Rhizobium lusitanum sp. nov. a bacterium that nodulates *Phaseolus vulgaris*

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The species *Phaseolus vulgaris* is a promiscuous legume nodulated by several species of the family *Rhizobiaceae*. During a study of rhizobia nodulating this legume in Portugal, we isolated several strains that nodulate *P. vulgaris* effectively and also *Macroptilium atropurpureum* and *Leucaena leucocephala*, but they form ineffective nodules in *Medicago sativa*. According to phylogenetic analysis of the 16S rRNA gene sequence, the strains from this study belong to the genus *Rhizobium*, with *Rhizobium rhizogenes* and *Rhizobium tropici* as the closest related species, with 99.9 and 99.2 % similarity, respectively, between the type strains of these species and strain P1-7^T. The *nodD* and *nifH* genes carried by strain P1-7^T are phylogenetically related to those of other species nodulating *Phaseolus*. This strain does not carry virulence genes present in the type strain of *R. rhizogenes*, ATCC 11235^T. Analysis of the *recA* and *atpD* genes confirms this phylogenetic arrangement, showing low similarity with respect to those of *R. rhizogenes* ATCC 11235^T (91.9 and 94.1 % similarity, respectively) and *R. tropici* IIB CIAT 899^T (90.6 % and 91.8 % similarity, respectively). The intergenic spacer (ITS) of the strains from this study is phylogenetically divergent from those of *R. rhizogenes* ATCC 11235^T and *R. tropici* CIAT 899^T, with 85.9 and 82.8 % similarity, respectively, with respect to strain P1-7^T. The tRNA profile and two-primer random amplified polymorphic DNA pattern of strain P1-7^T are also different from those of *R. rhizogenes* ATCC 11235^T and *R. tropici* CIAT 899^T. The strains isolated in this study can be also differentiated from *R. rhizogenes* and *R. tropici* by several phenotypic characteristics. The results of DNA–DNA hybridization showed means of 28 and 25 % similarity between strain P1-7^T and *R. rhizogenes* ATCC 11235^T and *R. tropici* CIAT 899^T, respectively. All these data showed that the strains isolated in this study belong to a novel species of the genus *Rhizobium*, for which we propose the name *Rhizobium lusitanum* sp. nov.; the type strain is P1-7^T (= LMG 22705^T = CECT 7016^T).

The common bean (*Phaseolus vulgaris*) is a legume indigenous to Mesoamerica and the Andean region of South America that is extensively cultivated throughout the world (Martínez-Romero, 2003). It constitutes a staple cultivation in many developing countries. This legume is considered as a promiscuous host, since it can be nodulated

by several rhizobial species. To date, six species belonging to the genus *Rhizobium* have been identified as endosymbionts of common bean (Amarger *et al.*, 1997; Jordan, 1984; Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993; Velázquez *et al.*, 2001b) but some other species, from this and other rhizobial genera, have been also described as infective under experimental conditions (Martínez-Romero, 2003). The diversity of rhizobia nodulating *P. vulgaris* has been widely studied, but, because of its promiscuous nature, novel endosymbionts of this legume should be expected as more ecological niches are studied. In the present work, we analysed several strains nodulating common bean in several soils from the region of Arcos de Valdevez, in the north-west of Portugal, where a traditional mixed farming system, with livestock and crops integrated into a single ecosystem, and local varieties of common beans have been used for centuries. A polyphasic study of these strains, including phenotypic and molecular taxonomic

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Abbreviations: LMW RNA, low molecular weight RNA; TP-RAPD, two-primer random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *nodD* and *nifH* gene sequences of strain P1-7^T are AY738130, AY943643 and AY943644, respectively.

A number of additional figures and a table detailing DNA–DNA hybridization results are available as supplementary material in IJSEM Online.

approaches, showed that they belong to a novel species of the genus *Rhizobium* phylogenetically close to *Rhizobium rhizogenes* and *Rhizobium tropici*.

