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Genetic Uniformity and Symbiotic Properties of Acid-Tolerant Alfalfa-Nodulating Rhizobia Isolated from Dispersed Locations throughout Argentina

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Abstract

Alfalfa-nodulating rhizobia from acidic soils (pH 5.52–6.2) in Argentina comprise a distinct group of acid-tolerant *Rhizobium* isolates which were found to be related to *Rhizobium* sp. strain Or 191 (Del Papa et al., 1999). Lipopolysaccharide profiles and 16S rDNA sequences demonstrated that all twelve acid-tolerant *Rhizobium* isolates belonged to one species. Furthermore, the group of acid-

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tolerant *Rhizobium* isolates was formed by one strain type, since they gave the same plasmid profile as well as the same IS- and ERIC-fingerprints. All acid-tolerant *Rhizobium* isolates were genetically identical even though they were isolated at very different locations in Argentina. The isolates nodulated a range of *Medicago*, *Melilotus* and *Trigonella* species, and *Phaseolus vulgaris*, but were weak in nitrogen fixation. *M. sativa* nodules induced by one of the acid-tolerant rhizobial isolates contained several bacteroids within a vesicle-like structure, in contrast to nodules induced by the reference strain *S. meliloti* 2011. Reisolation of bacteria from root nodules resulted in approx. 40 times more colony forming bacteria in case of the acid-tolerant strains. These strains mark the borderline between a symbiotic and a parasitic plant-microbe interaction.

Keywords: *Rhizobium*, acid-tolerance, genetical diversity, host range, pathogenicity, symbiotic effectiveness

1. Introduction

Symbiosis between bacteria of the genera *Azorhizobium*, *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* and their leguminous hosts results in nodule formation and nitrogen-fixation. Biological nitrogen fixation is critical to sustainable agriculture (Vance, 1997), but in many situations can be limited by environmental factors, particularly soil pH (Bordeleau and Prévost, 1994; Graham, 1992). Acidic soil conditions limit the persistence and survival of soil rhizobia, and reduce nodulation and nitrogen fixation (Brockwell et al., 1991). *Sinorhizobium meliloti* and *S. medicae*, the main microsymbionts of the perennial legume alfalfa *(Medicago sativa* L.) and other annual *Medicago* species, are extremely sensitive to acidic pH (Graham, 1992). A soil pH slightly below 6.0 leads to poor survival of many *S. meliloti* strains (Rice et al., 1977). Nevertheless, some strains of *S. meliloti* are effective in nodulating alfalfa grown on acidic soils (Rice, 1982). Howieson et al. (1988) isolated *S. meliloti* strains from acidic soils of Sardinia that persisted and nodulated *Medicago* spp. under somewhat acidic conditions. The commercial release of two of these strains (WSM419 and WSM540) resulted in the successful establishment of medic pastures in mildly acidic soils in Western Australia (Howieson et al., 1988).

Little is known about the mechanisms of acid-tolerance of *S. meliloti*. O'Hara et al. (1989) determined that the acid-tolerant strains WSM419 and WSM540 maintain an alkaline intracellular pH at low external pH, whereas acid-sensitive strains were unable to regulate intracellular pH at pH lower than pH 6.0. As many as 20 genes could be involved in the acid-tolerance of *S. meliloti* (Glenn and Dilworth, 1994), with a two-component sensor-regulator system involved in the acidic pH tolerance in this organism (Tiwari et al., 1996).

S. meliloti and *S. medicae* are not the only endosymbionts of alfalfa. Eardly et al. (1985) isolated rhizobia from nodules of alfalfa grown in moderately acidic soils in Oregon. They had the unique ability to nodulate both alfalfa and common bean (*Phaseolus vulgaris* L.). These isolates were closely related to *Rhizobium etli* (former *R. leguminosarum* bv. *phaseoli* type I), the principal microsymbiont of common bean (Eardly et al., 1992). The Oregon isolates grew in synthetic media at pH 5.2, but were reduced in nitrogen fixation with both alfalfa or common bean compared to their usual symbionts.

In Argentina alfalfa is an important forage crop, but acidic soil restricts alfalfa productivity in many parts of the country. One step to improve alfalfa cultivation under these conditions would be the identification and use of acid-tolerant rhizobial strains. Del Papa et al. (1999) described the isolation and initial characterisation of 465 strains of alfalfa-nodulating rhizobia from different locations in Argentina and Uruguay in a region spanning approximately 600 square kilometres. The isolates were grouped into acid-sensitive strains with growth up to pH 6.0, semi-acid-tolerant strains able to grow between pH 6.0 and 5.5 and acid-tolerant rhizobia still growing in the range of pH 5.5 to 5.0. The acid-sensitive and semi-acid-tolerant strains exhibited a high genetic diversity as usually observed among alfalfa-nodulating rhizobia (Del Papa et al., 1999; Segundo et al., 1999). Of the 465 isolates only 15 exhibited acid-tolerance. A preliminary characterisation of these acid-tolerant rhizobia gave initial evidence for a reduced nitrogen fixation capacity and a low genetic diversity in these isolates (Del Papa et al., 1999). In this paper, we present further data on nodulation and nitrogen fixation capacities in these rhizobia and provide additional details on strain diversity using their lipopolysaccharide profiles, and especially by different molecular fingerprinting methods. Furthermore, we analysed the ultrastructure of alfalfa root nodules induced by these isolates and investigated the viability of the bacteria reisolated from the nodules.

2. Material and Methods

Bacterial strains and growth media

The strains used in this study are listed in Table 1. The isolation and geographic origin of the acid-tolerant alfalfa-nodulating rhizobia has been described in detail by Del Papa et al. (1999).

