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Isolation and Characterization of Alfalfa-Nodulating Rhizobia Present in Acidic Soils of Central Argentina and Uruguay

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We describe the isolation and characterization of alfalfa-nodulating rhizobia from acid soils of different locations in Central Argentina and Uruguay. A collection of 465 isolates was assembled, and the rhizobia were characterized for acid tolerance. Growth tests revealed the existence of 15 acid-tolerant (AT) isolates which were able to grow at pH 5.0 and formed nodules in alfalfa with a low rate of nitrogen fixation. Analysis of those isolates, including partial sequencing of the genes encoding 16S rRNA and genomic PCR-fingerprinting with MBOREP1 and BOXC1 primers, demonstrated that the new isolates share a genetic background closely related to that of the previously reported *Rhizobium* sp. Or191 recovered from an acid soil in Oregon (B. D. Eardly, J. P. Young, and R. K. Selander, Appl. Environ. Microbiol. 58:1809–1815, 1992). Growth curves, melanin production, temperature tolerance, and megaplasmid profiles of the AT isolates were all coincident with these characteristics in strain Or191. In addition to the ability of all of these strains to nodulate alfalfa (*Medicago sativa*) inefficiently, the AT isolates also nodulated the common bean and *Leucaena leucocephala*, showing an extended host range for nodulation of legumes. In alfalfa, the time course of nodule formation by the AT isolate LPU 83 showed a continued nodulation restricted to the emerging secondary roots, which was probably related to the low rate of nitrogen fixation by the largely ineffective nodules. Results demonstrate the complexity of the rhizobial populations present in the acidic soils represented by a main group of N_2-fixing rhizobia and a second group of ineffective and less-predominant isolates related to the AT strain Or191.

Over 4 million ha of land throughout Argentina and Uruguay are used for the production of alfalfa (*Medicago sativa* L.) (38). Therefore, it is important to manage the N_2-fixing symbiosis to maximize the production of this crop. An important constraint to this aim results from the moderately low soil pH that affects the establishment of an effective symbiosis with indigenous and inoculated rhizobia. Large areas of arable lands in the central region of Argentina have progressively acidified over the last 10 to 20 years (21, 34), where the continuous cultivation over time without crop rotation has been identified as one of the main factors that favored the acidification of soils (21, 34).

It has been shown that the poor symbiosis at low pH results from a variety of influences upon the host plant (24, 27), the population of rhizobia (31), and the symbiotic interaction itself (11, 36, 41). Early studies by Munns (36, 37) compared the progress of symbiosis under neutral and acid conditions, concluding that early steps during preinfection are the more acid-sensitive events (36). This observation is in agreement with results reported by Caetano Anollés et al. in 1989, who showed a negative influence of low pH on the bacterial attachment to roots. Most of the fundamental research has sought to characterize the physiology of the interaction (11, 23, 25, 27, 28) and the effects of acidity in laboratory and in field experiments (1a, 39) and only more recently to address the identification of the bacterial determinants of acid tolerance (18, 43, 44). Particularly, *Sinorhizobium meliloti* strains are among the more acid-sensitive rhizobia (6, 19, 20). Most *S. meliloti* isolates tolerate acidity in the range between pH 5.5 and 6.0 (25). Although there is no basis to support that a higher acid tolerance of the bacteria will correspond to a better symbiotic performance under acidic conditions, it was found that acid-tolerant (AT) *S. meliloti* strains isolated from nodulated *Medicago* spp. collected in Sardinia enhanced the establishment of medic pastures in mildly acidic soils from Western Australia (23, 25). In any case, although symbiotic proficiency and acid tolerance of rhizobia are both desirable bacterial traits, they are not necessarily linked (17, 22, 25, 30, 31). While basic aspects of symbiosis have been extensively characterized, further work is still needed in order to increase our knowledge on the rhizobial ecology under suboptimal environmental conditions such as acidity.

The characterization of the populations of alfalfa-nodulating rhizobia from acid soils had shown the presence of alfalfa-specific nodulating *S. meliloti* and another lineage represented by strain Or191 isolated from Oregon which also nodulates the common bean (13, 15). Strain Or191 was also shown to be more tolerant to acidity on agar plates (pH 5.2). Results of the genetic characterization indicate that strain Or191 is related to a previously unrecognized taxonomic group that includes strains of *Rhizobium phaseoli* type I (15), since renamed *Rhizobium etli* (32, 45). The AT strain Or191 was ineffective in

