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# Characterization of rhizobia nodulating *Lotus subbiflorus* from Uruguayan soils

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## Abstract

Generation times, acid production, carbon utilization, immunological properties, plasmid content, protein profile and symbiotic properties of 15 isolates of rhizobia nodulating *Lotus subbiflorus* were studied. Based on specific growth rates, carbon source utilization and acid production, 13 out of the 15 isolates could be assigned to the slow-growing group of rhizobia (bradyrhizobia). Using antisera against whole cells of three isolates, we separated the 15 isolates into three serogroups. Only the slow-growing isolate Ls4 and the fast-growers Ls5 and Ls552 lacked cross-reactivity with any of the sera tested. Electrophoretic mobilities of whole cell protein from seven out of the eight isolates included in the serogroup represented by strain Ls31 were identical. Similarly, isolates Ls1B3 and Ls 1B4, both in serogroup Ls1B3, had the same pattern of cell proteins. In contrast, isolates Ls3 and Ls7, belonging to serogroup Ls7, differed in protein profile. Plant growth experiments carried out under bacteriologically controlled conditions revealed that all of the isolates effectively nodulated *L. subbiflorus* and *L. pedunculatus*, but were unable to form effective nodules on *L. tenuis* and *L. corniculatus*. All isolates showed similar effectiveness in symbiosis with *L. subbiflorus*, except isolate Ls7, which gave significantly higher plant dry weight.

*Abbreviations:* ELISA – enzyme linked immuno-sorbent assay, kDa – kiloDalton, MM – mineral medium, PBS – phosphate-buffered saline, RE – relative efficiency, SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis, YEM – yeast extract mannitol.

## Introduction

Within the Rhizobiaceae, bacteria capable of nodulating *Lotus* species include both fast-growing (*Rhizobium loti*) and slow-growing (*Bradyrhizobium* spp.) strains (Jarvis et al., 1982; Jordan, 1982, 1984). Strains of *R. loti* so far examined exhibit symbiotic promiscuity as they establish $N_2$-fixing associations not only with *Lotus* spp., but also with a variety of other legumes (Jordan et al., 1982; Pankhurst, 1977; Pankhurst et al., 1979).

Among *Lotus* spp. widely cultivated as pasture legume in Uruguay, *L. subbiflorus* is of special interest because of its ability to grow in soils of low water and nutrient availability and because its high quality and excellent persistence makes it a very promising variety to improve the forage production of natural pastures (Asuaga, 1994). In contrast to *L. corniculatus* and *L. tenuis*, which form effective $N_2$-fixing nodules when inoculated with fast-growing *R. loti* species, *L. subbiflorus* forms tumour-like structures that do not contain bacteroids (Monza et al., 1992a). It is therefore rather difficult to establish a pasture of *L. subbiflorus* in a field with a long *L. corniculatus* history. Only the commercial, fast-growing strain NZP2037, which forms effective nodules on *L. tenuis* and *L. pedunculatus* (Pankhurst, 1977), has been shown to fix atmospher-

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ic $N_2$ in symbiosis with *L. subbiflorus* (Russell et al., 1990). Currently, little is known about the native population of *L. subbiflorus* rhizobia in Uruguayan soils. The present study reports on the generation time, acid production, carbon utilization, immunological properties, plasmid content, protein profile and symbiotic properties of 15 indigenous rhizobia isolates nodulating *Lotus subbiflorus*.

## Materials and methods

### *Bacterial strains*

*Rhizobium loti* strain NZP2037 and *Bradyrhizobium* sp. (*Lotus*) strain NZP2309, used in this study as reference strains for fast- and slow-growing rhizobia, respectively, were obtained from the Department of Scientific and Industrial Research, Palmerston South, New Zealand, and from the Department of Microbiology, University of New South Wales, Kensington, Australia, respectively. Isolates of *L. subbiflorus* were obtained after screening of eight representative soils located in different areas in Uruguay (Baraibar et al., 1988). Isolates Ls11, Ls22, Ls31, Ls42, Ls71, Ls81, Ls82 and Ls552 were from the locality of Florida, and isolates Ls3, Ls4, Ls5, Ls7, LsS3, Ls1B3 and Ls1B4 from the locality of Durazno. Rhizobial isolates able to nodulate *L. subbiflorus* were not found in the remaining soil sites. Isolation procedures and characteristics of the soils have been described previously (Baraibar et al., 1988). Bacteria were maintained asymbiotically on yeast-extract mannitol (YEM) medium (Vincent, 1970).

