Rhizobium freirei sp. nov., a symbiont of *Phaseolus vulgaris* that is very effective at fixing nitrogen

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Common bean (*Phaseolus vulgaris* L.) can establish symbiotic associations with several *Rhizobium* species; however, the effectiveness of most strains at fixing nitrogen under field conditions is very low. PRF 81^T is a very effective strain, usually referred to as *Rhizobium tropici* and used successfully in thousands of doses of commercial inoculants for the common bean crop in Brazil; it has shown high rates of nitrogen fixation in all areas representative of the crop in the country. Here, we present results that indicate that PRF 81^T, although it belongs to the '*R. tropici* group', which includes 10 *Rhizobium* species, *R. tropici*, *R. leucaenae*, *R. lusitanum*, *R. multihospitium*, *R. miluonense*, *R. hainanense*, *R. calliandrae*, *R. mayense*, *R. jaguaris* and *R. rhizogenes*, represents a novel species. Several morpho-physiological traits differentiated PRF 81^T from related species. Differences were also confirmed in the analysis of rep-PCR (sharing less than 45 % similarity with the other species), MLSA with *recA*, *atpD* and *rpoB* genes, and DNA–DNA hybridization. The novel species, for which we propose the name *Rhizobium freirei* sp. nov., is able to establish effective root nodule symbioses with *Phaseolus vulgaris*, *Leucaena leucocephala*, *Leucaena esculenta*, *Crotalaria juncea* and *Macroptilium atropurpureum*. The type strain is PRF 81^T (=CNPSo 122^T=SEMIA 4080^T=IPR-Pv81^T=WDCM 440^T).

Common bean (*Phaseolus vulgaris* L.) represents a major source of protein for poor populations of South and Central America, Africa and India. An important characteristic of common bean is its capacity to establish symbiotic partnerships with a variety of *Rhizobium* species (Martínez-Romero, 2003). These symbioses include both Fix$^+$ (*Rhizobium leguminosarum* sv. phaseoli, *R. phaseoli*, *R. tropici, R. etli*, *R. leucaenae*, *R. giardinii* sv. phaseoli, *R. gallicum*, *R. lusitanum* and *R. pisi*) and Fix$^-$ (*R. giardinii* sv. giardinii and *R. miluonense*) species. This promiscuous nature (Michiels *et al.*, 1998) is probably responsible for the commonly reported low contribution of biological N_2 fixation to common bean nutrition, as the legume often nodulates with very competitive but inefficient indigenous rhizobia (Graham, 1981; Hardarson, 1993).

Strains belonging to the broad-host-range species *R. tropici* have been used as inoculants in the tropics because of their high tolerance to environmentally stressful conditions and high genetic stability (Hungria *et al.*, 2000, 2003). The most competitive and effective strain used in commercial inoculants for common bean in Brazil is PRF 81^T, which

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; IGS, intergenic spacer; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *recA* gene sequences of strain PRF 81^T are EU488742 and EU488827. The accession number of the whole genome shotgun sequencing project for strain PRF 81^T is AQHN00000000.

Four supplementary tables and two supplementary figures are available with the online version of this paper.

is closely related to *R. tropici* on the basis of 16S rRNA gene sequence analysis; however, it may be distinguished from *R. tropici* by phenotypic and genetic characteristics (Hungria *et al.*, 2000; Ribeiro *et al.*, 2009). A recent comparison of the genome of PRF 81^T to that of *R. tropici* CIAT 899^T highlighted the high similarity of their symbiotic plasmids, but considerable differences between the rest of their genomes, with low average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (DDH) (Ormeño-Orrillo *et al.*, 2012). High divergence of PRF 81^T from other rhizobial species has also been revealed in the reference proteomic map of this strain (Gomes *et al.*, 2012) and by multilocus sequence analysis (MLSA) (Ribeiro *et al.*, 2009).