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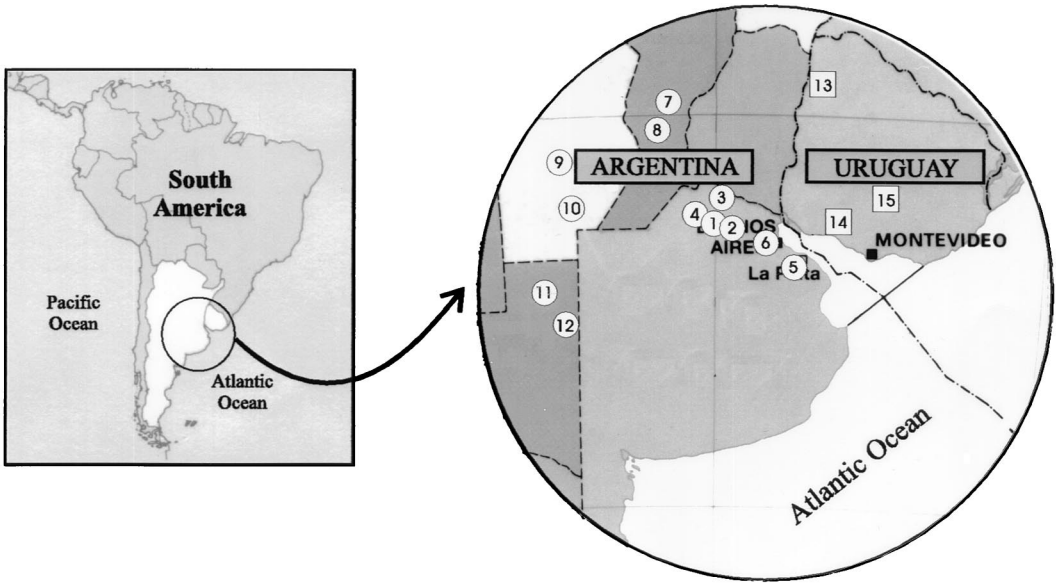

FIG. 1. Geographical distribution of representative locations where acid soils were sampled in Argentina and Uruguay. The sampled area extends over the provinces of Buenos Aires, Córdoba, La Pampa, and Santa Fe in Argentina and over four different Departments in Uruguay. Representative sampled locations in both countries are shown. Numbers in the map refer to the main cities located close to each place of sampling. Numbers in the map refer to the following cities: 1, Arrecifes; 2, Carmen de Areco; 3, San Pedro; 4, Pergamino; 5, La Plata; 6, Castelar; 7, Reconquista; 8, Rafaela; 9, San Francisco; 10, Manfredi; 11, Anguil; and 12, General Pico (all in Argentina); and 13, Paysandú; 14, Colonia; and 15, Canelones (all in Uruguay).

alfalfa (13) but had measurable levels of symbiotic nitrogen fixation (15).

So far, a detailed examination of the composition of native populations of alfalfa-nodulating rhizobia in soils from Argentina and Uruguay has not yet been carried out. In this work, we present results on the isolation and characterization of alfalfa-nodulating rhizobia from local acid soils and demonstrate the presence of two rhizobial populations with marked differences in their acid tolerance and symbiotic properties.

MATERIALS AND METHODS

Plants and microorganisms. Alfalfa (*Medicago sativa* L.) cv. CFU101 seeds were surface sterilized as previously described (9). The seeds were germinated on inverted water-agar Petri dishes and used in plant nodulation experiments. *S. meliloti* 2011 and *Rhizobium* sp. strain Or191 were obtained from J. Dénarié (Toulouse, France) and B. D. Eardly (Reading, Pa.), respectively. Alfalfa-nodulating rhizobia designed with the prefix LPU (Universidad Nacional de La Plata, La Plata, Argentina) or CE (Instituto Clemente Estable, Montevideo, Uruguay) correspond to isolates from Argentina and Uruguay, respectively.

Media and growth conditions. TY medium (2) or Luria-Bertani (LB) medium (35) were used for routine cultivation of rhizobia. The rhizobial growth curves and the screening of isolates to identify AT strains were both performed with glutamate-sucrose (GS) minimal medium (27.45 mM sucrose, 18.70 mM Na-glutamate, 0.15 mM $K_2HPO_4 \cdot 3H_2O$, 0.15 mM KH_2PO_4, 0.7 mM Na_2SO_4, 1.0 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM $CaCl_2 \cdot 2H_2O$, 2.95 μM thiamine-HCl, 4.2 μM Ca-pantothenate, 0.08 μM biotin, 48.0 μM H_3BO_3, 10.0 μM $MnSO_4$, 10.0 μM $ZnSO_4$, 48.0 μM $CuSO_4$, 0.5 μM $CoCl_2$, 1.0 μM $Na_2MoO_4 \cdot 2H_2O$, 1.0 μM $FeCl_3 \cdot 6H_2O$). Based on previous results by Howieson (22), GS medium was supplemented with 20 mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid] to adjust pH in the range of 5.5 to 6.0. Twenty millimolar PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] was added to the GS medium to control pH in the range of 6.5 to 7.0. Bacterial cultures in liquid medium were grown at 28°C and at 250 rpm. The kinetics of bacterial growth were studied in GS minimal medium supplemented with MES or PIPES depending on the actual pH of the experiment. The pH was approximated with HCl or KOH prior to autoclaving and adjusted more precisely after the addition of filter-sterilized vitamins and micronutrients. The pH of cultures during bacterial growth was monitored each time that the optical density was measured.