### *Plant material and growth conditions*

Seeds of *Lotus subbiflorus* var. Rincón, *L. corniculatus*, *L. tenuis* and *L. pedunculatus* were surface-sterilized, germinated and planted in autoclaved Leonard jar assemblies filled with vermiculite as previously indicated (Monza et al., 1992b). Plants were inoculated at sowing with 1 mL of a single commercial strain or isolate (approximately $10^8$ cells $mL^{-1}$) and provided with sterile N-free nutrient solution (Rigaud and Puppo, 1975). Plants (6 plants per jar) were grown under greenhouse conditions. A 16/8 h light/dark photoperiod and 25/18°C were used. Supplementary light was provided by Sylvania incandescent and cool-white lamps (500 $\mu$mol $m^{-2}$ $s^{-1}$, 400–700 nm, at the plant tops).

### *Generation times and acid production*

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

### *Carbon source utilization*

The utilization of monosaccharides (D-glucose, D-galactose, and D-xylose), polyols (glycerol and mannitol), disaccharides (lactose, raphinose, sucrose, maltose, cellobiose, and melibiose), carboxylic acids (succinate, piruvate, and fumarate) and aromatic compounds (phenolic acid, salicilic acid, ferulic acid and cumaric acid) was determined as described by Arias et al. (1979).

For determination of carbon source utilization, cells were cultured in liquid YEM medium, collected by centrifugation at 8000 *g* for 10 min at 4°C, washed twice with 20 m*M* sodium phosphate buffer, pH 7, containing 150 m*M* NaCl and 3 m*M* KCl (PBS buffer), and finally resuspended in the mineral medium (MM) described by Vincent (1970). Aliquots containing approximately $10^8$ cells were used to inoculate 5 mL of liquid MM medium containing 5 m*M* $KNO_3$ and the appropriate carbon source at a final concentration of 10% (w/v). When aromatic compounds were used as carbon sources, stock solutions were prepared in ethanol and added to the MM medium at a final concentration of 20 m*M*.

### *Antisera production and adsorption*

Antisera production and adsorption were carried out essentially as described by Monza et al. (1992b). Whole cells of isolates Ls31, Ls7 and Ls1B3 were used to elicit antibodies, separately. Bacteria were grown in liquid YEM, harvested by centrifugation and washed five times in PBS. A bacterial suspension ($10^3$ cells $mL^{-1}$) was emulsified with Freund's incomplete adjuvant (1:1), and then 1 mL was injected subcutaneously into five places on the rabbit's back. Rabbits were subcutaneously boosted 6 weeks later with 0.5 mL of the same bacterial suspension. Bleedings were carried out 1 week later. Undiluted antisera (0.8 mL) were treated with 0.2 mL of a concentrated suspension of the cor-

responding adsorbing cells ($10^{11}$ cells $mL^{-1}$) in PBS containing 0.05% $NaN_3$ overnight at 4°C. Cells were then sedimented by centrifugation (twice at 12000 *g* × 15 min) and the clear supernatant was tested by ELISA against the adsorbing and remaining strains or isolates.

*ELISA*

All strains and isolates were tested by the indirect ELISA method (Wright, 1986) using antisera raised against isolates Ls31, Ls7 and Ls1B3. The appropriate antigen suspended in PBS ($10^7$ cells $mL^{-1}$) was used for plate coating. Goat antirabbit-IgG conjugated with alkaline phosphatase and the substrate *ρ*-nitrophenyl phosphate were used. Optical densities (A = 405 nm) were read with a Titertek multiscan reader after 30 min at room temperature, using substrate solution as a blank. Appropriately diluted rabbit nonimmune serum served as the negative control.