Strain PRF 81^T was isolated in Irati county, State of Paraná, Brazil (25° 28′ 01″ S 50° 37′ 03″ W; Köppen's classification of climate *Cfb*; altitude 812 m), by one of us (D. S. A.) on 28 May 1992. The DNA G+C content of PRF 81^T was determined previously as 59.9 mol% (Ormeño-Orrillo *et al.*, 2012), which is within the range reported for *Rhizobium* (Jordan, 1984). In extensive common bean and *Leucaena* spp. rhizobial isolations performed in our laboratory, we have never found other strains showing high similarity with PRF 81^T in rep-PCR profiles; however, we have obtained isolates showing identical or nearly identical profiles to PRF 81^T (>90 % in rep-PCR) in several soils from Brazil (states of Pernambuco, Goiás, Santa Catarina, Paraná, Mato Grosso do Sul, Federal District). Examples of unique rep-PCR profiles obtained in our studies are given elsewhere (Pinto *et al.*, 2007; Stocco *et al.*, 2008). These isolates possessed sequences identical to PRF 81^T in their 16S rRNA and housekeeping genes (data not shown). This seemed to indicate that PRF 81^T is a member of a broadly distributed *Rhizobium* clonal group that nodulates common bean as well as leucaenas. Due to its relevant economic importance, we sought to establish the taxonomic position of PRF 81^T as a representative of this group, using a polyphasic approach with data published by us and new evidence obtained in this study.

16S rRNA gene sequences from all *Rhizobium* type strains were retrieved from the GenBank database and aligned with MUSCLE (Edgar, 2004). Neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) phylogenies were reconstructed with MEGA (Tamura *et al.*, 2011) using the Tamura–Nei model (Tamura & Nei, 1993). The 16S rRNA gene sequence of PRF 81^T was part of a clade with bootstrap support of 98 % (Figs 1 and S1, available in IJSEM Online) that included all species of the '*R. tropici* group' (Ribeiro *et al.*, 2012). The 16S rRNA gene sequence identity between PRF 81^T and *R. multihospitium* CCBAU 83401^T was 100 %, and identity ranged from 98.8 to 99.8 % with the remaining species of the clade (Table S1). It was surprising to find such level of identity with *R. multihospitium* CCBAU 83401^T, as the similarity between the BOX-PCR profiles of PRF 81^T and *R. multihospitium* CCBAU 83401^T was lower than 45 % (Fig. S2). It is known that phylogenetic analysis based exclusively on the 16S rRNA gene often does not reflect the correct taxonomic position of a species (Coenye *et al.*, 2001; Martens *et al.*, 2007; Menna *et al.*, 2009; Wang & Martínez-Romero, 2000). We tested whether the similarity between PRF 81^T and *R. multihospitium* also included other parts of the ribosomal (*rrn*) operon by comparing the sequences of their 16S–23S intergenic spacer (IGS) regions. The available IGS sequences of four *R. multihospitium* strains were identical to each other (Han *et al.*, 2008), as were the sequences of the three *rrn* operons in the PRF 81^T genome (Ormeño-Orrillo *et al.*, 2012). Interestingly, the PRF 81^T and *R. multihospitium* IGS sequences were only 76.4 % identical (Table S1), indicating that they may have recombined at their 16S rRNA gene loci at some point in their evolutionary history. PRF 81^T showed the highest IGS sequence identity to *R. hainanense* (77.5 % to the type strain), while *R. multihospitium* showed the highest identity to *R. tropici* (84.1 % to the type strain) (Table S1).

An MLSA approach with the *recA*, *atpD* and *rpoB* genes was used to explore the relationships of PRF 81^T and related species further. Sequences were retrieved from GenBank or extracted from the PRF 81^T genome, and phylogenies were constructed as described above for the