All *Rhizobium* isolates were grown in TY at 30°C. Acid-tolerance was tested in the following minimal medium: 27.5 mM mannitol, 2.7 mM sodium glutamate, 0.15 mM K_2HPO_4, 0.15 mM KH_2PO_4, 0.7 mM Na_2SO_4, 1.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2.95 µM thiamine-HCl, 4.2 µM Ca-pantothenate, 0.08 µM biotin,

Table 1. Alfalfa-nodulating rhizobial isolates from Argentina and reference strains used in this work

Strains	Place of strain isolation	pH of original soils	Minimal growth pH in MM	Reference
***Rhizobium* spp.**				
LPU81	Argentina	6.08	5.20	Del Papa et al., 1999
LPU83	Argentina	6.08	5.20	Del Papa et al., 1999
LPU84	Argentina	6.08	5.20	Del Papa et al., 1999
LPU154	Argentina	6.20	5.20	Del Papa et al., 1999
LPU158	Argentina	6.20	5.20	Del Papa et al., 1999
LPU250	Argentina	5.49	5.20	Del Papa et al., 1999
LPU264	Argentina	5.52	5.20	Del Papa et al., 1999
LPU266	Argentina	5.52	5.20	Del Papa et al., 1999
LPU275	Argentina	5.92	5.20	Del Papa et al., 1999
LPU284	Argentina	5.55	5.20	Del Papa et al., 1999
LPU285	Argentina	5.55	5.20	Del Papa et al., 1999
LPU286	Argentina	5.55	5.20	Del Papa et al., 1999
Reference strains				
S. meliloti 2011	Australia	–	5.80	Casse et al., 1979
S. meliloti AK631	France	–	5.80	Bánfalvi et al., 1981
S. medicae CC 169	Australia	–	5.80	Eardly et al., 1990
Rhiz. sp. Or 191	USA, Oregon	5.5–5.7	5.20	Eardly et al., 1992
Rhiz. sp. WN 1	Germany	–	–	This work

MM = minimal medium; *Rhiz.* = *Rhizobium*.

0.05 mM H_3BO_3, 0.01 mM $MnSO_4$, 0.01 mM $ZnSO_4$, 0.05 mM $CuSO_4$, 0.5 µM $CoCl_2$, 1.0 µM Na_2MoO_4, 1.0 µM $FeCl_3$. To control the pH in the range of 5.0–6.0, 20 mM MES buffer (2-N-morpholinoethane-sulfonic acid) and in the range of 6.5–7.0 20 mM PIPES buffer (1,4 piperazine diethanesulfonic acid) was added. The pH of the medium was adjusted as necessary with HCl/NaOH prior to and after autoclaving. Bacterial growth was detected by absorbance measurement at 600 nm.

Nodulation and acetylene reduction assay

Seeds were surface-sterilized by shaking in 32% (v/v) HCl for 15 min and washing six times with sterile water. The seeds were germinated on nitrogen-free agar plates. The plant tests with *Medicago sativa* Europe, *M. nigra* var.

nigra, *M. arabica*, *M. falcata*, *Melilotus alba* var. *alba*, *Melilotus indica*, *Melilotus messanensis*, *Melilotus officinalis*, and *Trigonella caerulea* were carried out as described by Rolfe et al. (1980). Five seedlings were grown on each Petri dish containing synthetic, nitrogen-free agar medium of pH 7.0. Each Petri dish was inoculated with 0.1 ml of culture. For each experiment five Petri dishes were analysed. *T. foenum-graecum* and *Phaseolus vulgaris* seedlings were planted in pots 7×7 cm filled with sterile vermiculite. Ten ml of a mineral solution of pH 7.0 (Rolfe et al., 1980) were added and the pots were inoculated with 0.5 ml bacterial culture. For each plant and rhizobial strain, five pots each containing two seedlings were examined. All plants were cultured in a growth chamber at 22°C with a 16 h photoperiod. For the acetylene reduction assay of *M. sativa* and *M. truncatula*, four independent measurements, each with two nodulated plants per rhizobial strain, were performed three weeks after inoculation, as described by Somasegaran and Hoben (1985). For the acetylene reduction assay of *P. vulgaris*, independent measurements of six whole roots per rhizobial strain were carried out four weeks after inoculation.

Light and electron microscopy

M. sativa Europe nodules were harvested 3 weeks after inoculation, cut longitudinally and fixed on ice for 2 h in 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer pH 7.2. They were dehydrated in a graded ethanol series, infiltrated and embedded in LR-White resin (Plano, Marburg, Germany). Semi-thin and ultra-thin sectioning were carried out as described by Kapp et al. (1990). Semi-thin sections of about 2 µm were fixed on microscope slides and stained with 0.1% (w/v) aqueous toluidine blue O (Sigma). Photographs were taken with an Olympus BH-2 microscope.

For electron microscopy, ultra-thin sections of about 70 nm were cut with a diamond knife and collected on Parlodion-carbon-coated 200-mesh nickel grids. Sections were stained for 5 min in 2% (w/v) aqueous uranyl acetate, followed by 1 min in Reynolds lead citrate (Reynolds 1963). Specimens were examined with a Zeiss EM 109 at 50 kV.

Reisolation of bacteria from root nodules

Nodules were surface-sterilized in 20% sodium hypochlorite and washed once in sterile water and then several times in phosphate buffer (50 mM, pH 7.2). Single nodules were squashed in 500 µl of the same buffer with a glass pestle. Serial dilutions of the squashed nodule mixture in TY medium were plated on TY agar. After incubation for three days at 28°C bacterial colonies were counted and colony forming cells (cfc) were calculated. Aliquots of the

same dilution series were stained by 1 µM DAPI (4,6-diamidino-2-phenylindole) and reisolated bacterial cells (rbc) were counted with a fluorescence microscope.

SDS-PAGE of lipopolysaccharides (LPS)

LPS was solubilised from rhizobial cells for sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) by boiling the pellet from an 1.5 ml overnight culture in 50 µl of 1 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.05% (w/v) bromophenol blue. Ten µl of a solution containing 2 mg proteinase K ml^{-1} were added to the lysis buffer followed by an incubation for 1 h at 60°C. The mixture was centrifuged for 10 min at 10,000 g. Two parts of the supernatant were mixed with one part of loading buffer (120 mM Tris-HCl (pH 6.8), 3% (w/v) SDS, 9% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, and 0.003% (w/v) bromophenol blue). Electrophoresis was carried out using a 16.5% (w/v) polyacrylamide gel and tricine buffer as described by Lesse et al. (1990). LPS reference samples from *Salmonella typhimurium* were obtained from Sigma (Deisenhofen, Germany). The gels were silver-stained according to the method of Tsai and Frasch (1982).