Isolation of alfalfa-nodulating rhizobia from acid soils. Preliminary information concerning the agricultural regions affected by low soil pH was obtained from the INTA (Instituto Nacional de Tecnología Agropecuaria)-Argentina and from INIA (Instituto Nacional de Investigaciones Agropecuarias)-Uruguay. Figure 1 shows the geographical locations of the 20 main sampled sites. Alfalfa plants from selected fields were examined for the presence of root nodules. The collected nodules were kept in closed containers over silica gel at room temperature until their isolation in the laboratory. Soil samples were also collected and investigated for the presence of rhizobia by using alfalfa as the trapping plant. Nodules collected in the field and in the laboratory were surface sterilized in 20 volumes of H_2O_2 (15 min), washed with distilled water, and crushed in 100 μl of Fåhraeus (16) mineral solution. Bacterial clones isolated from this suspension in TY (2) agar plates were used to inoculate alfalfa to confirm the nodulation phenotype.

Megaplasmid profiles. Cells were grown in rich medium to mid-log phase. A 100-μl portion of cell suspension was collected in a microcentrifuge tube and mixed with 500 μl of 0.3% Sarkosyl in TBE buffer (Tris base, 89 mM; H_3BO_3, 89 mM; EDTA, 2.5 mM; pH 8.0). The cell suspension was centrifuged 30 s at 14,000 × *g*, and the supernatant was discarded. The cell pellet was resuspended in 40 μl of loading buffer (10% sucrose, 0.01 mg of RNase A per ml, and 1 mg of lysozyme per ml) and applied to a 0.7% agarose gel containing 1% sodium dodecyl sulfate (SDS) in TBE buffer. Electrophoresis was run for 6 h at 80 V and 10°C. Plasmid bands were observed under UV illumination after the gel was stained with 0.5 to 1 μg of ethidium bromide per ml.

Production of melanin. Melanin production was determined by the method of Cubo et al. (12), with the following modifications. Bacterial isolates were streaked on TY agar medium supplemented with 600 mg of L-tyrosine and 40 mg of $CuSO_4 \cdot 5H_2O$ per liter and incubated at room temperature for 1 week. The presence of a diffusible dark brown pigment with or without the addition of 50 μl of 10% (wt/vol) SDS in TBE (pH 8.3) was scored as positive for melanin production.

Sensitivity of LPU and CE isolates to SDS. The ability of rhizobial isolates to grow in the presence of hydrophobic compounds was tested by streaking the bacteria in TY solid medium containing 0.1 g of SDS per liter. Bacterial growth at 28°C was scored 3 to 5 days after inoculation.

Nodulation tests. Two-day-old seedlings were transferred to gamma-irradiated sterilized plastic growth pouches (Mega Minneapolis International, Minneapolis, Minn.) containing 10 ml of nitrogen-free Howieson mineral solution (28) (pH 6.7). Three days later, primary roots were inoculated by dripping 100 μl of bacterial suspension onto the root from the tip toward the base. The positions of the root tips (RT) and the smallest emergent root hairs (EH) were marked on the plastic pouches immediately after inoculation with the aid of a dissecting microscope at a magnification of ×12. The plants were cultured in a growth chamber at 22°C with a 16-h photoperiod. To follow the nodulation kinetics, the number of nodules per individual plant in different locations along the root was periodically examined. The precise location of individual nodules to construct complete nodulation profiles was obtained and digitized with the aid of a magnetic tablet (Genitizer GT1212B) linked to a personal computer. Nodule coordinates were processed by using in-house-developed software to calculate the number of nodules per unit of root distance at different root positions (3). Nodulation assays with common beans cv. NAG12 (INTA) and *Leucaena leu-*

TABLE 1. Distribution of pH among the sampled soils from Argentina and Uruguay

Soil pH	Argentine soils		Uruguayan soils		Total	
	N[a]	%[b]	*N*	%[c]	*N*	%[d]
4.50–4.99	1	0.6	0	0.0	1	0.4
5.00–5.49	21	12.2	2	2.5	23	9.2
5.50–5.99	52	30.2	14	17.7	66	26.3
6.00–6.49	56	32.6	27	34.2	83	33.1
6.50–6.99	22	12.8	21	26.6	43	17.1
>7.00	20	11.6	15	19.0	35	13.9
Total	172	100	79	100	251	100

[a] *N*, number of soils with pH within the interval.
[b] Calculated with 172 soils as the 100% value (Argentine soils).
[c] Calculated with 79 soils as the 100% value (Uruguayan soils).
[d] Calculated with 251 soils as the 100% value (172 + 79).

cocephala were carried out in 200-ml plastic pots containing vermiculite and Jensen mineral solution, pH 7 (29). Surface-sterilized seeds were germinated on water-agar (1.5%, wt/vol), and two small seedlings were transferred to each pot. At sowing, the seedlings were inoculated with ca. 10^7 rhizobia/pot. Plant roots were analyzed for the presence of nodules 45 days after inoculation.