In this study, 22 strains (Table 1) were isolated from *P. vulgaris* plants growing in three soils from the north-west of Portugal (Arcos de Valdevez region) with different chemical characteristics (data not shown). For isolation of bacterial strains, nodules present in *P. vulgaris* roots were surface sterilized using a 2.5% aqueous solution of HgCl₂ for 2 min. The nodules were then washed ten times with sterile water, disrupted in sterile water and cultivated in YMA medium (Bergersen, 1961) at 28 °C for 4 days. To test the symbiotic characteristics of the new bacterial isolates, *P. vulgaris*, *Leucaena leucocephala*, *Macroptilium atropurpureum* and *Medicago sativa* plants were inoculated as described by Velázquez *et al.* (2001b), using *R. tropici* CIAT 899^T as a control. All the novel strains were able to elicit effective nodules in *P. vulgaris*, *Macroptilium atropurpureum* and *L. leucocephala* and ineffective nodules in *Medicago sativa*. The ability to induce the production of roots on discs of *Daucus carota* was examined as described previously (Moore *et al.*, 1979). The strains isolated in this study were unable to produce these symptoms.

The plasmid content was determined by the method of Plazinski *et al.* (1985), with the modifications described by Rivas *et al.* (2002b), using *Ensifer meliloti* GR4 as a reference (Toro & Olivares, 1986). The results obtained (see

Supplementary Fig. S1 in IJSEM Online) showed that the strains from this study present seven different plasmid profiles (see Table 1) containing between one and four plasmids, the sizes of which ranged from approximately 90 to 1700 kb, some of which are megaplasmids (>1000 kb).

The *nodD* and *nifH* genes harboured by strain P1-7^T were amplified and sequenced as described previously (Rivas *et al.*, 2002b). According to *nodD* and *nifH* gene sequences, the closest relative of strain P1-7^T is *Devosia neptuniae* J1^T, showing 99.6 and 99.5% similarity, respectively (Supplementary Figs S2 and S3). The same genes from *R. tropici* CIAT 899^T showed 99.2 and 99.5% similarity, respectively. In a previous work (Rivas *et al.*, 2002b), we have already shown the high similarity of the *nodD* and *nifH* genes carried by *D. neptuniae* J1^T and those of *R. tropici* CIAT 899^T. These results are in agreement with those obtained in the analysis of the host range of strain P1-7^T, because it was able to nodulate *Phaseolus* and *Leucaena*, two common hosts for *R. tropici* (Martínez-Romero *et al.*, 1991). Nevertheless, the *nodD* and *nifH* genes recently sequenced in *R. rhizogenes* ATCC 11325^T are phylogenetically distant from those of strain P1-7^T, strongly supporting our previous hypothesis that *P. vulgaris* was not the ancestral host of *R. rhizogenes* (Velázquez *et al.*, 2005).

The presence of the *virA* gene in strain P1-7^T was analysed using the primers and PCR conditions described previously (Velázquez *et al.*, 2005). The results were negative,

Table 1. Differential genotypic characteristics of the novel strains (*Rhizobium lusitanum* sp. nov.) and phylogenetically closely related *Rhizobium* species

Strain	TP-RAPD type	RAPD type	ITS profile	LMW RNA profile	Sequence identity (ungapped) to strain P1-7 ^T *	Plasmid profile
<i>Rhizobium lusitanum</i> sp. nov.						
P1-7 ^T , P6-18, P6-20	A	I	a	1	100, 100, 100, 100	A
P1-16, P1-17, P1-18, P1-19, P1-21, P1-22	A	I	a	1	100, 100, 100, 100	B
P3-12, P3-13, P3-15, P3-16, P3-17, P3-18, P3-19	A	II	a	1	100, 99.5, 98.5, 98.5	C
P3-20, P3-21, P3-25	A	I	a	1	100, 100, 100, 100	D
P3-24	A	I	a	1	100, 100, 100, 100	E
P6-7	A	I	a	1	100, 100, 100, 100	F
P6-8	A	I	a	1	100, 100, 100, 100	G
<i>Rhizobium tropici</i>						
CFN 299 (IIA)	B	VIII	b	4	99.6, 84.3, 92.3, 92.0	Acosta-Durán & Martínez-Romero (2002)
CIAT 899 ^T (IIB)	D	VI	c	3	99.2, 82.8, 91.8, 90.6	Acosta-Durán & Martínez-Romero (2002)
Br859 (IIB)	D	VII	c	3	ND, 83.9, 92.4, 90.7	ND
<i>Rhizobium rhizogenes</i>						
IAM 13571	C	III	a	2	ND, 87.1, 93.7, 91.8	ND
163C	C	IV	a	2	99.9, 89.8, 93.4, 91.8	Velázquez <i>et al.</i> (2005)
ATCC 11325 ^T	C	V	a	2	99.9, 85.9, 94.1, 91.9	Velázquez <i>et al.</i> (2005)

ND, No data available.