Agarose gel electrophoresis of plasmids

A modification of the Eckhardt technique (Eckhardt, 1978) was used for gel electrophoretic analysis of plasmids. Gels were prepared in Tris-borate buffer with 0.8% (w/v) agarose and 0.2% (w/v) SDS. About 1.5×10^8 exponential-phase rhizobial cells were transferred to a sterile Eppendorf tube and then kept on ice for 5 min. After centrifugation at 10,000 g, the supernatant was removed and the pellet suspended in 30 µl of lysis solution containing 0.2 mg lysozyme ml^{-1}, 10 mg RNAse ml^{-1}, 25% (w/v) sucrose, and 1.5% (w/v) Ficoll 400000 in TB-R buffer (33.85 mM Tris-HCl, 157.5 mM boric acid, 2.5 mM EDTA, pH 7.0). This solution was immediately loaded into the wells of the gel. Twenty volts were applied until turbidity disappeared, then the gel was run at 80 volts for 4 h. After electrophoresis, the gels were stained with ethidium bromide.

Southern hybridisation

Total DNA of the *Rhizobium* isolates was isolated as described by Simon et al. (1991). After *Eco*RI digestion the DNA was electrophoretically separated on an 0.8% (w/v) Tris-acetate agarose gel and vacuum-blotted onto nylon filters. DNA probes for IS*Rm2011-1* and IS*Rm2011-2*, originally isolated from *S. meliloti* (Simon et al., 1991) and the *S. meliloti nod*H gene were obtained by

Table 2. Nucleotide sequences of primers

Primer	Sequence	Reference
16S rRNA-1	5'-TGGCTCAGAACGAACGCTGGCGGC-3'	Eardly et al., 1992
16S rRNA-2	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'	Eardly et al., 1992
IS1-1	5'-GGCAAGAAGACGATGGAGAG-3'	This work
IS1-2	5'-ATGAAGCAGGAGCCGTTGTCC-3'	This work
IS2-1	5'-GCGGTATGTATTTAAGCCCG-3'	This work
IS2-2	5'-TAGAGCGGGATGCATTTAGG-3'	This work
nodH-1	5'-CCTCAGCCATTTGCAATCCT-3'	This work
nodH-2	5'-CAGTCGTTAGCAAGCTCAA-3'	This work
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	De Bruijn, 1992

PCR amplification with total DNA of *S. meliloti* 2011 as the template. Primers used for the PCR amplification are listed in Table 2. To obtain the *mucR* probe, the plasmid pJE (Bertram-Drogatz et al., 1997) was labelled using the DIG DNA Labeling Kit (Boehringer Mannheim, Germany). Hybridisation was carried out at high stringency conditions (68°C, 5 X SSC). Filters were washed twice for 5 minutes each, in 2 X SSC, 0.1% (w/v) SDS at room temperature and twice for 15 minutes each, in 0.1 X SSC, 0.1% (w/v) SDS at 68°C. The hybridisation signal was detected according to the instructions of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Germany).

ERIC (enterobacterial repetitive intergenic consensus) PCR fingerprinting

ERIC-PCR fingerprints of strains were obtained with the primer ERIC2 (De Bruijn, 1992) as described by Niemann et al. (1997). Eight µl of the PCR reactant were separated on a 2% (w/v) tris-acetate agarose gel and visualised by ethidium bromide staining.

Sequencing of 16S rDNA

PCR of the 16S rRNA gene fragment was carried out as described by Eardly et al. (1992), with the total DNA of the acid-tolerant *Rhizobium* spp. isolates as templates and the primers 16S rRNA-1 and 16S rRNA-2 (Table 2). The PCR products were cloned into pUC18 with the SureClone Ligation Kit (Pharmacia Biotech, Sweden). The plasmid DNA was isolated with the QIAGEN Plasmid Mini Kit (Qiagen, Germany) and the nucleotide sequence was determined with an ALFexpress DNA Sequencer and the AutoRead Sequencing Kit (Pharmacia, Sweden).

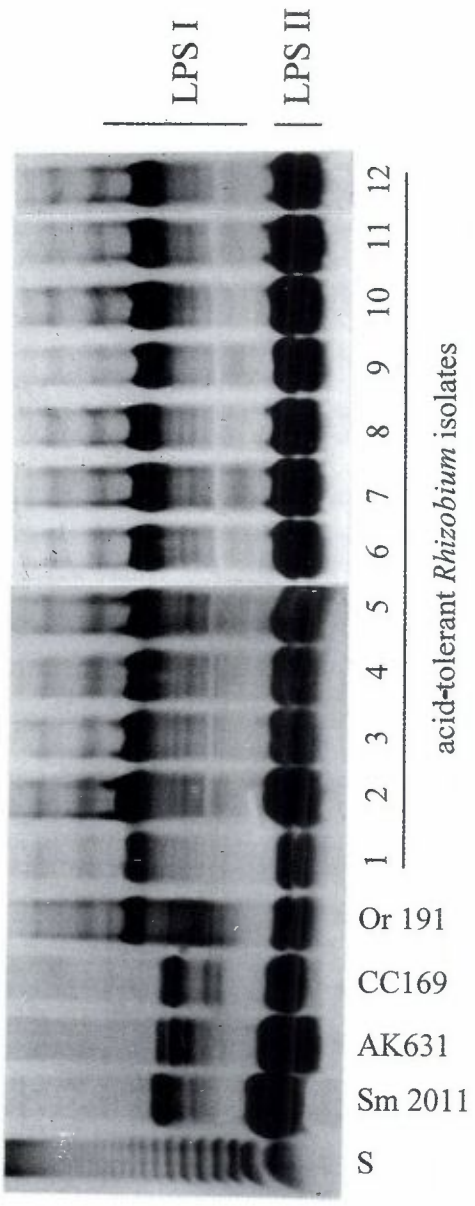

Figure 1. Silver stained SDS-PAGE profiles of lipopolysaccharides of proteinase K-treated cell lysates of the acid-tolerant *Rhizobium* isolates and the reference strains Sm 2011, CC 169, AK631 and Or 191. LPS from smooth *S. typhimurium* (S) were used as standard. Bars indicate the positions of LPS I and LPS II. Lanes 1 to 12, LPU 81, LPU 83, LPU 84, LPU 154, LPU 158, LPU 250, LPU 264, LPU 266, LPU 275, LPU 284, LPU 285, LPU 286.