Oligonucleotide primers and PCR conditions. (i) DNA amplification fingerprints. Total DNA amplification fingerprints were performed with MBOREP1 and BOXC1 primers as previously described by Versalovic et al. (46, 47) with minor modifications. The deoxyoligonucleotide primers were synthesized by DNAgency (Malvern, Pa.). The sequences of the primers were as follows: MBOREP1 (3′-CCG CCG TTG CCG CCG TTG CCG CCG-5′) (47) and BOXC1 (3′-TGC GGC TASG CTT CCT AGT TTG C-5′) (47). PCR mixtures of 25 μl contained 50 mM Tris (pH 8.3), 500 μg of bovine serum albumin (BSA) per ml, 3 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Promega Corp.), a 10 μM concentration of primer MBOREP1 or BOXC1, and 10 μl of template DNA, obtained previously by heating a freshly isolated bacterial colony in 50 μl of distilled water to 100°C for 15 min. The amplifications were carried out in capillary tubes in an Idaho 1605 Air Thermo Cycler (Idaho Technology). The cycling conditions were as follows: 94°C for 7 min, followed by 30 cycles at 94°C for 10 s, at 52°C for 10 s (MBOREP1) or 60 s (BOXC1), and at 72°C for 2 min. After the reaction, 10 μl of the PCR products was separated in 1% agarose gels containing 0.5 to 1 μg of ethidium bromide per ml and photographed by using Polaroid type 667 film.

(ii) Amplification of *nifH* sequences. A primer pair to amplify a 511-bp DNA fragment of the *nifH* gene from different *Rhizobium* species was used. The sequences of the primers are as follows: NIFHα, 5′-ATT TCC TTG AAG AGA ACG GTG C-3′; and NIFHβ2, 5′-AGT TCG GCC AGC ATC TGC TCG T-3′ (1). PCR mixtures of 25 μl contained the following: 50 mM Tris, pH 8.3; 500 μg of BSA per ml; 3 mM $MgCl_2$; 200 μM deoxynucleoside triphosphates; 1 U of *Taq* polymerase (Promega Corp.); a 0.5 μM concentration of each primer; and 10 μl of template DNA. Cycling conditions were as follows: 94°C for 15 s, followed by 35 cycles at 94°C for 10 s, at 55°C for 10 s, at 72°C for 20 s, and at 72°C for 1 min. Amplification products were visualized as described above.

Partial sequencing of the 16S rDNA. Total DNA from the AT rhizobia was isolated as by Meade et al. (33). The partial nucleotide sequences of the 16S rRNA gene (rDNA) were determined by direct sequencing of appropriate PCR products. A DNA region corresponding to nucleotides 20 to 338 of *Escherichia coli* 16S rDNA was amplified from each strain with the universal primers Y1 (5′-TGG CTC AGA ACG AAC GCT GGC GGC-3′) and Y2 (5′-CCC ACT GCT GCC TCC CGT AGG AGT-3′) as previously described for proteobacteria (48). The nucleotide sequence of the PCR products was determined for both strands with an Automatic Laser Fluorescent DNA Sequencer (Pharmacia).

RESULTS

Isolation of alfalfa-nodulating rhizobia from moderately acid soils in central Argentina and Uruguay. Alfalfa fields from central Argentina and Uruguay were sampled and investigated for the presence of alfalfa-nodulating rhizobia (Fig. 1). Since sampling sites were selected without prior knowledge of the actual pH of the soils, data presented in Table 1 reflect the distribution of pH among fields cultured with alfalfa in the studied region. Approximately 70% of the soil samples had a pH below 6.5. All soil samples were examined in the laboratory for the presence of indigenous alfalfa-nodulating rhizobia by using alfalfa as a trap host. The collection of rhizobia includes a total of 466 isolates from 251 soil samples. Alfalfa plants growing in the field were also sampled and examined for the presence of root nodules. In most cases, few nodules could be obtained from alfalfa plants collected in fields of Argentina (10% of the isolates). In contrast, it was found that about 48% of the isolates from Uruguay were obtained from alfalfa plants growing in the field.

Screening for the presence of AT isolates. In order to characterize the acid tolerance of the alfalfa-nodulating isolates, their ability to grow in acid minimal medium was assessed. It was found that by using sodium glutamate as a nitrogen source instead of ammonium chloride, growth was initiated by some isolates at pH 5.0. Therefore, sodium glutamate was routinely used in the medium for our screening. According to the level of acid tolerance, the isolates were grouped into different categories as follows: acid-sensitive (AS) rhizobia, which include isolates able to grow at starting pH above 6.0; and AT rhizobia, which include isolates that grow at pH 5.0 or lower. A third phenotype of mid-AT (MAT) rhizobia, which includes isolates able to grow between pH 5.0 and 6.0, was also considered. However, classification of isolates as either AS or MAT was not simple due to variations in the growth behavior of many strains at pH 6.0 ± 0.3. In some cases long lag phases were observed below pH 6. It was found that about 95% of the isolated rhizobia correspond to the AS or MAT category, whereas a rather small proportion was found to be AT (only 15 of 466 isolates). All AT isolates were recovered from acidic soils with a pH between 5.0 and 6.5, and most AS or MAT isolates were obtained from soils with pH above 6.5. Figure 2 shows the growth curves of a representative AT isolate, LPU83, compared to the AS control strain *S. meliloti* 2011. The isolate LPU83 had similar growth rates in the range of pH between 5.0 to 7.0, whereas strain *S. meliloti* 2011 was unable to grow at pH 5.5, and viability decreased at lower pH. The number of viable cells of *S. meliloti* 2011 did not change at pH 5.5, but some metabolic activity still appeared to be present. The optical density of the culture slowly increased, suggesting the production of extracellular metabolites (Fig. 2B). Although cultures were buffered to maintain the initial pH, an increase in the acidity of the extracellular medium of about 0.2 to 0.6 pH units was found with isolate LPU83 (pH variations, Fig. 2). The rest of the AT isolates behaved similarly to LPU83 under all pH conditions (not shown).