*Values respectively represent similarity for the 16S rRNA gene, ITS and *atpD* and *recA* genes.

coinciding with the inability of our strains to induce pathogenicity symptoms in plants.

The diversity of strains isolated in this study was assessed by randomly amplified polymorphic DNA (RAPD) pattern analysis using the primer M13 (5'-GAGGGTGGCG-GTTCT-3') as described previously (Igual *et al.*, 2003). Two different RAPD patterns were found among the 22 strains isolated from the three studied soils (Supplementary Fig. S4). Most of them showed RAPD pattern I (lanes 1–15), whereas seven strains showed pattern II (lanes 16–22). The remaining strains used in this study (lanes 23–28) presented different RAPD patterns (see Table 1).

The RAPD patterns are strain dependent and therefore they are useful in analysing the intraspecific diversity of a rhizobial population (Corich *et al.*, 2001; Moschetti *et al.*, 2005). Other PCR-based profiles, such as two-primer RAPD (TP-RAPD) patterns, are not strain dependent and, according to our previous studies (Rivas *et al.*, 2001, 2004; Zurdo-Piñeiro *et al.*, 2004), are useful in differentiating among rhizobial species. TP-RAPD patterns were analysed according to the method described by Rivas *et al.* (2002a) using the primers 879F (5'-GCCTGGGGAG-TACGGCCGCA-3') and 1522R (5'-AAGGAGGTGATC-CANCCRCA-3'), which correspond to *Escherichia coli* positions 879–898 and 1509–1522, respectively. The DNA patterns obtained contain a band that corresponded to the fragment of the 16S rRNA gene amplified with these two primers and several others produced by random amplification on the total DNA (Rivas *et al.*, 2001). In a previous work, we demonstrated that all strains that show identical TP-RAPD pattern belong to the same species. No variations were observed in these patterns in strains from the same species with different plasmid profiles, and they do not vary with the growth phase (Rivas *et al.*, 2001). All the strains isolated in this study presented the same TP-RAPD pattern (see Table 1 and Supplementary Fig. S5) and, therefore, all of them belong to the same bacterial species. This pattern (lanes 1–22) was different from those from strains of *R. tropici* IIB (lanes 25 and 28), *R. tropici* IIA (lane 23) and *R. rhizogenes* (lanes 24, 26 and 27), which are the closest phylogenetically related species on the basis of the 16S rRNA, *recA* and *atpD* gene sequences as well as the 16S–23S intergenic spacer (ITS) sequences (see below).

Strains P1-7^T and P3-13, which presented different RAPD patterns, were selected for analysis of 16S rRNA, *atpD* and *recA* gene sequences, analysis of the ITS sequence and DNA–DNA hybridization experiments.

Nearly complete 16S rRNA gene sequences were obtained in this study according to the method described previously by Rivas *et al.* (2002b). Sequences of the *recA* and *atpD* genes were obtained according to Gaunt *et al.* (2001). The ITS region was amplified and sequenced as described by Kwon *et al.* (2005). These sequences were compared with those held in GenBank using the BLASTN program (Altschul *et al.*, 1990). 16S rRNA gene sequences were aligned using the CLUSTAL W software (Thompson *et al.*, 1997) and distances were calculated according to the models of Jukes & Cantor (1969), Kimura (1980), Tajima & Nei (1984) and Tamura & Nei (1993). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987), minimum evolution (Rzhetsky & Nei, 1993) and parsimony analysis (Felsenstein, 1983). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses. As no significant topological differences were found among the phylogenetic trees obtained by the different methods assayed, only those trees constructed by using the neighbour-joining method after distance analysis of aligned sequences according to Kimura's two parameters (ITS and 16S rRNA gene) or Tamura–Nei (*recA* and *atpD* genes) models are shown.

The 16S rRNA gene sequences of strains P1-7^T and P3-13 exhibit 100 % similarity and, thus, only the type strain was included in the phylogenetic analysis. The resulting neighbour-joining tree corresponding to 16S rRNA gene sequences is shown in Fig. 1 (a fuller phylogenetic tree is available as Supplementary Fig. S6). The results of the phylogenetic analysis indicate that the strains from this study are related to members of the genus *Rhizobium* within the family *Rhizobiaceae*. According to the 16S rRNA gene sequences, the closest relative to strain P1-7^T is *R. rhizogenes* ATCC 11325^T, showing 99.9 % similarity, followed by *R. tropici* IIB CIAT 899^T, with 99.2 % similarity. *R. tropici* IIA CFN 299 showed 99.6 % similarity (95.0 % when an insertion of 72 nucleotides at the beginning of the 16S rRNA gene is considered).