*SDS-PAGE of whole cell proteins*

Protein profile of each isolate was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacteria were grown in liquid YEM medium, collected by centrifugation and washed twice with 10 m*M* Tris-HCl buffer, pH 7.0. Samples containing 10–20 mg protein $mL^{-1}$ were prepared as described by Wright et al. (1986). For SDS-PAGE, the discontinous buffer system of Laemmli (1970) was used. The stacking gel was 5% (w/v) acrylamide and the resolving SDS-containing gel was 10% (w/v) acrylamide.

*Plasmid content*

Plasmid content of the isolates was analyzed by a modification (Rosemberg et al., 1981) of the Eckhardt procedure (1978) in which 0.7% agarose vertical gels were used. *R. loti* NZP2037 carrying a 240 MDa plasmid (Pankhurst et al., 1983) and *R. meliloti* GR4 containing three plasmids of 114 MDa, 140 MDa and > 1000 MDa (Toro and Olivares, 1986) were used as reference strains.

*Hybridization experiments*

Physical verification of *hup* genes in each isolate was carried out by DNA hybridization analysis (Sambrook et al., 1989). Isolation of total DNA from bacteria was performed essentially as described by Corbin et al. (1982). After isolation, genomic DNA was enzymatically digested to completion with *Eco*RI. The probe used was the internal *Sal*I fragment containing *hup* structural genes of *R. leguminosarum* bv. *viceae* UPM791 (Leyva et al., 1987). Hybridization was performed with a non-radioactive detection kit (Boehringer, Mannheim, Germany), and the chemiluminescence method was used to detect hybridization bands.

*Physiological assays and symbiotic effectiveness*

Nitrogenase activities were measured on detached root systems excised at the cotyledonary node of 45-day-old *L. subbiflorus* plants. Hydrogen evolution in air and $C_2H_2$-dependent $C_2H_4$ production ($C_2H_2$ reduction) were assayed sequentially within 35 min after shoot excision, when $C_2H_2$ reduction activities were linear. A gas chromatograph equipped with a thermistor detector was operated with a Molecular Sieve 5A (80–100 mesh) column (0.5 × 185 cm) to measure $H_2$. Oven temperature was 90°C, and Ar flowing at 80 mL $min^{-1}$ served as the carrier gas. Ethylene was measured on the same gas chromatograph equipped with a flame ionization detector. A Porapak Q (60–80 mesh) column (0.5 × 90 cm), oven temperature 50°C, and $N_2$ as carrier gas at 60 mL $min^{-1}$ were used. Root systems were incubated for 10 min in 100-mL bottles before measuring $H_2$. The bottles were then opened to air for 30 s before sealing with serum caps. Ten mL of the internal atmosphere were removed from each bottle and replaced with 10 mL of $C_2H_2$ in air. Bottles were sampled for $C_2H_4$ after 10 and 20 min. Relative efficiency (RE) of nitrogenase was calculated according to the equation RE = 1-hydrogen evolved in air/acetylene reduced (Schubert and Evans, 1976). Plant dry weight was determined on plants that had been heated at 80°C for 48 h.

## Results and discussion

*Generation times and acid production*

Although previous work established that fast-growing *R. loti* strains can nodulate *L. subbiflorus* plants (Russell et al., 1990), our results show that most (87%) native rhizobia isolates nodulating *L. subbiflorus* were slow-growers. Out of the 15 isolates tested, only two of them, Ls5 and Ls552, showed generation times (3–3.5 h) similar to that of the fast-growing *R. loti* NZP2037

*Table 1.* Physiological and immunological characteristics of native isolates and commercial inoculant *R. loti* strains nodulating *L. subbiflorus*