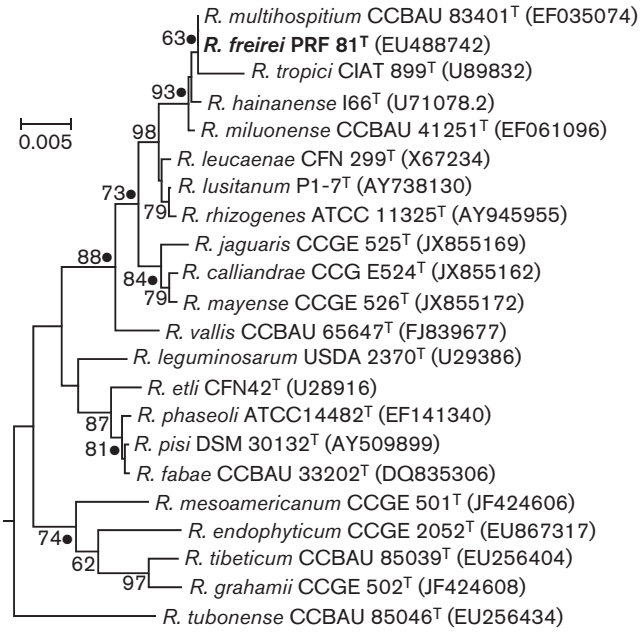

Fig. 1. Part of a neighbour-joining phylogeny of 16S rRNA gene sequences (1308 nt) of *R. freirei* sp. nov. PRF 81^T and other *Rhizobium* species (the complete phylogram is presented in Fig. S1). GenBank accession numbers are given in parentheses. Bootstrap support values based on 500 resamplings are shown as percentages at nodes only when ⩾60 %. Nodes with adjacent filled circles also had ⩾60 % support in a separate maximum-likelihood phylogeny. Bar, 0.005 substitutions per nucleotide position.

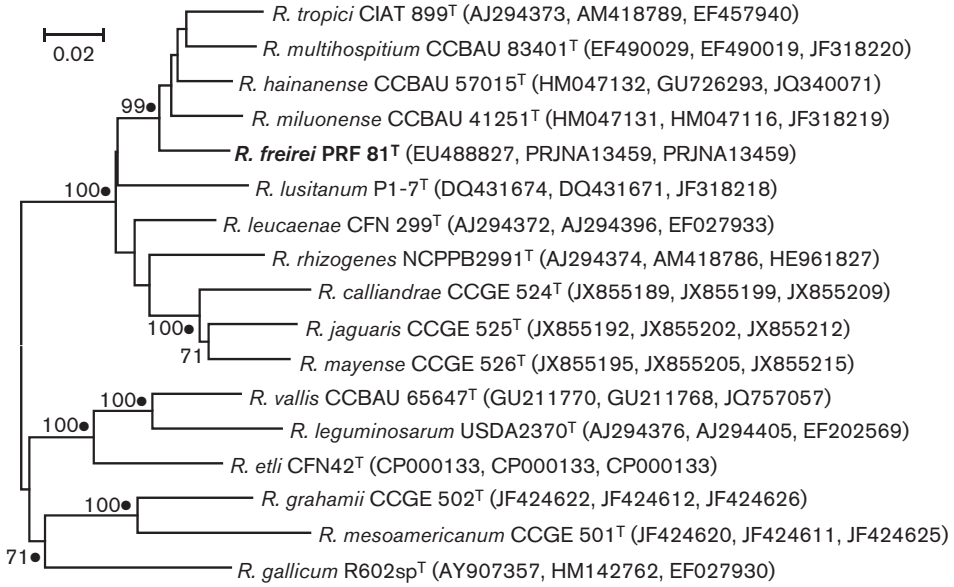

Fig. 2. Neighbour-joining phylogeny based on a concatenated alignment of *recA* (442 nt), *atpD* (437 nt) and *rpoB* (650 nt) gene sequences of *R. freirei* sp. nov. PRF 81^T and closely related *Rhizobium* species. GenBank accession numbers are given in parentheses (*recA*, *atpD*, *rpoB*). Bootstrap support values based on 500 resamplings are shown as percentages at nodes only when $\geqslant$60 %. Nodes with adjacent filled circles also had $\geqslant$60 % support in a separate maximum-likelihood phylogeny. Bar, 0.02 substitutions per nucleotide position.