3. Results

Identity of all acid-tolerant isolates with Rhizobium sp. Or 191 by LPS profiles and rDNA sequence analysis

Only about 2% of the 465 alfalfa-nodulating rhizobia collected at different sites in central Argentina and Uruguay exhibited an enhanced acid-tolerance (Del Papa et al., 1999). In the liquid medium used in this work twelve isolates were able to grow at pH values as low as 5.2 (Table 1). These isolates were used for further analysis.

Lipopolysaccharide (LPS) profiles were used to characterise the diversity of the acid-tolerant isolates (Fig. 1). All acid-tolerant *Rhizobium* isolates as well as the reference strain Or 191 had the same LPS pattern, which was different from those of *S. meliloti* strains 2011 and AK631 and *S. medicae* strain CC 169. The variability between the LPS patterns was mainly displayed by LPS I, which showed a ladder-like pattern in case of the acid-tolerant isolates.

Del Papa et al. (1999) revealed that a partial 16S rDNA sequence of strain LPU 83 was identical to that of strain Or 191. We analysed a fragment of the 16S rDNA of all Argentinean acid-tolerant isolates using primers specific for the 16S rDNA. No differences were found in the nucleotide sequences of the 16S rDNA fragments of the reference *Rhizobium* sp. strain Or 191 (GenBank accession numbers X91211 and M55236) and the acid-tolerant isolates. Sequence alignment indicated highest level of identity (99%) with *Rhizobium etli* (GenBank accession number U47303), formerly *Rhizobium leguminosarum* type I. The alignment with the 16S rDNA of *S. meliloti* (GenBank accession numbers D14509 and D12783) and *R. leguminosarum* bv. *phaseoli* (GenBank accession number U29388) revealed an identity of 96%.

Genetic uniformity of the acid-tolerant alfalfa-nodulating isolates

All acid-tolerant *Rhizobium* isolates and the *Rhizobium* sp. strain Or 191 possessed at least 3 plasmids (data not shown). The size of the largest plasmid was in the range of those of the two megaplasmids of *S. meliloti* strain Sm 2011. *S. meliloti* strain AK631 and *S. medicae* strain CC 169 had a third plasmid in addition to the two megaplasmids.

The acid-tolerant *Rhizobium* isolates were further analysed by hybridisation with a *S. meliloti nodH* gene probe. In fact, all acid-tolerant *Rhizobium* isolates hybridised with the *S. meliloti nodH* probe (data not shown). The size of the hybridising *Eco*RI fragments was in the range of 10–15 kb. Hybridisation also was performed with the *S. meliloti mucR* gene probe. The *mucR* gene is known to regulate the exopolysaccharide biosynthesis in

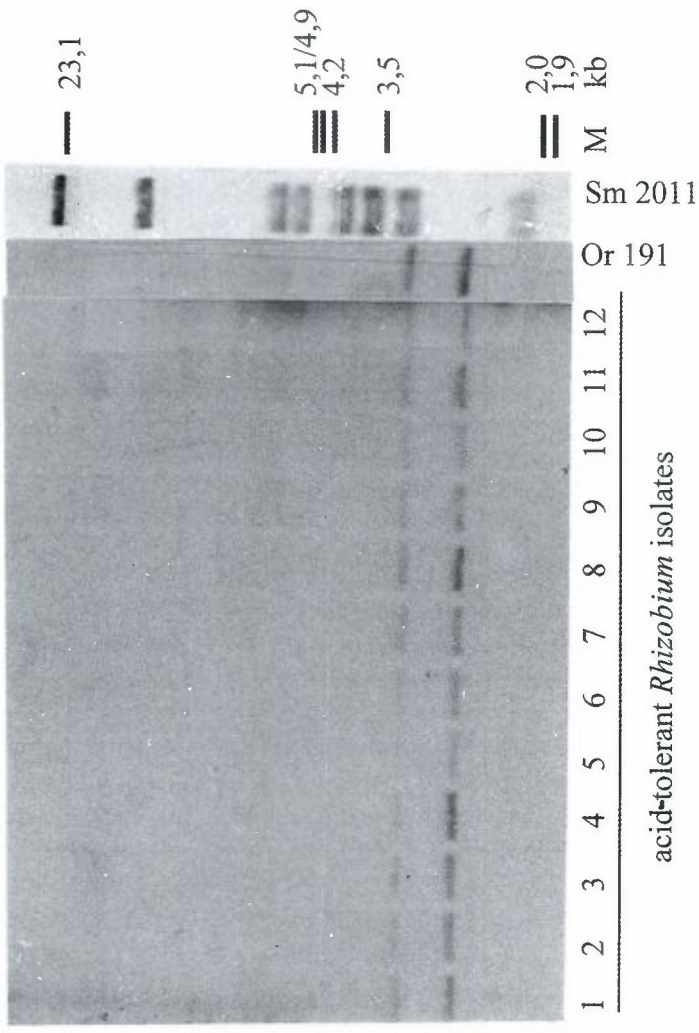

Figure 2. Southern hybridization of *Eco*RI digested total DNA of the acid-tolerant *Rhizobium* isolates and the reference strains Sm 2011 and Or 191 by using the DIG-labeled IS*Rm2011-1* probe. Lane M, λ DNA *Eco*RI/*Hind*III digested, lanes 1 to 12, LPU 81, LPU 83, LPU 84, LPU 154, LPU 158, LPU 250, LPU 264, LPU 266, LPU 275, LPU 284, LPU 285, LPU 286.

S. meliloti (Keller et al., 1995). All acid-tolerant *Rhizobium* isolates and strain Or 191 did not hybridise with the *mucR* probe (data not shown).