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strain P1-7^T (*Rhizobium lusitanum* sp. nov.) and closely related species within the genus *Rhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 5 substitutions per 1000 nt.

According to the results of Gaunt *et al.* (2001), *recA* and *atpD* gene-based phylogenies of members of the family *Rhizobiaceae* are congruent with the 16S rRNA gene-based phylogeny. Although more studies on the variability of these genes will be necessary to establish the similarity range between strains from the same species and those of different species from the same genus within the family *Rhizobiaceae*, it seems that they are very useful in the classification and identification of rhizobial isolates (Vinuesa *et al.*, 2005a, b). Thus, to confirm the phylogenetic position of the strains isolated in this study, we sequenced their *recA* and *atpD* genes (Supplementary Figs S7 and S8). The results obtained completely confirm the phylogenetic position of these strains within the group *R. rhizogenes*–*R. tropici* and, moreover, the *recA* and *atpD* sequence similarities between strains P1-7^T and P3-13 were near to 98.5 % for both genes, demonstrating that these two strains are not clones. The sequence similarities between strain P1-7^T and *R. rhizogenes* ATCC 11325^T, *R. tropici* IIA CFN 299 and *R. tropici* IIB CIAT 899^T were, respectively, 91.9, 92.0 and 90.6 % for *recA* and 94.1, 92.3 and 91.8 % for *atpD*. These values are similar to those found between other phylogenetically close species of the genera *Rhizobium* and *Ensifer*, such as between *Rhizobium leguminosarum* and *Rhizobium etli* or between *E. meliloti* and *Ensifer medicae* (Gaunt *et al.*, 2001). These similarity values with respect to *R. rhizogenes* and *R. tropici* suggest that the strains isolated in this study may belong to a novel species.

Comparison of 16S–23S rRNA ITS regions provides a fast tool to assess relatedness between closely related rhizobial strains (Kwon *et al.*, 2005). This region was sequenced in the strains P1-7^T and P3-13, representative of each of the two RAPD groups. The length of the fragment obtained in both strains was 1281 bp and their sequence similarity was 99.5 %. The same length was obtained for *R. rhizogenes* ATCC 11325^T; however, the ITS regions of *R. tropici* IIA CFN 299 and *R. tropici* IIB CIAT 899^T were, respectively, 130 and 190 bp shorter (Supplementary Fig. S9). After a pairwise analysis, the ITS sequence of strain P1-7^T showed 85.9 % (73.0 % including gaps), 84.3 % (70.9 % including gaps) and 82.8 % (66.3 % including gaps) similarity with respect to those of *R. rhizogenes* ATCC 11325^T, *R. tropici* IIA CFN 299 and *R. tropici* IIB CIAT 899^T, respectively. In agreement with the phylogenetic analyses based on the 16S rRNA, *atpD* and *recA* gene sequences, phylogenetic analysis of ITS sequences also demonstrated that the strains from this study belong to the *R. rhizogenes*–*R. tropici* cluster (Supplementary Fig. S10). However, differences in the nucleotide sequence between the type strains of these two species and strain P1-7^T suggest that the strains isolated in this study belong to a different species, which is in concordance with other results from this work.

Although discordant phylogenies have been reported within the *rrn* (16S rRNA) locus in some rhizobial species (van Berkum *et al.*, 2003), the results here obtained on the basis of *rrn*, *recA* and *atpD* sequence analyses show a complete