Table 1. Strains used in this study for phenotypic characterization

Strain	Other strain nomenclature	Host species	Geographical origin	Reference or source
R. freirei sp. nov. PRF 81^T	CNPSo 122^T=SEMIA 4080^T=IPR-*Pv*81^T=WDCM 440^T	*Phaseolus vulgaris*	Paraná, Brazil	Hungria *et al.* (2000)
R. leucaenae CFN 299^T	USDA 9039^T=LMG 9517^T=UMR1026^T=CENA 183^T=SEMIA 4083^T=CNPSo 141^T	*P. vulgaris*	Brazil	Ribeiro *et al.* (2012)
R. tropici CIAT 899^T	USDA 9030^T=ATCC 49672^T=UMR1899^T=TAL 1797^T=HAMBI 1163^T=CM0^T=SEMIA 4077^T=DSM 11418^T=BR 322^T=CNPSo 142^T	*P. vulgaris*	Colombia	Martínez-Romero *et al.* (1991)
R. rhizogenes ATCC 11325^T	DSM 30148^T=LMG 150^T=IFO 13257^T=IAM 13570^T=CNPSo 1976^T	Apple	NK	Velázquez *et al.* (2010)
R. hainanense I66^T	BCRC 15793^T=CCRC 15793^T= CECT 4658^T=DSM 11917^T=ICMP 13690^T= LMG 18074^T=USDA 3588^T=CNPSo 2450^T	*Desmodium sinuatum*	Hainan, China	Chen *et al.* (1997)
R. lusitanum P1-7^T	CECT 7016^T=CIP 109524^T=LMG 22705^T= CNPSo 2055^T	*P. vulgaris*	Arcos de Valdevez, Portugal	Valverde *et al.* (2006)
R. multihospitium CCBAU 83401^T	HAMBI 2975^T=LMG 23946^T=LMG 24298^T=CNPSo 2054^T	*Halimodendron halodendron*	Xinjiang, China	Han *et al.* (2008)
R. miluonense CCBAU 41251^T	HAMBI 2971^T=LMG 24208^T=CNPSo 2056^T	*Lespedeza chinensis*	Hunan, China	Gu *et al.* (2008)
R. calliandrae CCGE 524^T	ATCC BAA-2435^T=CIP110456^T=LBP2-1^T= CNPSo 2466^T	*Calliandra grandiflora*	Chiapas, Mexico	Rincón-Rosales *et al.* (2013)
R. mayense CCG E526^T	CIP 110454^T=NSJP1-1^T=CNPSo 2464^T	*C. grandiflora*	Chiapas, Mexico	Rincón-Rosales *et al.* (2013)
R. jaguaris CCG E525^T	ATCC BAA-2445^T=CIP 110453^T=SJP1-2^T= CNPSo 2465^T	*C. grandiflora*	Chiapas, Mexico	Rincón-Rosales *et al.* (2013)

NK, Not known.

Table 2. Distinctive phenotypic features of *Rhizobium freirei* sp. nov. PRF 81^T and type strains of phylogenetically related species of the '*R. tropici* group'

Strains: 1, *R. freirei* PRF 81^T; 2, *R. leucaenae* CFN 299^T; 3, *R. tropici* CIAT 899^T; 4, *R. lusitanum* P1-7^T; 5, *R. multihospitium* CCBAU 83401^T; 6, *R. miluonense* CCBAU 41251^T; 7, *R. rhizogenes* ATCC 11325^T; 8, *R. hainanense* I66^T; 9, *R. calliandrae* CCGE 524^T; 10, *R. mayense* CCGE 526^T; 11, *R. jaguaris* CCGE 525^T. +, Growth, −, no growth; W, weak growth; ND, not determined. Data were obtained in this study. All strains are sensitive to tetracycline (30 μg).