Since no differences were detected between the acid-tolerant isolates after genetic analyses, a more sensitive fingerprinting method which analysed the

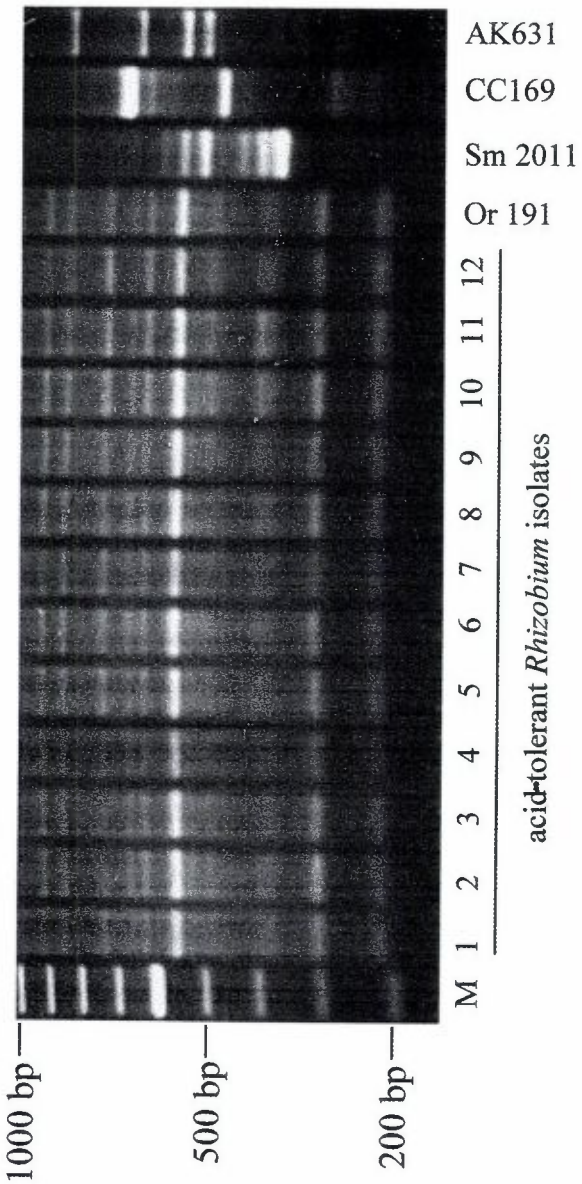

Figure 3. ERIC PCR fingerprint patterns of genomic DNA of the acid-tolerant *Rhizobium* isolates and the reference strains Sm 2011, CC 169, AK631 and Or 191. The PCR reaction was done by using the ERIC2 oligonucleotide as a primer. The amplified DNA fragments were separated on a 2% agarose gel. Lane M, 100 bp DNA marker, lanes 1 to 12, LPU 81, LPU 83, LPU 84, LPU 154, LPU 158, LPU 250, LPU 264, LPU 266, LPU 275, LPU 284, LPU 285, LPU 286.

distribution of insertion sequence (IS) elements IS*Rm2011-1* and IS*Rm2011-2* was used. All acid-tolerant isolates and the *Rhizobium* sp. strain Or 191 gave two hybridisation signals of low intensity with the IS*Rm2011-1* probe, leading to the assumption that an IS element of low homology exists in these isolates. Eight signals of higher intensity were obtained with strain *S. meliloti* Sm 2011 (Fig. 2). In contrast, IS*Rm2011-1* or a homologous IS element was not detectable in strains AK631 and CC 169. No hybridization signal was observed for the acid-tolerant *Rhizobium* isolates nor for strain Or 191 with the IS*Rm2011-2* probe.

We employed the ERIC PCR fingerprinting method in order to identify the acid-tolerant isolates on the strain level. Identical patterns were obtained for the twelve acid-tolerant isolates and also for strain Or 191 (Fig. 3). The patterns differed strongly from those of *S. meliloti* strains Sm 2011, AK 631 and *S. medicae* strain CC 169. These results provided evidence that the acid-tolerant *Rhizobium* isolates obtained from very different locations in Argentina were homologous to each other and to *Rhizobium* sp. strain Or 191.

Host-range and symbiotic properties of the acid-tolerant alfalfa-nodulating rhizobia

We tested the host range of the twelve acid-tolerant *Rhizobium* isolates on different species of three common host genera of *S. meliloti* including *Medicago*, *Melilotus* and *Trigonella*. As demonstrated previously (Del Papa et al., 1999) *M. sativa* plants inoculated with LPU 83 grew poorly on nitrogen-free medium, which argues for an ineffective symbiosis. In this work we examined the symbiotic efficiency on a broader host range of plants. The acid-tolerant strains were able to nodulate the species *Medicago sativa*, *M. truncatula*, *M. nigra* var. *nigra*, *M. arabica*, *M. falcata*, *Melilotus alba* var. *alba*, *Melilotus indica*, *Melilotus messanensis*, *Melilotus officinalis*, *Trigonella foenum-graecum*, and *T. caerulea*. But the leaves of inoculated plants became yellow and most nodules induced by the acid-tolerant isolates were white, some with brown necrotic areas. A nodulation assay using common bean (*Phaseolus vulgaris*) revealed that all acid-tolerant *Rhizobium* isolates had the ability to nodulate this plant but the light green leaves also indicated a much reduced effectiveness.