Characterization of the AT alfalfa-nodulating rhizobia. We have found that isolate LPU83 was unable to grow either at 37°C or on LB medium. These are features that clearly differentiated between LPU83 and *S. meliloti*. This result and the results presented above prompted us to undertake a phenotypic characterization of our 15 AT isolates. The results of the study are summarized in Table 2. The phenotypic characteristics were found to be similar among all 15 AT isolates, as well as similar to those of strain Or191, isolated in Oregon (13, 15) (Table 2, isolate Or191).

Symbiotic characterization of the AT alfalfa-nodulating rhizobia. Nodulation assays to characterize the host range of the indigenous AT isolates showed that they were able to nodulate *Phaseolus vulgaris* as it was also previously described for the AT strain Or191 (13) (Table 2). We have also found that the AT isolates here reported and strain Or191 were all able to nodulate *Leucaena leucocephala* (Table 2). In order to characterize the nodulation of alfalfa by AT isolates, the time course of nodule formation in different parts of the root was evaluated (nodulation kinetics, Fig. 3). The pattern of nodulation induced by isolate LPU83 (AT) was compared with those of strain *S. meliloti* 2011 (our AS control) and isolate *S. meliloti* LPU63 (an indigenous isolate from a local acid soil

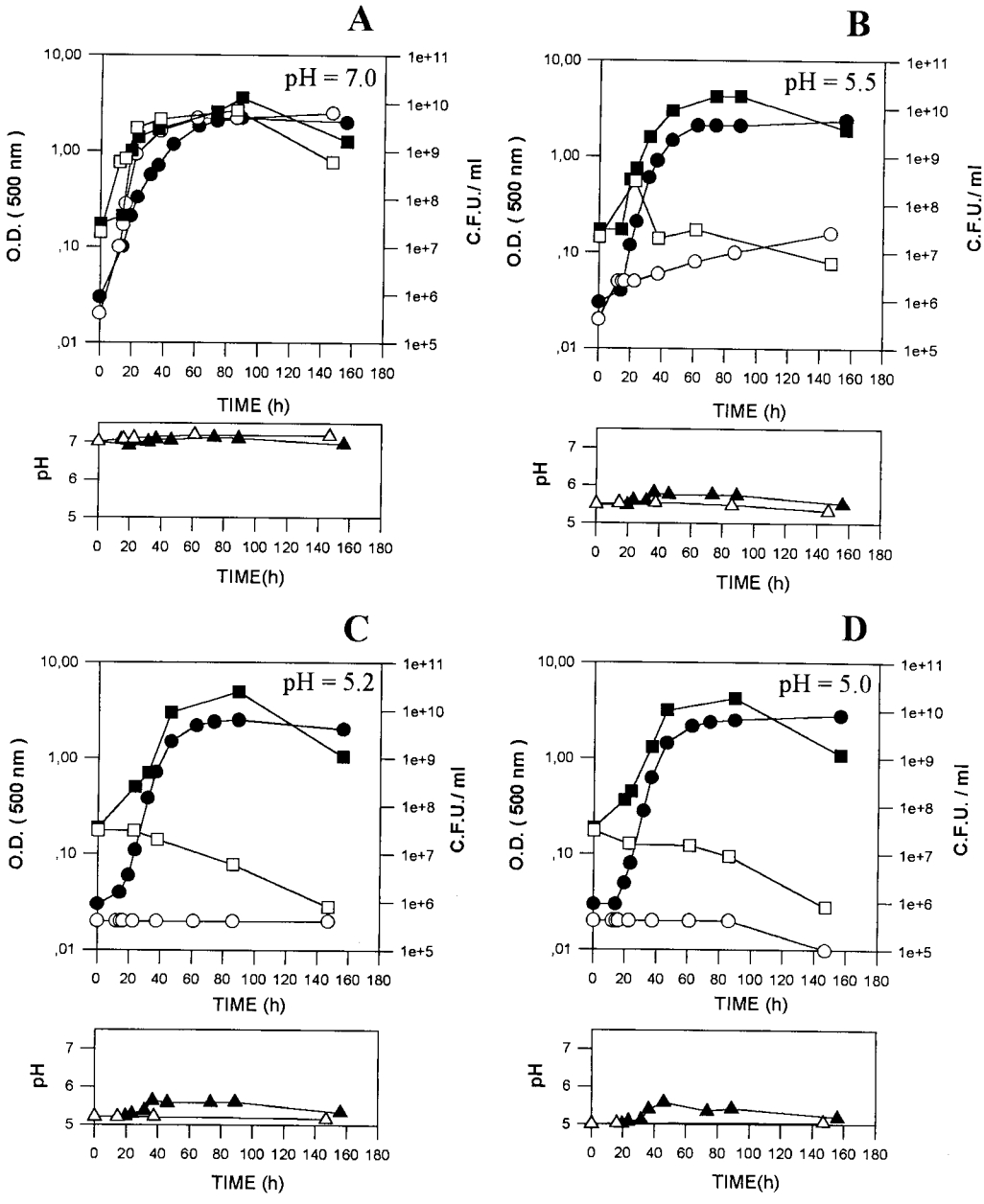

FIG. 2. Growth curves of the AT strain LPU83 and the AS *S. meliloti* 2011 at different pHs in buffered minimal medium. Panels A through D present the growth curves of strain LPU83 (solid symbols) and strain 2011 (open symbols) at pH 7.0, 5.5, 5.2, and 5.0, respectively. Circles, optical density; squares, CFU/ml. Changes in the pH along each experiment were no higher than 0.6 U. The pH variation induced by strain LPU83 (◆) and strain 2011 (◁) in each culture is presented below the corresponding set of growth curves. The given values of CFU per milliliter are the numerical averages of the number of colonies from at least two replica plates. Differences among replica plates were lower than 10%. Strains were grown to saturation in GS minimal medium at pH 7.0 and then inoculated into fresh GS medium at the different pH values to get an initial concentrations of 10^6 to 10^7 CFU/ml. Cultures were incubated at 28°C and at 250 rpm.

found to be a competitive and effective N_2 fixer in both neutral and moderately acid conditions). The number of nodules induced by all rhizobia were similar during the first 2 weeks but increased steadily during the following 3 weeks only in the case of isolate LPU83 (Fig. 3A). Nodulation by strain LPU83 did not reach a plateau. The nodulation kinetics on primary roots was found to be similar for the three rhizobia (Fig. 3B). However, the nodulation kinetics on secondary roots by isolate LPU83 differed from that of *S. meliloti* 2011 and