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Carbon source											
Glycerol	+	+	+	+	+	+	W	+	+	W	+
Erythritol	+	−	+	+	+	+	+	+	+	+	+
D-Arabinose	W	+	+	W	+	+	+	+	W	W	W
D-Xylose	W	+	+	+	+	+	W	+	W	W	W
L-Xylose	+	+	−	+	+	+	+	+	W	W	W
D-Adonitol	+	+	+	+	+	W	+	+	W	W	W
Methyl β-D-xylopyranoside	W	+	+	+	+	+	+	+	W	+	W
D-Glucose	+	+	+	+	+	+	W	+	+	W	W
D-Fructose	W	+	+	+	+	+	W	+	+	W	W
D-Mannose	+	+	+	+	+	+	W	+	+	W	W
L-Sorbose	W	−	−	W	W	+	W	W	−	−	−
L-Rhamnose	W	+	+	+	+	+	W	+	W	+	+
Dulcitol	−	W	−	−	W	W	W	W	W	−	−
Inositol	W	+	+	+	+	+	W	W	W	W	W
D-Mannitol	W	+	+	W	+	+	W	+	+	+	W
D-Sorbitol	W	−	−	W	W	+	W	+	−	W	W
Methyl α-D-mannopyranoside	−	−	−	W	W	−	−	−	−	−	−
Methyl α-D-glucopyranoside	W	−	+	W	+	+	+	+	W	W	W
N-Acetylglucosamine	+	−	−	−	+	+	+	W	−	W	−
Amygdalin	−	−	−	W	W	W	+	+	−	W	−
Arbutin	+	−	W	+	+	+	+	+	W	W	W
Salicin	+	−	W	+	+	+	+	W	W	W	W
Cellobiose	W	W	+	+	+	+	+	+	+	+	W
Maltose	W	W	+	+	+	+	W	+	W	W	W
Lactose	W	W	W	+	+	+	W	W	W	W	W
Melibiose	W	W	W	+	+	+	W	W	W	W	W
Sucrose	W	W	+	+	+	+	+	W	W	W	W
Trehalose	W	W	+	+	+	+	+	+	W	W	W
Melezitose	−	−	−	−	−	−	−	W	−	−	−
Raffinose	W	W	W	+	+	+	W	W	W	W	W
Glycogen	+	−	−	+	+	+	+	+	+	+	+
Xylitol	W	−	−	W	W	+	W	+	−	W	−
Gentiobiose	W	W	W	+	+	+	+	+	W	W	W
D-Turanose	W	W	W	W	+	+	W	+	W	W	W
D-Lyxose	W	+	+	+	+	+	+	+	+	+	+
D-Tagatose	W	W	−	W	W	+	+	W	W	−	−
D-Arabitol	W	+	+	W	+	+	W	+	W	W	W
L-Arabitol	+	−	W	W	W	W	+	+	−	−	−
Resistance to:											
Nalidixic acid (30 μg)	+	+	+	+	+	+	−	W	+	+	+
Ampicillin (10 μg)	W	−	+	−	+	+	−	−	−	−	−
Chloramphenicol (30 μg)	−	−	+	−	+	−	W	+	W	−	−
Cephuroxyn (30 μg)	+	−	+	+	+	+	−	−	−	−	−
Erithromycin (15 μg)	+	+	+	+	+	+	+	+	+	+	−
Streptomycin (10 μg)	−	−	+	−	W	W	W	+	−	−	−
Neomycin (30 μg)	W	−	−	W	−	W	−	W	W	+	W
Penicillin (10 U)	+	−	+	+	+	+	+	+	+	W	W
Growth in/at:											
PY without Ca medium	W	−	+	+	+	+	+	+	−	−	−

Table 2. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
LB medium	W	−	+	+	+	−	−	+	−	−	−
37 °C	+	+	+	+	+	+	−	+	−	−	−
40 °C	+	W	+	−	−	−	−	+	−	−	−
1 % NaCl	+	−	+	W	+	−	−	+	−	−	−
pH 4	+	W	+	−	+	−	ND	−	−	−	−

16S rRNA gene. In the concatenated-alignment phylogeny, PRF 81^T was grouped with *R. tropici*, *R. multihospitium*, *R. hainanense* and *R. miluonense* in a subclade with bootstrap support of 99 % (Fig. 2) that was part of a clade that included all species of the '*R. tropici* group'. The range of nucleotide identity between described species of this clade, calculated from the concatenated genes, was 85.4–96 % (Table S1). Konstantinidis *et al.* (2006) found that 96 % ANI in concatenated housekeeping genes is equivalent to 70 % DDH. PRF 81^T showed the highest identity with *R. hainanense* (95.5 % to the type strain), while it showed 95.2 % identity or less to all other species, indicating that this strain is a member of a species different from all others of the '*R. tropici* group'. In order to ascertain that PRF 81^T represents a distinct species, DDH experiments were performed using a filter hybridization methodology (Martínez-Romero *et al.*, 1991) to estimate the overall DNA relatedness with type strains of related species. DDH values obtained were lower than 70 % with all type strains tested, including those of *R. multihospitium* and *R. hainanense* (Table S2), indicating that PRF 81^T represents a novel species.