The nitrogen fixation capacity of the twelve acid-tolerant *Rhizobium* isolates was analysed by acetylene reduction assay on *M. sativa*, an agronomically important plant and *M. truncatula*, a model legume for the study of plant microbe-interactions. Their fixation rates were significantly lower than those of the reference strains *S. meliloti* 2011, AK631 and *S. medicae* CC 169, but similar to those of the reference strain *Rhizobium* sp. Or 191 (Fig. 4). On *M. truncatula* even lower rates than those on *M. sativa* were measured. All the

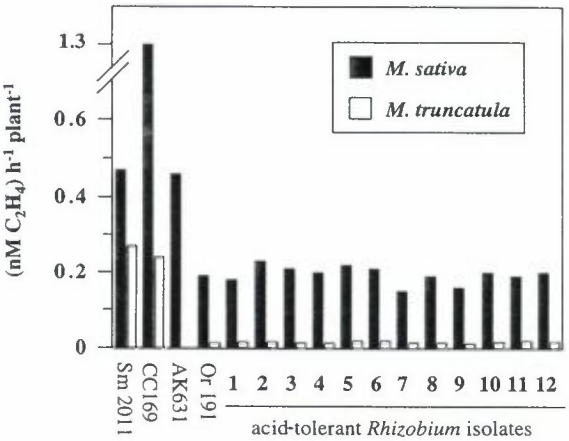

Figure 4. Acetylene reduction assay of *M. sativa* and M. *truncatula* plants inoculated with acid-tolerant *Rhizobium* isolates from Argentina and with the reference strains Sm 2011, CC 169, AK631 and Or 191. Acetylene reduction was measured three weeks after inoculation. The fixation rate was calculated as nM C_2H_4 $hour^{-1}$ $plant^{-1}$. Each bar reflects the mean value of four measurements each done with two plants. The variation within each set of eight plants was less than 10%. Lane 1 to 12, LPU 81, LPU 83, LPU 84, LPU 154, LPU 158, LPU 250, LPU 264, LPU 266, LPU 275, LPU 284, LPU 285, LPU 286.

Table 3. Acetylene reduction capacity of *Rhizobium* sp. isolate LPU 83 in comparison to reference strains

	Acetylene reduction capacity (nM C_2H_4 h^{-1} $plant^{-1}$ ±SD)			Fixation rate of *Rhizobium* sp. LPU 83 in comparison to reference strains[a]
	Reference strains			
	Sm 2011	WN 1	LPU 83	
M. sativa	0.48±0.03	n.t.	0.19±0.015	40%
M. truncatula	0.28±0.02	n.t.	0.02±0.01	7%
P. vulgaris	n.t.	45.99±4.77	13.98±4.03	30%

[a]In order to allow a direct comparison of the symbiosis with the different plant species, the fixation rate of isolate LPU 83 is also given in percentage of the fixation rate of the particular reference strains. SD = standard deviation; n.t. = not tested.

acid-tolerant *Rhizobium* isolates as well as reference strain Or 191, yielded values in the range of 0.03 nM ethylene per hour per plant (Fig. 4), which is

only 7% of that observed with *S. meliloti* 2011. Nodules induced by the reference strain AK631 turned out to be deficient in nitrogen fixation on *M. truncatula*. Since all the acid-tolerant *Rhizobium* isolates tested behaved very similar on *M. sativa* and *M. truncatula*, the acetylene reduction assay of *P. vulgaris* was carried out with only one isolate, LPU 83. Similar to the results obtained with *M. sativa* and *M. truncatula*, the nitrogen fixation capacity of isolate LPU 83 was low in combination with *P. vulgaris*. Its fixation rate was only 30% of that observed after the inoculation of *P. vulgaris* with the effective wild-type isolate *Rhizobium* sp. WN 1 (Table 3).

Symbiosome structure and vitality of reisolated acid-tolerant alfalfa-nodulating rhizobia

To investigate whether the defect in nitrogen fixation was related to a defect in nodule development, alfalfa root nodules induced by the acid-tolerant isolate LPU 83 and the reference strain Sm 2011 were analysed by light and electron microscopy. The central tissue of alfalfa root nodules induced by the reference strain Sm 2011 constituted of noninfected and infected host cells in a ratio of approximately 1:1, with numerous bacteroids in the latter (Fig. 5A). In contrast, alfalfa root nodules induced by isolate LPU 83 were characterised by a lower number of infected plant cells, which often appeared as clusters separated from one another by layers of uninfected parenchyma (Fig. 5B). Root nodules with a ratio of noninfected to infected cells similar to those of the reference strain were also observed. Both types of cells accumulated numerous starch granules, consistent with an ineffective symbiosis. No normal symbiotic zone appeared to be established, and the senescence zone was enlarged.

The colonization of infected nodule cells was further studied by electron microscopy. The bacteroids in plant cells infected by *Rhizobium* sp. LPU 83 were smaller in size than those formed by fully effective strains like *S. meliloti* 2011 and contained areas of electron-lucent material, probably poly-β-hydroxy-butyrate (Fig. 5C). The most striking morphological feature was the appearance of more than one bacteroid (up to six) within the contours of a single peribacteroid membrane, while in nodules occupied by strain Sm 2011 bacteroids were enveloped singly. The peribacteroid space, which in case of strain Sm 2011 is small with little material (not shown), was massively enlarged and filled with large amounts of fibrillar material surrounding the LPU83 bacteroids (Fig. 5C). Three weeks after inoculation, alfalfa nodules induced by *S. meliloti* 2011 showed little or no senescence. In contrast, root nodules induced by *Rhizobium* sp. LPU 83 had a greatly enlarged senescence zone and the plant cells were filled with numerous vesicles suggesting a rapid lysis of bacteroids (Fig. 5D).

Since bacteroids formed by the acid-tolerant strains exhibited morphological features resembling those of free living bacteria, we reisolated these cells and tested their ability to form colonies on TY-medium. For this purpose three week old alfalfa nodules were cut from the roots, surface-sterilised and squashed in buffer. The DNA-specific DAPI staining method was used to visualise bacterial cells which were counted under a fluorescence microscope. The fraction of colony forming cells was determined by plating the same samples on agar plates containing TY-medium. From alfalfa root nodules formed by the reference strain *S. meliloti* 2011 2×10^7 (mean of five nodules analysed) bacterial cells were recovered. Approx. 0.16% of these cells were able to grow on TY medium and gave rise to colonies. In contrast to the reference strain 2011, 1.5×10^7 (mean of five nodules analysed) bacterial cells were isolated from root nodules induced by the acid-tolerant strain LPU83, but 8.9% of which were able to form colonies on TY medium.

4. Discussion

The initial study of Del Papa et al. (1999) attested a low diversity among the acid-tolerant alfalfa nodulating rhizobia isolated from very different regions in Argentina. We have now characterized them in detail. The identical LPS-patterns observed for all acid-tolerant isolates as well as the identical 16S rDNA sequences argue that the acid-tolerant alfalfa-nodulating isolates including Or 191 represent only one species. In a phylogenetic tree based on the analysis of 16S rRNA gene sequences, the acid-tolerant isolates and strain Or 191 share one branch with *Rhizobium etli* and other bean-nodulating bacteria (Martinez-Romero and Caballero-Mellado, 1996).