LPU63 (Fig. 3C). Most of the late nodules were located on secondary roots and continued emerging at the same rate even 3 weeks postinoculation (Fig. 3B and C), suggesting a deficiency in the systemic autoregulatory plant response that controls nodulation (8, 10). Similar results were also found under more acidic conditions, suggesting that the increased nodulation in secondary roots is a strain-dependent phenotype. Plant inoculation experiments demonstrated that all AT isolates were unable to support plant growth in N-free medium (ineffective phenotype). Indeed, 4 weeks after inoculation the dry weight of the aereal part of the alfalfa plants showed no significant difference with the uninoculated control. Symptoms of nutrient deficiencies similar to those found in the control were evident 3

TABLE 2. Phenotypic and genotypic characteristics of representative alfalfa-nodulating rhizobia isolated from acid soils of Argentina and Uruguay

Isolate[a]	Place of isolation[b]	pH of original soils	Growth in:			Melanin production	*nifH*	MBOREP profile[c]	Plasmid profile (Eckhardt gels)[c]	Nodulation on:		
			JMM medium at pH 5.0	LB medium (28°C)	TY medium (37°C)					*M. sativa*	*L. leucocephala*	Common bean
LPU5	Castelar, Arg.	NA[d]	−	+	+	−	+	*e*	*m*	+	ND	ND
LPU7	Castelar, Arg.	5.60	−	+	+	+	+	*b*	*m*	+	ND	ND
LPU11	Castelar, Arg.	5.96	−	+	+	+	+	*g*	ND[e]	+	ND	ND
LPU18	Castelar, Arg.	5.60	−	−	−	−	+	*b*	*n*	+	ND	ND
LPU30	Rafaela, Arg.	6.04	−	+	+	−	+	*c*	*o*	+	ND	ND
LPU63	Castelar, Arg.	5.89	−	+	+	+	+	*c*	*q*	+	ND	ND
LPU283	Santa Fe, Arg.	5.68	−	+	+	+	+	*b*	*o*	+	−	ND
LPU119	Arrecifes, Arg.	6.64	−	+	+	+	+	*b*	*m*	+	ND	ND
CE15	Colonia, Uru.	6.39	−	+	+	−	+	ND	*q*	+	ND	ND
CE31	Paysandú, Uru.	6.23	−	+	+	+	+	ND	*q*	+	ND	ND
CE47	Soriano, Uru.	5.72	−	+	+	−	+	*c*	*q*	+	ND	ND
LPU81	Castelar, Arg.	6.08	+	−	−	−	+	*a*	*m*	+	+	+
LPU83	Castelar, Arg.	6.08	+	−	−	−	+	*a*	*m*	+	+	+
LPU84	Castelar, Arg.	6.08	+	−	−	−	+	*a*	*m*	+	+	+
LPU154	Arrecifes, Arg.	6.20	+	−	−	−	+	*a*	*m*	+	+	+
LPU158	Arrecifes, Arg.	6.20	+	−	−	−	+	*a*	*m*	+	+	+
LPU250	Carlos Casares, Arg.	5.49	+	−	−	−	+	*a*	*m*	+	ND	ND
LPU264	S. A. de Areco, Arg.	5.52	+	−	−	−	+	*a*	*m*	+	ND	ND
LPU266	S. A. de Areco, Arg.	5.52	+	−	−	−	+	*a*	*r*	+	+	+
LPU275	S. A. de Areco, Arg.	5.92	+	−	−	−	+	*a*	*m*	+	ND	ND
LPU284	S. A. de Areco, Arg.	5.55	+	−	−	−	+	*a*	*m*	+	+	+
LPU285	S. A. de Areco, Arg.	5.55	+	−	−	+	+	*a*	*m*	+	+	+
LPU286	S. A. de Areco, Arg.	5.55	+	−	−	−	+	*a*	*m*	+	ND	ND
CE20	Colonia, Uru	5.96	+	−	−	−	+	*a*	*m*	+	ND	ND
CE26	Colonia, Uru	5.95	+	−	−	−	+	*a*	*m*	+	ND	ND
Rhizobium sp. strain Or191	United States (B. Eardly)	5.5–5.7	+	−	−	−	+	*a*	*m*	+	+	+
S. meliloti 2011	France (J. Denarié)	NA	−	+	+	−	+	*b*	*q*	+	−	−
S. meliloti L530	France (J. Denarié)	NA	−	+	+	−	+	ND	ND	+	ND	ND
R. etli CE3	Mexico (E. Martínez)	NA	+	ND	ND	ND	+	*e*	*r*	−	−	+
R. tropici CIAT 899 (type IIB)	Colombia (CIAT)	NA	+	ND	ND	ND	+	*f*	*s*	−	+	+