| Strain or isolate | Generation time (h)[a] | Antiserum[b] | | |
|---|---|---|---|---|
| | | Ls31 | Ls7 | Ls1B3 |
| NZP2037 | 5.0 ± 0.9 | 0.02 ± 0.01 | 0.0 | 0.0 |
| NZP2309 | 13.5 ± 0.4 | 0.13 ± 0.03 | 0.0 | 0.0 |
| Ls31 | 11.5 ± 1.0 | 0.72 ± 0.15 | 0.0 | 0.0 |
| Ls4 | 12.0 ± 0.8 | 0.09 ± 0.03 | 0.0 | 0.0 |
| Ls11 | 12.2 ± 0.4 | 0.65 ± 0.08 | 0.0 | 0.0 |
| Ls22 | 11.2 ± 1.1 | 0.78 ± 0.15 | 0.0 | 0.0 |
| Ls42 | 12.4 ± 0.5 | 0.87 ± 0.09 | 0.0 | 0.0 |
| Ls71 | 12.0 ± 1.1 | 0.80 ± 0.05 | 0.0 | 0.0 |
| Ls81 | 12.1 ± 0.2 | 0.65 ± 0.15 | 0.0 | 0.0 |
| Ls82 | 9.3 ± 1.0 | 0.77 ± 0.03 | 0.0 | 0.0 |
| LsS3 | 13.0 ± 0.8 | 0.53 ± 0.04 | 0.0 | 0.0 |
| Ls7 | 10.1 ± 1.0 | 0.09 ± 0.06 | 0.87 ± 0.02 | 0.0 |
| Ls3 | 8.2 ± 1.0 | 0.10 ± 0.01 | 0.74 ± 0.02 | 0.0 |
| Ls1B3 | 13.3 ± 1.2 | 0.0 | 0.0 | 0.74 ± 0.03 |
| Ls1B4 | 11.0 ± 1.1 | 0.0 | 0.0 | 0.81 ± 0.02 |
| Ls5 | 3.0 ± 0.9 | 0.10 ± 0.08 | 0.0 | 0.0 |
| Ls552 | 3.4 ± 0.8 | 0.0 | 0.0 | 0.0 |

[a]Values are means of four cultures ± S.D.
[b]ELISA test was performed with bacterial cells as antigens and the indicated isolates as primary antibodies. Values ($OD_{450}$ nm) are means of four wells following substraction of the negative control reading ± S.D.

(5 h) used as a reference strain (Table 1). The remaining 13 isolates, on the contrary, exhibited growth rates ranging from 8.2 to 13.3 h, which are close to that of the slow-growing reference strain NZP2309 (13.5 h) (Table 1).

Only the strain NZP2037 and the isolates Ls5 and Ls552 produced an acid reaction in YEM medium. This is consistent with previous results that indicate that fast-growing *R. loti* strains are able to acidify the growth medium (Monza et al., 1992b). All other isolates, as well as the slow-growing reference strain NZP2309 did not produce any significant changes in pH (data not shown).

*Immunological cross-reactivity of strains and isolates*

Eight out of the thirteen slow-growing isolates showed cross-reactivity with the antiserum raised against isolate Ls31 (Table 1). This indicates that they are closely related antigenically and, therefore, could be classified as belonging to the same serogroup. Among the remaining slow-growing isolates, on one hand, two of them, Ls3 and Ls7 and, on the other hand, isolates Ls1B3 and Ls1B4, can be grouped together according to their cross-reactivity with Ls7 and Ls1B3 antiserum, respectively (Table 1). As indicated by the ELISA test, the slow-growing isolate Ls4 and the fast-growing Ls5 and Ls552, as well as the reference strains NZP2037 and NZP2309, did not share detectable antigenic determinants against the three antisera used (Table 1).

*Carbon source utilization*

No differences in the utilization of monosaccharides were observed among isolates (Table 2). All of them grew well with either D-glucose, D-galactose or D-xylose as the only C source. Similarly, all rhizobial cells tested were able to grow on mineral medium with either glycerol or mannitol (Table 2). Although it has been shown that fast- and slow-growing species of the Rhizobiaceae can use a variety of disaccharides (Stower, 1985), none of the *L. subbiflorus* isolates grew on lactose, raphinose or sucrose as the only C source. All of them, nevertheless, utilized maltose to support their growth. A clear-cut difference was observed in relation to the utilization of cellobiose and melibiose as the fast-growing isolates Ls5 and Ls552 utilized those compounds, whereas the slow-growing isolates did not