concordance in the phylogenetic location of the strains isolated in this study within the cluster *R. rhizogenes*–*R. tropici*. In order to confirm these phylogenetic data we also analysed the low-molecular-weight (LMW) RNA profiles of the isolated strains (Supplementary Fig. S11), as was described recently (Velázquez *et al.*, 2006). These profiles contain three clearly distinguishable zones. The 5S rRNA zone is identical in species from the same genus, as occurs in the case of strain P1-7^T, *R. rhizogenes* ATCC 11325^T, *R. tropici* IIB CIAT 899^T and *R. tropici* IIA CFN 299 (Supplementary Fig. S11, lanes 1–4, respectively). The class 1 and 2 tRNAs are different in different species from the same or different genera and the differences are related to phylogenetic distances on basis of the 16S rRNA gene sequences (Velázquez *et al.*, 2001d). As expected, the tRNA profiles of all of the novel strains were identical (data not shown). As can be seen in Supplementary Fig. S11, the tRNA profile of strain P1-7^T (lane 1) has only one band different from *R. rhizogenes* ATCC 11325^T (lane 2), whereas many differences are observed with respect to the tRNA profiles of *R. tropici* IIB CIAT 899^T (lane 3) and *R. tropici* IIA CFN 299 (lane 4). These results confirmed the placement of the strains from this study in the group of *R. rhizogenes* and *R. tropici*, being the most closely related species to the novel strains. The existence of a different tRNA band between the novel strains and *R. rhizogenes* supports the hypothesis that the strains isolated in this study do not belong to *R. rhizogenes*, since a single difference in tRNA profiles has been proposed to be diagnostic in bacterial species differentiation (Höfle, 1990). Moreover, the different LMW RNA profiles showed by the strains belonging to the subgroups IIA and IIB of *R. tropici*, in addition to the results from ITS, 16S rRNA, *recA* and *atpD* sequence analyses, as well as the DNA–DNA hybridization results (see below), indicate that they belong to different species.

DNA–DNA hybridization was carried out by using the method of Ezaki *et al.* (1989), following the recommendations of Willems *et al.* (2001). Strains P1-7^T and P3-13 were hybridized with two strains from the subgroup IIB of *R. tropici*, CIAT 899^T and Br859 (Martínez-Romero *et al.*, 1991), which presented the same TP-RAPD pattern (Supplementary Fig. S5, lanes 25 and 28) but different RAPD pattern (Supplementary Fig. S4, lanes 26 and 27), with *R. tropici* IIA CFN 299 (Supplementary Figs S5 and S4, lanes 23 and 28, respectively) and with three strains of *R. rhizogenes*, IAM 13571 (de Oliveira *et al.*, 1999), ATCC 11325^T and 163C (Velázquez *et al.*, 2005), which presented the same TP-RAPD pattern (Supplementary Fig. S5, lanes 24, 26 and 27) but different RAPD patterns (Supplementary Fig. S4, lanes 23–25). Strains P1-7^T and P3-13 showed hybridization values of 90–100 % (Supplementary Table S1). DNA–DNA hybridization between either of these strains and strains belonging to the species *R. rhizogenes* or *R. tropici* always yielded values of 43 % or lower. These results indicate that the strains from this study do not belong to either of these recognized species when the recommendation of a threshold value of 70 % DNA–DNA

relatedness for species definition is considered (Wayne *et al.*, 1987).

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). The G+C content of DNA of strain P1-7^T was determined using the thermal denaturation method (Mandel & Marmur, 1968) as 65.15 mol%. This value is similar to those obtained for other *Rhizobium* species (Jordan, 1984).

Phenotypic characterization of the strains was based on growth with different carbon and nitrogen sources, the production of exoenzymes and resistance to different antibiotics as described previously (Kerstens & De Ley, 1984, Velázquez *et al.*, 2001a, b; Zurdo-Piñeiro *et al.*, 2004) and by using an API 20NE kit according to the manufacturer's instructions (bioMérieux). For testing antibiotic resistance, the following antibiotics were used: ampicillin (2 µg), erythromycin (2 µg), ciprofloxacin (5 µg), penicillin (10 IU), polymyxin (300 IU), cloxacillin (1 µg), oxytetracycline (30 µg), gentamicin (10 µg), cefuroxime (30 µg) and neomycin (5 µg) (Becton Dickinson). The basal medium was YMA (Vincent, 1970) supplemented with 10 g yeast extract l⁻¹. The temperature range for growth was determined by incubating cultures in YMA medium between 4 and 45 °C. The pH range was determined in the same medium with a final pH between 4.0 and 10.0. Salt tolerance was studied in YMA medium containing 0–5 % (w/v) NaCl. *R. rhizogenes* ATCC 11325^T, *R. tropici* IIB CIAT 899^T and *R. tropici* IIA CFN 299 were used as reference strains. All of the novel strains had the same phenotypic characteristics, but they showed several differences compared with their closest relatives, *R. rhizogenes* and *R. tropici* (Table 2). They differ from *R. rhizogenes* in growth at 35 °C, growth in Luria–Bertani (LB) medium, resistance to penicillin, oxytetracycline and cefuroxime and in the ability to induce hairy roots in *Daucus carota*. They differ from subgroup IIB of *R. tropici* in growth at 40 °C, assimilation of adipate as a carbon source, production of α -fucosidases and resistance to oxytetracycline and polymyxin B. Finally, they differ from subgroup IIA of *R. tropici* in growth in PY without calcium and LB media, production of β -galactosaminidases, β -cellobiases, α -fucosidases and β -maltosidases and resistance to erythromycin, oxytetracycline and cefuroxime.