[a] LPU, La Plata University; CE, Clemente Estable Uruguay.
[b] The geographical location of each place is shown in Fig. 1. Arg, Argentina; Uru., Uruguay.
[c] Different letters in the column correspond to different plasmid-fingerprinting profiles in agarose gels.
[d] NA, information not available.
[e] ND, not determined.

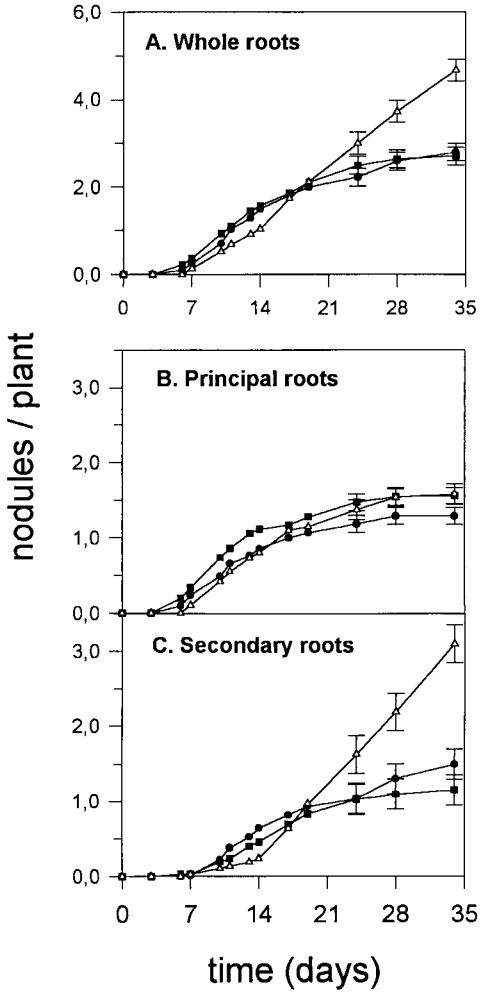

FIG. 3. Kinetics of nodulation of alfalfa roots by *Rhizobium* sp. strain LPU83, *S. meliloti* 2011, and *S. meliloti* LPU63. Sets of 74, 88, and 80 plants were inoculated with 2.96×10^6, 4.90×10^6, and 4.91×10^6 bacteria per plant of strains LPU83 (◁), 2011 (●), and LPU63 (■), respectively. Nodules on the whole root (A), primary root (B), and secondary roots (C) were scored at the indicated times. The results are given as the average numbers of nodules per plant. Where present, the error bars indicate the standard deviation ($\sigma/\sqrt{n}$). Results are taken from a representative experiment among a set of three.

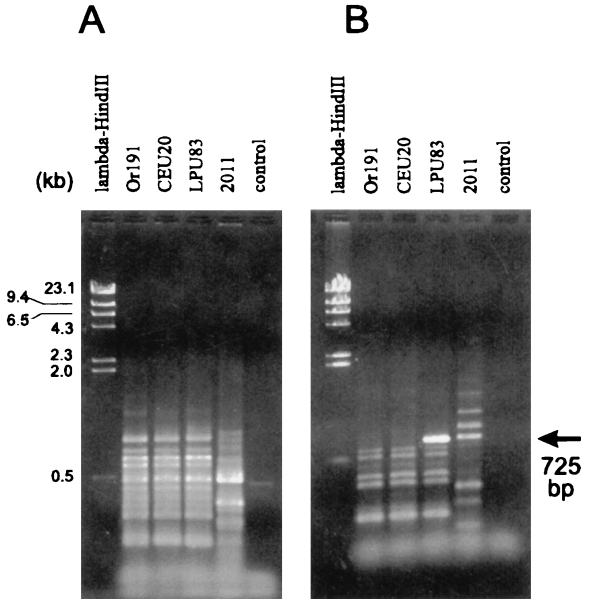

FIG. 4. MBOREP1 and BOXC1 PCR-fingerprinting patterns of representative AT isolates from central Argentina and Uruguay. Panels A and B show the patterns of PCR products generated by using chromosomal DNA of the indicated strains and primer MBOREP1 or BOXC1, respectively. The DNA molecular-weight standard corresponding to restriction fragments of the lambda phage digested with *Hin*dIII is indicated on the left side of each panel. The arrow on the right side designates the differential BOXC1 amplicon of ca. 725 bp, which was only detected in the AT isolate LPU83. Agarose gels containing ethidium bromide were photographed with Polaroid type 667 film under UV illumination. Pictures were scanned with a Hewlett-Packard ScanJet4C (high resolution), composed with Corel Draw 5, and printed with a Rainbow-3M dye sublimation primer.

weeks after inoculation. Plants inoculated with the AT isolates died during the fifth week postinoculation.