*Table 2.* Growth of native isolates and commercial inoculant *R. loti* strains nodulating *L. subbiflorus* on some carbon sources

| Carbon source | Strain or isolate | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NZP2037 | NZP2309 | Ls31 | Ls4 | Ls11 | Ls22 | Ls42 | Ls71 | Ls81 | Ls82 | LsS3 | Ls7 | Ls3 | LslB3 | Ls1B4 | Ls5 | Ls552 |
| *Monosaccharides* | | | | | | | | | | | | | | | | | |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Galactose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Xylose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Polyols* | | | | | | | | | | | | | | | | | |
| Glycerol | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Mannitol | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Disaccharides* | | | | | | | | | | | | | | | | | |
| Maltose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Lactose | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Raphinose | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Sucrose | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Cellobiose | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| Melibiose | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| *Carboxylic acids* | | | | | | | | | | | | | | | | | |
| Succinate | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Citrate | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| Piruvate | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fumarate | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Aromatic compounds* (acid forms, except for phenol) | | | | | | | | | | | | | | | | | |
| Salicilic | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Phenol | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Ferulic | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + |
| Cumaric | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | - | - |

(Table 2). Since disaccharide utilization has been used as a criterion to distinguish between *Rhizobium* and *Bradyrhizobium* strains (Jordan, 1984; Martinez de Drets et al., 1974), and given the growth rate data presented in Table 1, isolates Ls5 and LS552 could be tentatively ascribed to *Rhizobizium* species, whereas the remaining isolates could be considered as *Bradyrhizobium* species.

Among carboxylic acids, succinate allowed growth of both slow- and fast-growing isolates, whereas pyruvate and fumarate did not serve as C source for any of them. Citrate supported growth of the slow-growing isolates, and was not used by the fast-growing isolates Ls5 and Ls552 (Table 2). Although utilization of phenol or salicilic acid was not achieved by any of the isolates, they all grew well with ferulic acid. It is interesting to note that the slow-growers utilized cumaric acid, which could not be metabolized by the fast-growers (Table 2). This is consistent with other reports that indicate that the ability to use aromatic compounds is widespread among slow-growing species of the Rhizobiaceae (Stower, 1985).

*Whole cell protein profiles*

Electrophoretic mobilities of whole cell proteins have been used not only to identify rhizobial strains (Fabiano and Arias, 1990; Roberts et al., 1980), but also to differentiate among isolates within the same serogroup (Broughton et al., 1987). One-dimensional SDS-PAGE revealed that seven out of the eight isolates included in serogroup represented by Ls31 showed a similar banding pattern (Fig. 1, lane A); only LsS3, within serogroup Ls31, had different protein profile to that of all other isolates in the serogroup (Fig. 1, lane B). Isolates Ls7 and Ls3 belonging to serogroup Ls7 differed

