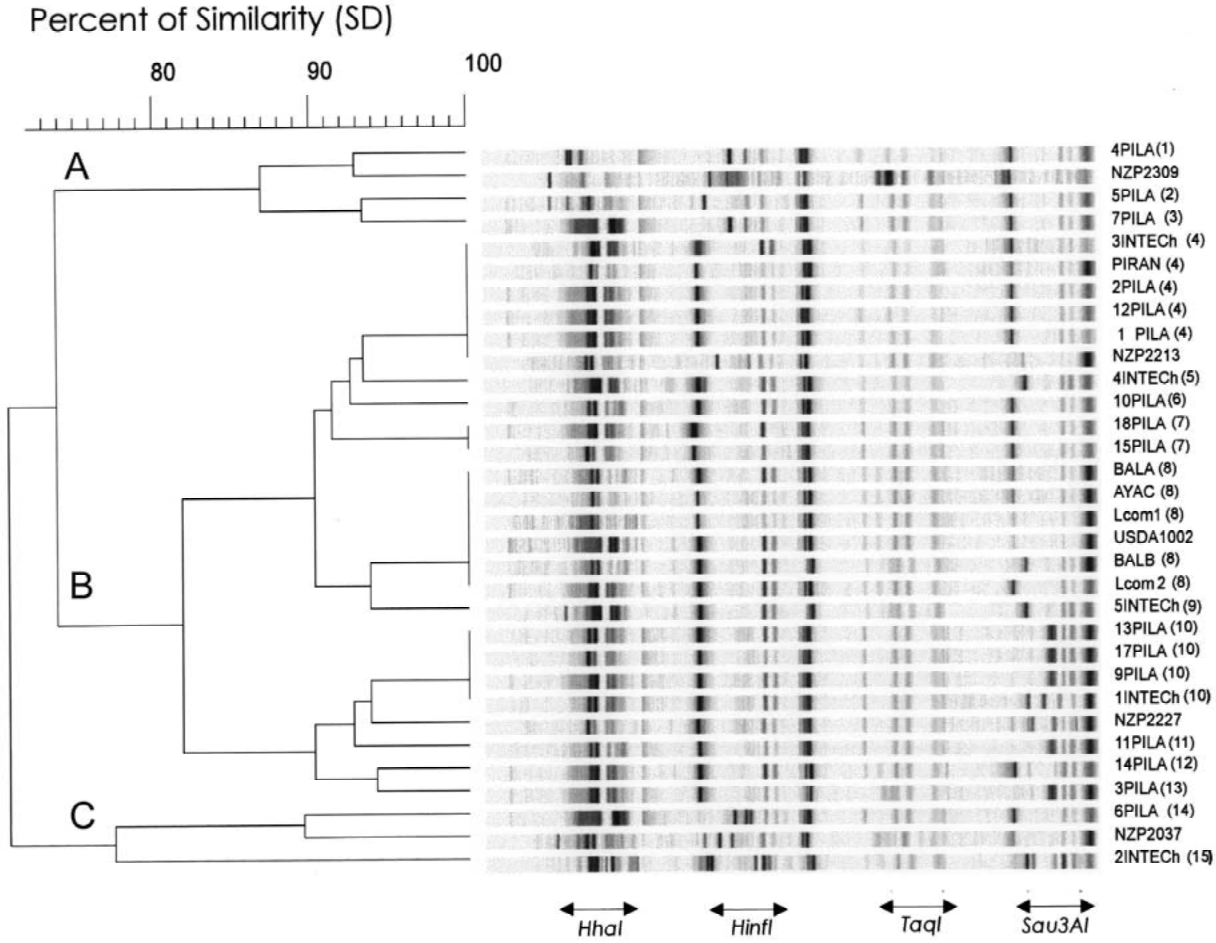

Figure 2. Dendrogram derived from cluster analysis (UPGMA) of the combined *Hha*I, *Hinf*I, *Taq*I, and *Sau*3AI restriction patterns of amplified 16S rDNA from the rhizobial strains. The 16S rDNA genotypes (1 to 15) are indicated in parentheses.

of similarity with USDA1002, was considered as a separate group (number 9). Strains 13-*PILA*, 17-*PILA*, 9-*PILA* and 1-*INTECh*, all which shared a 94% similarity to *R. loti* NZP 2227, were assigned to group 10. Strains 11-*PILA* and NZP 2227 formed group 11 at the 93% similarity level. Strains 14-*PILA* and 3-*PILA* were considered as groups 12 and 13 respectively, both having a 90% similarity to reference strain NZP 2227. On the other hand, groups 14 (6-*PILA*) and 15 (2-*INTECh*) were 91 and 78% similar to *R. loti* NZP 2037. These three strains conformed a single cluster (C) with a similarity to clusters A and B lower than 74% as also shown in Figure 2.