Therefore, the novel group can be differentiated genotypically and phenotypically from previously described species and we therefore propose to name it *Rhizobium lusitanum* sp. nov.

Description of *Rhizobium lusitanum* sp. nov.

Rhizobium lusitanum (lu.si.ta'num. L. neut. adj. *lusitanum* of *Lusitania*, the Roman name of Portugal, where the strains reported in this study were isolated).

Gram-negative rods, as for the other species of the genus. Colonies are small, pearl white on YMA at 28 °C, which is the optimal growth temperature. The optimum pH is 7–7.5.

Table 2. Differential characteristics of the novel strains (*Rhizobium lusitanum* sp. nov.) and the closest related species

Taxa: 1, *R. tropici* IIB; 2, *R. tropici* IIA; 3, *R. rhizogenes*; 4, *R. lusitanum* sp. nov. Data were taken from Amarger *et al.* (1997), Jordan (1984), Kersters & De Ley (1984), Martínez-Romero *et al.* (1991) and Young *et al.* (2001) and this study. +, Positive; –, negative; W, weak; pNP, *p*-nitrophenyl; ND, no data available.

Characteristic	1	2	3	4
Growth at/in:				
35 °C	+	+	–	+
40 °C	+	–	–	–
LB medium	+	–	–	+
PY medium lacking calcium	+	–	+	+
Use of adipate as a carbon source	+*	–†	–‡	–
Cleavage of:				
pNP β -D-galactosaminide	+*	–†	+‡	+
pNP β -D-cellobioside	W*	–†	+‡	+
pNP α -L-fucopyranoside	–*	–†	+‡	+
pNP β -D-maltopyranoside	W*	–†	+‡	+
pNP thio- β -D-glucopyranoside	+*	–†	+‡	+
Resistance to:				
Penicillin	+*	+†	–‡	+
Erythromycin	+	–	+‡	+
Oxytetracycline	–	–	–‡	+
Cefuroxime	+*	–†	–‡	+
Polymyxin B	+	–	–‡	–
Root formation on discs of <i>D. carota</i>	ND	ND	+	–

*Data from Zurdo-Piñeiro *et al.* (2004) confirmed in this study.

†Data from this study for *R. tropici* IIA CFN 299.

‡Data from this study for several strains included in Velázquez *et al.* (2001c).

Strains may grow between 10 and 37 °C, pH 5 and 8 and weakly up to 1 % (w/v) NaCl. Denitrification is carried out by the strains from this study. The strains produce β -galactosidase and urease and hydrolyse aesculin in the API 20NE system. They also produce α - and β -arabinosidases, α - and β -galactosidases, β -galactosaminidases, β -cellobiases, α - and β -xylosidases, α -maltosidases and β -glucosaminidases using chromogenic *p*-nitrophenyl substrates. They do not produce indole, arginine dihydrolase or gelatinase in API 20NE. They use glucose, melezitose, ethanol, *meso*-erythritol, L-arabinose, mannose, fructose, galactose, L-rhamnose, xylose, *N*-acetylglucosamine, maltose, sucrose, cellobiose, raffinose, melibiose, trehalose, salicin, inositol, mannitol, sorbitol, malate, gluconate and citrate as carbon sources. They grow on L-histidine, aspartate, glutamate and betaine as carbon and nitrogen sources. They grow weakly on L-lysine, xylitol and L-sorbose. They do not grow on caprate, adipate, phenylacetate, glucuronate, propionate, valine or L-alanine. All strains are resistant to ampicillin, erythromycin, cloxacillin, penicillin, cefuroxime and oxytetracycline. They do not grow in the presence of ciprofloxacin, polymyxin B or gentamicin. Growth is weak

in the presence of neomycin. The G + C content of strain P1-7^T is 65.15 mol%.