Comparative phenotypic characterization of PRF 81^T and strains of related species (Table 1) was performed by using previously described methodologies (Hungria *et al.*, 2001; Ribeiro *et al.*, 2012). All tests were performed in triplicate. Differential phenotypic characteristics are displayed in Table 2. Additionally, Table S3 shows the differences found between PRF 81^T and well-known symbionts of common bean. It is interesting to observe that PRF 81^T could be easily distinguished from *R. tropici*, the species in which it is usually classified, from *R. multihospitium*, which has an identical 16S rRNA gene sequence, and from *R. hainanense*, which shares 95.5 % ANI. PRF 81^T differs from *R. tropici* CIAT 899^T in mucus production, colour and morphology of colonies (Table S3); likewise, it grows very poorly in PY minus Ca and LB medium, while *R. tropici* CIAT 899^T grows abundantly in those media. The two latter characteristics also distinguished PRF 81^T from *R. multihospitium* CCBAU 83401^T and *R. hainanense* 166^T, as well as its ability to growth at 40 °C, in contrast to *R. multihospitium* CCBAU 83401^T. Additionally, differential utilization of 24, 23 and 21 out of the 49 carbon sources tested with the API 50CH kit can be used to distinguish PRF 81^T from *R. tropici* CIAT 899^T, *R. multihospitium* CCBAU 83401^T and *R. hainanense* I66^T, respectively, as well as different tolerance to four of five antibiotics.

The fatty acid profiles of PRF 81^T, *R. tropici* CIAT 899^T, *R. multihospitium* CCBAU 83401^T and *R. hainanense* I66^T were obtained with the MIDI system using FAME library TSBA6 of a culture grown for 5 days at 28 °C on YM agar plates. The results shown in Table S4 indicate that all strains had summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$) as a major fatty acid, like other *Rhizobium* strains (Tighe *et al.*, 2000); however, differences in the relative amounts of some fatty acids seem to be useful to distinguish the strains. For example, PRF 81^T had significant less $C_{19:0}$ cyclo $\omega8c$ than *R. tropici* CIAT 899^T and *R. multihospitium* CCBAU 83401^T, and about half the content of summed feature 2 ($C_{14:0}$ 3OH/16 : 1 iso I) compared with *R. hainanense* I66^T (Table S4).

In view of the genotypic and phenotypic data presented here, which clearly indicate the distinctiveness of PRF 81^T in relation to all described species of *Rhizobium*, we propose to create a separate species, named *Rhizobium freirei* sp. nov., to accommodate this strain.

Description of *Rhizobium freirei* sp. nov.

Rhizobium freirei (frei′re.i. N.L. masc. gen. n. *freirei* of Freire, named after Professor João Ruy Jardim Freire, a distinguished Brazilian rhizobiologist).

Cells are Gram-negative, aerobic, non-spore-forming rods. Colonies on YMA medium are circular, convex, rose, opaque and gummy, with moderate production of mucus, and usually 2–4 mm in diameter within 2–3 days of incubation at 28 °C. It acidifies YMA medium after 3 days. It can tolerate 40 °C and grow at pH 4; optimum growth occurs at pH 5–7 and at 25–28 °C. Grows weakly in LB medium and PY minus Ca medium and is sensitive to chloramphenicol (30 μg), streptomycin (10 μg) and tetracycline (30 μg) and resistant to nalidixic acid (30 μg), cephuroxyn (30 μg), erythromycin (15 μg) and penicillin (10 U). As sources of carbon, uses glycerol, erythritol, L-arabinose, D-ribose, L-xylose, D-adonitol, D-galactose, D-glucose, D-mannose, *N*-acetylglucosamine, arbutin, aesculin, salicin, starch, glycogen, D- and L-fucose and L-arabitol; weakly utilizes D-arabinose, D-xylose, methyl β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, inositol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose and D-arabitol. Induces the formation of root nodules and fixes N_2 with *Phaseolus vulgaris*, *Leucaena leucocephala*,

Leucaena esculenta, *Crotalaria juncea* and *Macroptilium atropurpureum*.

The type strain is PRF 81^T (=CNPSo 122^T=SEMIA 4080^T=IPR-Pv81^T=WDCM 440^T), isolated from an effective nodule of *Phaseolus vulgaris* in Paraná State, Brazil. The DNA G+C content of the type strain is 59.9 mol%.

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