The apparent genetic uniformity of the Argentinean acid-tolerant *Rhizobium* isolates was strengthened by the fact that even with the very sensitive ERIC fingerprinting method, no differences could be found. ERIC fingerprinting can be used to distinguish and classify even closely related *Rhizobium* isolates (De Bruijn, 1992). In contrast and as expected, the acid-sensitive and semi-acid-tolerant *S. meliloti* isolates exhibited the high genetic diversity (Segundo et al., 1999). For this reason it is interesting that all acid-tolerant *Rhizobium* isolates collected at different sites in Argentina possessed the same ERIC pattern, identical to that of strain Or 191.

The absence of diversity between the twelve acid-tolerant *Rhizobium* isolates is remarkable since large genetic diversity is common among rhizobial isolates. Martinez-Romero and Caballero-Mellado (1996) suggested that a wide geographical distribution of rhizobia may be related to their high genetic diversity, but such was not the case in our study. It was further speculated that bacterial genetic diversity is related to ecological factors and

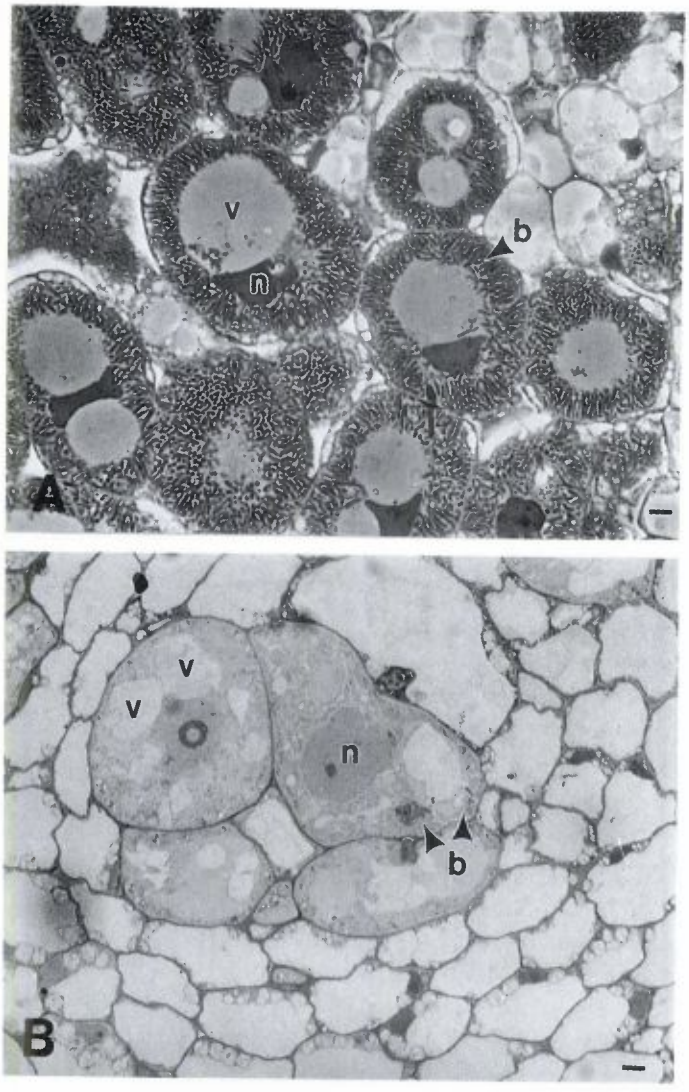

Figure 5. Light and electron microscopic comparison of *M. sativa* root nodules induced by the reference strain *S. meliloti* 2011 and by the acid-tolerant *Rhizobium* isolate LPU 83 three weeks post inoculation.
(A) Light microscopic view of the plant cells within the symbiotic zone of a *M. sativa* root nodule induced by *S. meliloti* 2011. Bacteroids (b), nucleus (n), vacuole (v). Bar represents 10 µm.
(B) Light microscopic view of the symbiotic zone of a *M. sativa* root nodule induced by the acid-tolerant isolate LPU 83. Bacteroids (b), nucleus (n), vacuole (v). Bar represents 10 µm.