The results reported in the present work indicate that the native isolates from Salado River Basin nodulating *L. tenuis* are genetically and phenotypically differentiated, including fast, intermediate and slow-growing strains; although the first two types are predominant. Moreover, the grouping obtained with the PCR-RFLP of 16S rDNA indicates the existence of considerable genotypic diversity among the isolates. Nevertheless, amplified rDNA restriction analysis reveals that most of the native strains are closely related to reference strains *R. loti* NZP 22123, *R. loti* NZP 2227, and *R. meliloti* USDA 1002, with a few isolates being closely related to the slow-growing strain NZP 2309 of *B. loti.*

Previous reports indicated that *L. tenuis* is only nodulated by fast growing rhizobia and that the slow-growing strains were effective for other *Lotus* species, such as *L. pedunculatus.* Interestingly, in the present work we characterized slow growing native strains of *Rhizobium* nodulating *L. tenuis*. These strains are relevant for the development of specific inoculants for this forage species in the Salado River Basin.

Acknowledgements

The authors wish to thank Dr Mario Aguilar from the Instituto de Bioquímica y Biología Molecular, Universidad Nacional de la Plata (Argentina) for invaluable help in the use of the GelCompar system; and Dr Marco Bazzicalupo from the Dipartamento di Biologia Animale e Genetica, Universita degli studi di Firenze (Italy) for helpful discussion on the manuscript. This work was supported by grants from the Comisión de Investigaciones Científicas (CIC, Buenos Aires) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT No. 01-0674-B). MJE is a professional research assistant from CIC. MF and AAI are members of the research centre of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

References

Arias A, Cerveñansky C, Gardiol A & Martinez-Drets G (1979) Phosphoglucose isomerase mutant of *Rhizobium meliloti.* J. Bacteriol.137: 409–414

Dupuy, N, Willems A, Pot B, Dewettinck D, Vandenbruaene L, Maestrojuan G, Dreyfus B, Kersters K, Collins MD, & Gillis (1994) Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. Int. J. Syst. Bacteriol. 44: 461–473

Estrella MJ, Pieckenstain F, Ugalde RA, & Iglesias AA (1997) Studies on the symbiotic potential of *Rhizobium loti* strains native from Chascomús on *Lotus pedunculatus* and *Lotus corniculatus* var. *hirsutus* plants. Lotus Newslett. 28 http://www/psu.missouri.edu/lnl

Gao JL, Sun JG Li Y, Wang ET & Chen WX (1994) Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan province, China. Int. J. Syst. Bacteriol. 44: 151–158

Irisarri P, Milnitsky F, Monza J & Bedmar EJ (1996) Characterization of rhizobia nodulating *Lotus subbiflorus* from Uruguayan soil. Plant Soil: 180: 39–47

Jarvis BD, Pannkhurst CE & Patel JJ (1982) *Rhizobium loti*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. 32: 378–380

Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanam 1980 to *Bradyrhizobium japonicum* gen nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32: 136–139.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685.

Laguerre G, Allard MR, Revoy F & Amarger N (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S r RNA genes. Appl. Environ. Microbiol. 60: 56–63

Martinez de Drets G, Arias A & Rovira de Cutinela M (1974) Fast and slow-growing rhizobia: differences in sucrose utilization and invertase activity. Can. J. Microbiol. 20: 605–609

Moreira FM, Gillis SM, Pot B, Kersters K & Franco AA (1993) Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis of their total proteins. Syst. Appl. Microbiol. 16: 135–146

Montes L (1987) Current research on *Lotus tenuis* in Balcarce (Argentina) Lotus Newslett 18: 13

Monza J, Fabiano E & Arias A (1992) Characterization of an indigenous population of rhizobia nodulating *Lotus corniculatus*. Soil Biol. Biochem. 24: 241–247

Nei M & Li W. H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269–5273

Roberts GP, Leps WT, Silver LE & Brill WJ (1980) Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. Appl. Environ. Microbiol. 39: 414–422

Sambrook J, Fritsch EF & Maniatis TA (1989) Molecular cloning: a laboratory manual 2nd. edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NewYork

Sneath PHA & Sokal RR (1973) Numerical Taxonomy. WH Freeman & Co., San Francisco

Stower MD (1985) Carbon metabolism in *Rhizobium* species. Annu. Rev. Microbiol. 39: 89–108

Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. I.B.P. Handbook No.15. Blackwell, Oxford

Vinuesa P, Rademaker W, De Bruijn FJ & Werner D (1998) Genotypic characterization of *Bradyrhizobium* strains nodulating endemic wood legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting and partial 16S rDNA sequencing. Appl. Environ. Microbiol. 64: 2096–2104

Weisburg WG, Barns SM, Pelletier D & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703

Wright SF, Foster GJ & Bennett OL (1986) Production and use of monoclonal antibodies for identification of strains of *Rhizobium trifolii*. Appl. Environ. Microbiol. 52: 119–123