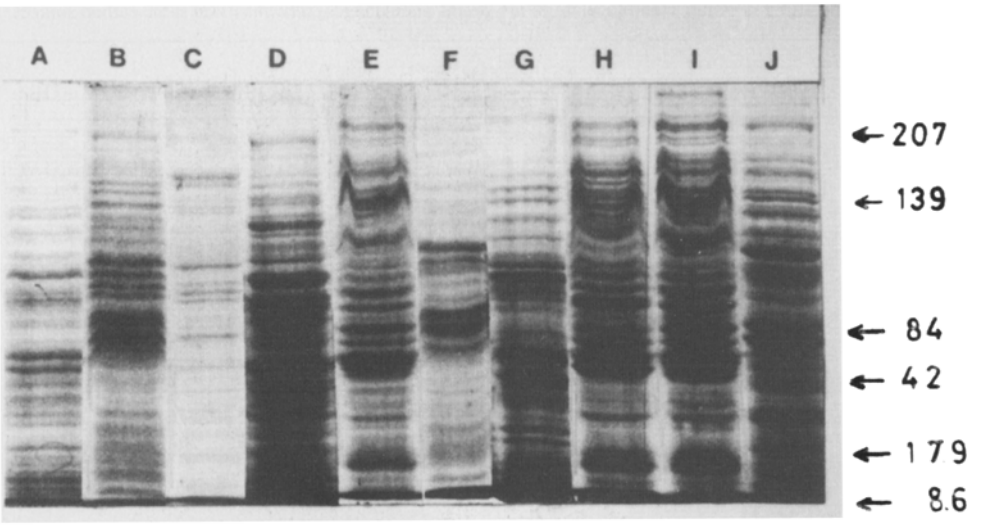


*Figure 1.* Whole cell proteins pattern (SDS-PAGE) of native isolates and commercial inoculant strains nodulating *L. subbiflorus*. Lanes A–H, isolates Ls31, LsS3, Ls7, LS3, Ls1B3, Ls5, Ls552, and Ls4, respectively; lane I, strain NZP2037, lane J, strain NZP2309. Numbers indicate in kDa the molecular masses of standard proteins: 207, myosin; 1.39, $\beta$-galactosidase; 84, bovine serum albumin; 42, carbonic anhidrase; 17.9, lysozyme; 8.6, aprotinin.

in protein profile (Fig. 1, lanes C and D, respectively), which also were clearly distinguishable from that of Ls31 and LsS3. In contrast to serogroup Ls7, isolates Ls1B3 and Ls1B4, both within serogroup Ls1B3, had an identical pattern of whole cell proteins (Fig. 1, lane E) which was clearly different from those of isolates in serogroups Ls31 and Ls7. The protein profiles of the fast-growing isolates Ls5 (Fig. 1, lane F) and Ls552 (Fig. 1, lane G), and that of the slow-growing Ls4 (Fig. 1, lane H) were different from one another as well as from those of isolates included in each serogroup. No similarities were found between electrophoretic mobilities of proteins from *L. subbiflorus* isolates and those of the proteins obtained from the commercial strains NZP2037 and NZP2309 (Fig. 1, lanes I and J, respectively). Results on both immunochemical assays and electrophoretic cell protein profiles indicate that only 8 out of the 13 slow-growing isolates could be considered as different strains of rhizobia. It is clear, however, that fast- and slow-growing isolates possess a different genetic background. These results contrast with those previously published (Monza et al., 1992b), that indicate that electrophoretic mobilities of cell proteins of each of the the 15 fast-growing isolates from root nodules of *L. corniculatus* were unique, suggesting that they represent different strains of rhizobia.

*Plasmid content*

Using the Eckhardt agarose gel procedure, no plasmid band could be seen in any of the isolates (Fig. 2, lane C). Simultaneous electrophoresis of *R. meliloti* GR4 (Fig. 2, lane A) and *R. loti* NZP2037 (Fig. 2, lane B), containing plasmids of known molecular weight, allowed visualization of the corresponding plasmid bands as previously described (Pankhurst et al., 1983; Toro and Olivares, 1986). These results contrast with those that indicate that most *R. loti* isolated from nodules of *L. corniculatus* harbour at least one plasmid (Monza et al., 1992b). Thus, although plasmid profiles have been reported as a convenient characteristic for grouping *R. meliloti* strains (Hartmann and Amarger, 1991), the absence of plasmids resolvable by electrophoresis did not allow the further identification of isolates from *L. subbiflorus*. The lack of plasmids in our isolates resembles that from other slow-growing rhizobia (bradyrhizobia), where the presence of plasmids is less frequent (Masterson et al., 1982).

*Symbiotic effectiveness and physiological assays*

Besides nodulating *L. subbiflorus*, all isolates used in this study formed effective nodules on *L. pedunculatus*, but did not nodulate either *L. corniculatus* or *L. tenuis*. Similarly, plants of *L. subbiflorus* were also effectively nodulated by rhizobial bacteria isolated from root