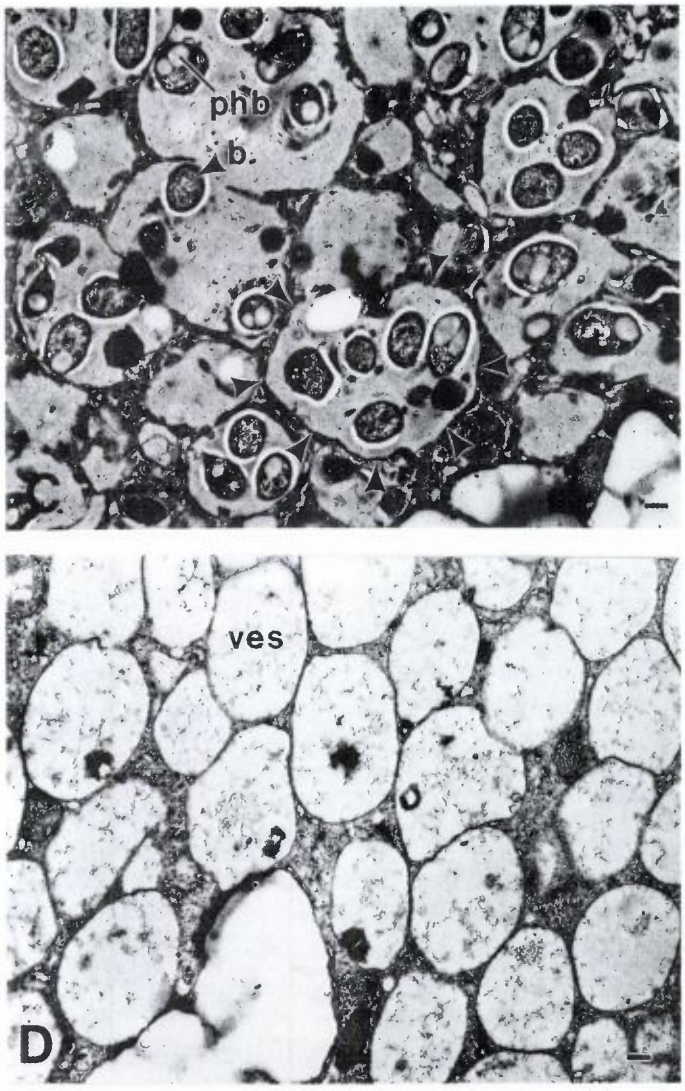

Figure 5. Continued.
(C) Electron microscopy of plant cells within the symbiotic zone of a *M. sativa* root nodule induced by the acid-tolerant isolate LPU 83. Up to six bacteroids (b) can be found within the contour of a single peribacteroid membrane, as indicated by arrows. Bacteroids inside these vesicles were separated by matrix material. Some bacteroids contain polyhydroxybutyrate (phb). Bar represents 2 μm.
(D) Electron microscopy of plant cells within the enlarged senescence zone of a *M. sativa* root nodule induced by LPU 83. Same preparation as in Fig. 2C. The plant cells are filled with numerous vesicles (ves) containing small amounts of fibrillar material. Bar represents 2 μm.

niche specialization, so that groups with little genetic variability most likely occupy specific habitats (Hildebrand et al., 1982). The ecological factor(s) that limit the diversity of the Argentinean strain collection is so far unknown. An obvious reason might be the increase of acidic soils as a consequence of industrial agriculture, which occured very recently compared to the evolutionary time scale necessary to develop genetic diversity. Interestingly Harrison and colleagues (1989) also found reduced genetic diversity among *R. leguminosarum* strains under acidic soil conditions. Further, the low number of IS elements found in this group compared to other rhizobia might be causal for the remarkable stability observed.

Acid-tolerant strains nodulate different *Medicago, Melilotus* and *Trigonella* species and *Phaseolus vulgaris*. In the last few years several *Rhizobium* isolates with a broad host-range have been reported. For example, the *Rhizobium* sp. strain NGR234 nodulates different legume genera and also the non-legume *Parasponia* (Stanley and Cervantes, 1991). Gao and Yang (1995) isolated a *S. meliloti* strain in China that fixed nitrogen in association with alfalfa and soybean. Furthermore, strains belonging to the species *Rhizobium etli* and *R. tropici* and other bean-nodulating strains are broad-host-range rhizobia (Hernandez-Lucas et al., 1995). These results led Martinez-Romero and Caballero-Mellado (1996) to the assumption that broad host-range is probably more prevalent among *Rhizobium* and *Sinorhizobium* than previously supposed.

With the plant species tested, the twelve acid-tolerant *Rhizobium* isolates were much reduced in symbiotic performance as reflected by their nodule morphology and nitrogen fixation rate. The reduced infection in the central tissue could be due to reduced infection thread development or slow proliferation of infecting bacterial cells. Strongly reduced or even no release of bacteria from the infection threads was first described for auxotrophic mutants of *S. meliloti* (Truchet et al., 1980). Most interestingly, bacteroids of the isolate LPU 83 were enclosed multiply within one peribacteroid membrane, separated by a medium electron-dense material. A similar observation was made by Eardly et al. (1992) for the *Rhizobium* sp. strain Or 191. Symbiosomes of this type are typical for determinate nodules, as it was shown in the *B. japonicum*-soybean symbiosis (Newcomb, 1981). Streeter et al. (1992) demonstrated that the matrix material within symbiosomes of the *B. japonicum*-soybean symbiosis represents an exopolysaccharide secreted by the bacteria. The production of exopolysaccharide within the symbiosome is widely distributed among specific serotypes of *B. japonicum*. Nevertheless, there is no correlation with the symbiotic effectiveness of this symbiosis (Streeter et al., 1992). In case of the *S. meliloti-M. sativa* symbiosis only one bacteroid is normally surrounded by the peribacteroid membrane, without significant matrix material in the peribacteroidal space (Vasse et al., 1990). The genes responsible for the

biosynthesis of the major *S. meliloti* exopolysaccharide, EPS I, are not transcribed in this developmental stage (Reuber et al., 1991). This could indicate that exopolysaccharides may interfere with the functions of symbiosomes in *M. sativa*.

Furthermore, bacteroids of strain LPU83 exhibited morphological features of free living rhizobia as small, road-shaped cells and secretion of exopolysaccharides. These findings are consistent with the observation that a high portion of bacterial cells reisolated from alfalfa nodules were living. In general, only a small portion of the bacterial cells within alfalfa root nodules are able to re-differentiate to the free living state and grow on TY-medium.

The described phenomenon that symbiotically ineffective rhizobia exist in soil has been observed (Barber, 1980; Bromfield, 1984). The isolation of alfalfa-nodulating rhizobia from different regions in Oregon revealed that 80–100% of the alfalfa-nodulating bacterial population was able to induce root nodules but exhibited poor or no nitrogen fixation activity in alfalfa stands established more than 6 years earlier and 0–60% in younger alfalfa stands (Barber, 1980). *Rhizobium* sp. strain Or 191 was isolated together with other rhizobia from ineffective nodules of alfalfa plants grown on moderately acidic soils in Oregon (Eardly et al., 1985). Bromfield (1984) examined soils from Canada with an indigenous, ineffective, alfalfa-nodulating *Rhizobium* population. The acid-tolerant strains described in this study displayed a wide host range, poor nitrogen fixation capacity and a high vitality after reisolation. Taking these observations together it can be speculated that a subpopulation of *Rhizobium* may behave parasitically rather than symbiotically (Vance, 1983). From an agricultural point of view these strains may present a possible risk since with further soil acidification they will probably gain more dominance which might result in alfalfa stands with ineffective symbiosis and therefore with low yields. Thus, it seems necessary to develop *Rhizobium* isolates which nodulate alfalfa effectively in acidic soil and overcome indigenous acid-tolerant but hardly effective strains.

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