The type strain, P1-7^T (= LMG 22705^T = CECT 7016^T), was isolated from effective nodules of *Phaseolus vulgaris* in Portugal.

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References

- Acosta-Durán, C. & Martínez-Romero, E. (2002). Diversity of rhizobia from nodules of the leguminous tree *Gliricidia sepium*, a natural host of *Rhizobium tropici*. *Arch Microbiol* **178**, 161–164.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Amarger, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* **47**, 996–1006.
- Bergersen, F. J. (1961). The growth of *Rhizobium* in synthetic media. *Aust J Biol* **14**, 349–360.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.
- Corich, V., Giacomini, A., Carlot, M., Simon, R., Tichy, H. V., Squartini, A. & Nuti, M. P. (2001). Comparative strain typing of *Rhizobium leguminosarum* bv. viciae natural populations. *Can J Microbiol* **47**, 580–584.
- de Oliveira, V. M., Coutinho, H. L., Sobral, B. W., Guimaraes, C. T., van Elsas, J. D. & Manfio, G. P. (1999). Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE). *Lett Appl Microbiol* **28**, 137–141.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1983). Parsimony in systematics: biological and statistical issues. *Annu Rev Ecol Syst* **14**, 313–333.
- Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.
- Höfle, M. G. (1990). Transfer RNAs as genotypic fingerprints of eubacteria. *Arch Microbiol* **153**, 299–304.
- Igual, J. M., Valverde, A., Rivas, R., Mateos, P. F., Rodríguez-Barrueco, C., Martínez-Molina, E., Cervantes, E. & Velázquez, E. (2003). Genomic fingerprinting of *Frankia* strains by PCR-based techniques. Assessment of a primer based on the sequence of 16S rRNA gene of *Escherichia coli*. *Plant Soil* **254**, 115–123.
- Jordan, D. C. (1984). Family III. *Rhizobiaceae*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–242. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. London: Academic Press.
- Kerstens, K. & De Ley, J. (1984). Genus III. *Agrobacterium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 244–254. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001). *Molecular Evolutionary Genetics Analysis Software*. Tempe, AZ: Arizona State University.
- Kwon, S. W., Park, J. Y., Kim, J. S., Kang, J. W., Cho, Y. H., Lim, C. K., Parker, M. A. & Lee, G. B. (2005). Phylogenetic analysis of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Simorhizobium* on the basis of 16S rRNA gene and internally transcribed spacer region sequences. *Int J Syst Evol Microbiol* **55**, 263–270.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.
- Martínez-Romero, E. (2003). Diversity of *Rhizobium*–*Phaseolus vulgaris* symbiosis: overview and perspectives. *Plant Soil* **252**, 11–23.
- Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. & Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41**, 417–426.
- Moore, L. W., Warren, G. & Strobel, G. (1979). Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid* **2**, 617–626.
- Moschetti, G., Peluso, A., Protopapa, A., Anastasio, M., Pepe, O. & Defez, R. (2005). Use of nodulation pattern, stress tolerance, *nodC* gene amplification, RAPD-PCR and RFLP-16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar viciae. *Syst Appl Microbiol* **28**, 619–631.
- Plazinski, J., Chen, Y. H. & Rolfe, B. G. (1985). General method for the identification of plasmid species in fast-growing soil micro-organisms. *Appl Environ Microbiol* **48**, 1001–1003.
- Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F. & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis* **22**, 1086–1089.
- Rivas, R., Velázquez, E., Palomo, J.-L., Mateos, P. F., García-Benavides, P. & Martínez-Molina, E. (2002a). Rapid identification of *Clavibacter michiganensis* subspecies *sepedonicus* using two primers random amplified polymorphic DNA (TP-RAPD) fingerprints. *Eur J Plant Pathol* **108**, 179–184.
- Rivas, R., Velázquez, E., Willems, A., Vizcaino, N., Subba-Rao, N. S., Mateos, P. F., Gillis, M., Dazzo, F. B. & Martínez-Molina, E. (2002b). A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L. f.) Druce. *Appl Environ Microbiol* **68**, 5217–5222.
- Rivas, R., Willems, A., Palomo, J. L., García-Benavides, P., Mateos, P. F., Martínez-Molina, E., Gillis, M. & Velázquez, E. (2004). *Bradyrhizobium betae* sp. nov., isolated from roots of *Beta vulgaris* affected by tumour-like deformations. *Int J Syst Evol Microbiol* **54**, 1271–1275.
- Rzhetsky, A. & Nei, M. (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Mol Biol Evol* **10**, 1073–1095.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