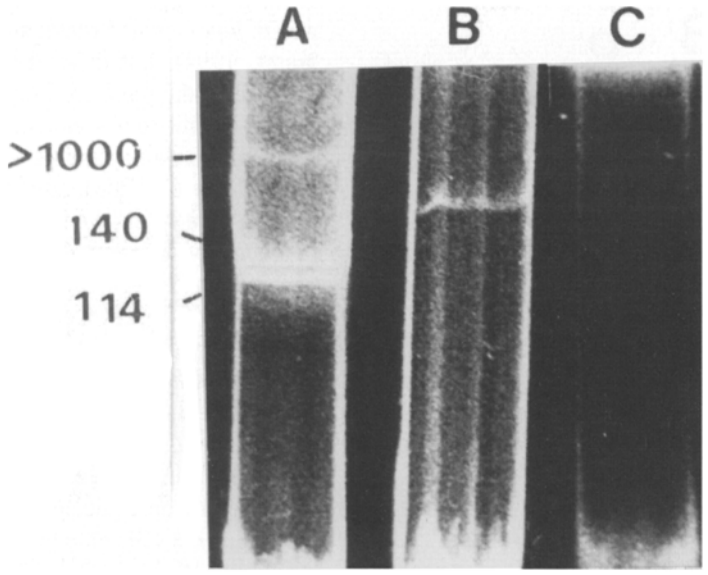


*Figure 2.* Plasmid profiles of lysates from *R. meliloti* GR4 (lane A), *R. loti* NZP2037 (lane B), and from any of the isolates tested (lane C). Numbers indicate in MDa the molecular size of plasmid bands (Pankhurst et al., 1983; Toro and Olivares, 1986).

*Table 3.* Symbiotic properties of native isolates and commercial inoculant *R. loti* strains nodulating *L. subbiflorus*

| Strain or isolate | $C_2H_2$ reduction[a] | $H_2$ production[a] | Relative efficiency[b] | Plant dry weight (mg) |
|---|---|---|---|---|
| | ($\mu$mol $C_2H_4$ or $H_2$ $h^{-1}$ $g^{-1}$ nfw)[c] | | | |
| NZP2037 | ND[d] | ND | ND | 10.8 ± 1.4 |
| NZP2309 | ND | ND | ND | 9.8 ± 1.1 |
| Ls31 | 13.8 ± 3.1 | 4.8 ± 1.0 | 0.65 | 12.0 ± 2.1 |
| Ls4 | 10.0 ± 2.6 | 3.2 ± 0.2 | 0.68 | 11.2 ± 0.5 |
| Ls11 | 10.6 ± 1.6 | 4.0 ± 0.6 | 0.62 | 14.2 ± 1.8 |
| Ls22 | 15.2 ± 3.9 | 5.2 ± 0.9 | 0.66 | 10.3 ± 2.0 |
| Ls42 | 12.0 ± 3.6 | 3.9 ± 0.6 | 0.68 | 9.8 ± 1.6 |
| Ls71 | 16.9 ± 4.0 | 4.4 ± 1.4 | 0.74 | 11.0 ± 1.7 |
| Ls81 | 9.9 ± 0.9 | 2.7 ± 0.3 | 0.73 | 9.9 ± 2.8 |
| Ls82 | 9.6 ± 1.6 | 3.6 ± 0.9 | 0.63 | 11.7 ± 3.5 |
| LsS3 | 11.3 ± 2.5 | 2.3 ± 0.6 | 0.80 | 12.7 ± 1.5 |
| Ls7 | 16.6 ± 4.7 | 3.0 ± 0.3 | 0.82 | 16.0 ± 2.8 |
| Ls3 | 9.2 ± 0.6 | 3.4 ± 1.4 | 0.63 | 13.0 ± 1.1 |
| Ls1B3 | 9.6 ± 1.0 | 3.4 ± 0.6 | 0.65 | 10.5 ± 3.1 |
| Ls1B4 | 15.4 ± 1.9 | 3.1 ± 0.8 | 0.80 | 15.0 ± 2.2 |
| Ls5 | 15.0 ± 1.9 | 4.1 ± 1.2 | 0.73 | 10.4 ± 0.9 |
| Ls552 | 10.2 ± 2.8 | 3.8 ± 0.8 | 0.63 | 10.2 ± 2.4 |

[a] Values are means of five replicates ± S.D.
[b] Relative efficiency was calculated as 1 - $H_2$ / $C_2H_4$.
[c] nfw = nodule fresh weight.
[d] ND = not determined.

nodules of *L. pedunculatus*. However, isolates from both *L. tenuis* and *L. corniculatus* formed ineffective pseudonodules of vivid red colour on *L. subbiflorus*. This type of tumour-like structure, which contains no *Rhizobium*-infected plant cells, has been previously