Genomic characterization of the AT isolates LPU83 and CE20: DNA amplification fingerprinting and analysis of 16S rDNA. The degree of similarity among the AT rhizobia and strain Or191 was investigated by partial sequencing of the 16S rDNA, and by PCR-fingerprinting methods. The 16S nucleotide sequence of the AT isolates LPU83 and CE20 was determined and compared to that of strain Or191. CE20 is a representative AT isolate from Uruguay with characteristics similar to isolate LPU83 (Table 2). Pairwise comparisons were made between homologous 260-bp 16S gene segments and showed that the rDNA sequences of isolates LPU83 and CE20 were identical to each other, as well as to the published sequence of strain Or191 (15). To determine whether this similarity could be extended to the whole genome, total DNA from the AT isolates was compared by using PCR-fingerprinting methods. The results of this analysis with primers MBOREP1 and BOXC1 are shown in Fig. 4A and B, respectively. MBOREP1 fingerprint profiles were identical among isolates LPU83, CE20, and Or191 but were different from that of *S. meliloti* 2011 (Fig. 4A) and that of strain *R. etli* CE3 (Table 2). Similar results were obtained with primer BOXC1, although a differential amplicon was present in the amplification profile of isolate LPU83 (Fig. 4B, arrow-labeled band of ca. 700 bp). Taken together, these results demonstrate that the AT rhizobia have a high degree of similarity among them and are closely related to strain Or191.

DISCUSSION

In this work we examined the population of alfalfa-nodulating rhizobia recovered from acid soils of Argentina and Uruguay. Of a collection of 466 indigenous alfalfa-nodulating isolates, 15 were AT with the ability to grow under laboratory conditions at pH 5.0. It has been shown elsewhere (20, 25) that the sensitivity of *S. meliloti* to acidity is generally observed in the range of pH between 5.6 and 6.0, depending on the strain and the level of Ca^{2+} in the culture medium. Although a group of rhizobia isolated in Oregon and designated *S. meliloti* were able to nodulate alfalfa and to grow at pH 5.0 (1), the actual species assignment of those isolates remains to be established.

The AT isolates described in this work were unrelated to *S. meliloti* and found to be similar to the strain Or191 (13). All AT isolates were retrieved from soils with a pH between 5.5 and 6.5. Our results demonstrate the existence of similar populations of AT alfalfa-nodulating rhizobia in geographically distant regions that have soil acidity as a common feature. The occurrence of isolates similar to strain Or191 may relate to a better adaptation of these rhizobia to acidity. The genetic

analysis of the AT isolates by PCR-fingerprinting with MBO-REP1 and BOXC1 primers demonstrated a very homogeneous genetic background, which appears also to be related to that of strain Or191. The means by which a population of Or191-like isolates characterized by a limited genetic diversity have spread geographically still remain to be elucidated. Although a distinctive aspect of all of the AT isolates is their broad host range for nodulation of legumes, neither the common bean nor *L. leucocephala* can be considered as natural hosts for the AT isolates in the region of central Argentina and Uruguay. It is not known whether a legume exists with which Or191-like rhizobia are able to establish a fully efficient symbiotic association in nature.

Although the number of Or191-like isolates in local soils represents a rather low proportion of the total bacterial population able to nodulate alfalfa, it has to be considered that over time the proportion of AT isolates may increase and thus also their significance within the indigenous alfalfa-compatible rhizobia. In this regard, at least two characteristics of the AT isolates have to be considered: (i) the marked tolerance to acidity of Or191-like isolates that might favor their persistence under acidity in free-living conditions, and (ii) the abnormal increased nodulation in secondary roots, a phenotype that might also help the bacteria to evade the acidic environment. We have also previously shown that under acidic conditions isolate LPU83 was highly competitive for the nodulation of alfalfa against *S. meliloti* (42). The persistence of ineffective alfalfa-nodulating rhizobia is a common yet unexplained observation in acid soils (1, 4, 5, 14, 40). As previously suggested, this may be associated with either a gradual loss of the strain effectiveness as a consequence of soil acidity (7) or with the presence of promiscuous *Rhizobium* species which can ineffectively nodulate alfalfa, as is the case for strain Or191 (13). A number of questions arise. Do acid soils favor the establishment of the Or191-like rhizobia? If so, are these rhizobia more saprophytically competent than *S. meliloti*? Answers to these questions will be required to assess the actual agricultural significance of the presence of inefficient AT rhizobia in acidic soils cultivated with alfalfa. It remains to be examined whether the occurrence of Or191-like rhizobia in acid soils is a general phenomenon in alfalfa-growing areas of the world.

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