- Segovia, L., Young, J. P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 374–377.
- Tajima, F. & Nei, M. (1984). Estimation of evolutionary distance between nucleotide sequences. *Mol Biol Evol* **1**, 269–285.
- Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *J Mol Evol* **10**, 512–526.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Toro, N. & Olivares, J. (1986). Characterization of a large plasmid of *Rhizobium meliloti* involved in enhancing nodulation. *Mol Gen Genet* **202**, 331–335.
- van Berkum, P., Terefework, Z., Paulin, L., Suomalaie, S., Lindström, K. & Eardly, B. D. (2003). Discordant phylogenies within the *rrn* loci of rhizobia. *J Bacteriol* **185**, 2988–2998.
- Velázquez, E., Igual, J. M., Willems, A. & 9 other authors (2001a). Description of *Mesorhizobium chacoense* sp. nov. that nodulates *Prosopis alba* in the Chaco Arido region (Argentina). *Int J Syst Evol Microbiol* **51**, 1011–1021.
- Velázquez, E., Martínez-Romero, E., Rodríguez-Navarro, D. N., Trujillo, M. E., Daza, A., Mateos, P. F., Martínez-Molina, E. & Van Berkum, P. (2001b). Characterization of rhizobial isolates of *Phaseolus vulgaris* by staircase electrophoresis of low molecular weight RNA. *Appl Environ Microbiol* **67**, 1008–1010.
- Velázquez, E., Palomo, J. L., Lastra, B., Mateos, P., García-Benavides, P. & Martínez-Molina, E. (2001c). Rapid identification of *Agrobacterium* species by staircase electrophoresis of low molecular weight RNA profiles. *Eur J Plant Pathol* **107**, 931–938.
- Velázquez, E., Trujillo, M. E., Peix, A., Palomo, J. L., García-Benavides, P., Mateos, P. F., Ventosa, A. & Martínez-Molina, E. (2001d). Stable low molecular weight RNA analyzed by staircase electrophoresis, a molecular signature for both prokaryotic and eukaryotic microorganisms. *Syst Appl Microbiol* **24**, 490–499.
- Velázquez, E., Peix, A., Zurdo-Piñeiro, J. L. & 7 other authors (2005). The coexistence of symbiosis and pathogenicity-determining genes in *Rhizobium rhizogenes* strains enables them to induce nodules and tumors or hairy roots in plants. *Mol Plant Microbe Interact* **18**, 1325–1332.
- Velázquez, E., Rivas, R., Villar, M., Valverde, A., Peix, A., Mateos, P. F., Velázquez, E. & Martínez-Molina, E. (2006). A new approach for separating LMW RNA molecules by staircase electrophoresis in non-sequencing gels. *Electrophoresis* **27**, 1732–1738.
- Vincent, J. M. (1970). The cultivation, isolation and maintenance of rhizobia. In *A Manual for the Practical Study of Root-Nodule Bacteria*, pp. 1–13. Edited by J. M. Vincent. Oxford: Blackwell Scientific.
- Vinuesa, P., Silva, C., Lorite, M. J., Izaguirre-Mayoral, M. L., Bedmar, E. J. & Martínez-Romero, E. (2005a). Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from *rrs*, *atpD*, *recA* and *nifH* sequences, and their use in the classification of *Sesbania* microsymbionts from Venezuelan wetlands. *Syst Appl Microbiol* **28**, 702–716.
- Vinuesa, P., Silva, C., Werner, D. & Martínez-Romero, E. (2005b). Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Mol Phylogenet Evol* **34**, 29–54.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., De Lajudie, P. & Gillis, M. (2001). DNA–DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.
- Young, J. M., Kuykendall, L. D., Martínez-Romero, E., Kerr, A. & Sawada, H. (2001). A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int J Syst Evol Microbiol* **51**, 89–103.
- Zurdo-Piñeiro, J. L., Velázquez, E., Lorite, M. J., Brelles-Mariño, G., Schröder, E. C., Bedmar, E. J., Mateos, P. F. & Martínez-Molina, E. (2004). Identification of fast-growing rhizobia nodulating tropical legumes from Puerto Rico as *Rhizobium gallicum* and *Rhizobium tropici*. *Syst Appl Microbiol* **27**, 469–477.