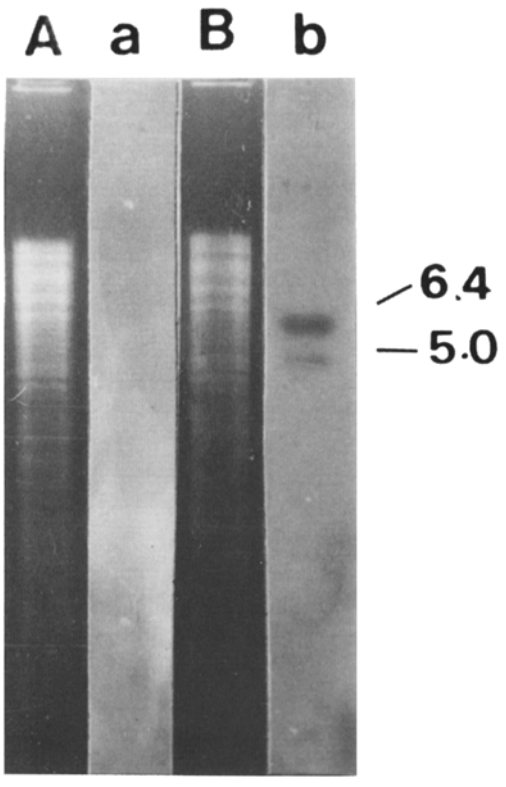


*Figure 3.* Agarose gel electrophoresis of *Eco*RI-restricted total DNA from isolate Ls11 (lane A) and *R. leguminosarum* bv. *viciae* UPM791 (lane B). Lanes a and b show the autoradiograms of dry gels A and B, respectively, after hybridization with the *Sal*I DNA fragment containing *hup* specific genes from *R. leguminosarum* bv. *viciae* UPM791. Since this *Sal*I fragment contains an internal *Eco*RI site, two hybridizing fragments of 5 and 6.4 Kb, respectively, appear for the UPM791 *Eco*RI digest in lane b (Leyva et al., 1987).

described for *L. pedunculatus* (Pankhurst et al., 1979) and *L. subbiflorus* (Monza et al., 1992a).

Only the inoculation of isolate Ls7 resulted in significantly ($p \leq 0.05$) higher plant dry weight than inoculation with the remaining isolates (Table 3). $H_2$ evolution in air and $C_2H_2$ reduction of root nodules from *L. subbiflorus* plants in symbiosis with each single isolate are indicated in Table 3. Given the criticisms on the traditional acetylene reduction assay (Minchin et al., 1994; Vessey, 1994), the values in Table 3 are intended for comparative purposes only, to show relative differences in nitrogenase activities of the different isolates.

Bacteria forming nodules on legume roots may synthetise uptake hydrogenase which recycles the $H_2$ evolved by nitrogenase in the nodules (Evans et al., 1987; Maier, 1986), and contribute to the overall efficiency of the $N_2$ fixation process (Evans et al., 1988). These bacteria include *R. leguminosarum* bv. *viciae* and *B. japonicum* (Brito et al., 1994; Vignais and Toussaint, 1994), but there are no data concerning the absence or the presence of genetic determinants (*hup* genes) in rhizobia nodulating *Lotus* sp. DNA-DNA hybridization experiments revealed that none of the *L. subbiflorus* isolates, as exemplified by isolate Ls11 (Fig. 3, lane a), showed DNA sequences homologous to the *hup* probe containing the hydrogenase structural genes from *R. leguminosarum* bv. *viciae*. UPM791. The possibility, however, that any of the isolates may contain *hup* genes cannot be ruled out as more than one type of *hup* sequence may occur within rhizobia (Murillo et al., 1989).

In conclusion, our results, based on plant specificity, doubling time, carbon source utilization and acid production indicate that native isolates present in Uruguayan soils able to nodulate *L. subbiflorus* are predominantly slow-growers. Among them, eight diffferent strains were characterized after immunochemical assays and protein profiles. Analysis of plasmid content of the isolates and on the presence of genes coding for hydrogenase activity did not further allow distinction among isolates. Although more information about those native isolates is required, they may be a useful source of strains to resolve practical problems in field inoculation of *L. subbiflorus* pastures.

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