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# *Rhizobium favelukesii* sp. nov., isolated from the root nodules of alfalfa (*Medicago sativa* L)

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Strains LPU83$^T$ and Or191 of the genus *Rhizobium* were isolated from the root nodules of alfalfa, grown in acid soils from Argentina and the USA. These two strains, which shared the same plasmid pattern, lipopolysaccharide profile, insertion-sequence fingerprint, 16S rRNA gene sequence and PCR-fingerprinting pattern, were different from reference strains representing species of the genus *Rhizobium* with validly published names. On the basis of previously reported data and from new DNA-DNA hybridization results, phenotypic characterization and phylogenetic analyses, strains LPU83$^T$ and Or191 can be considered to be representatives of a novel species of the genus *Rhizobium*, for which the name *Rhizobium favelukesii* sp. nov. is proposed. The type strain of this species is LPU83$^T$ (=CECT 9014$^T$=LMG 29160$^T$), for which an improved draft-genome sequence is available.

Rhizobia – a group designation for members of the genera *Azorhizobium*, *Rhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium*, *Bradyrhizobium*, *Neorhizobium*, *Phyllobacterium*, *Microvirga*, *Devosia* (belonging to the *Alphaproteobacteria*) and *Burkholderia* and *Cupriavidus* (belonging to the *Betaproteobacteria*) – are soil and rhizosphere bacteria of agronomic significance because they carry out nitrogen-fixing symbioses with leguminous plants (Gyaneshwar *et al.*, 2011; Mousavi *et al.*, 2014, 2015; Ormeño-Orrillo *et al.*, 2015; Peix *et al.*, 2014).

Alfalfa (*Medicago sativa*) is the most widely cultivated forage legume for cattle and other farm animals, encompassing about 32 million hectares worldwide (Michaud *et al.*, 1988).

A particular characteristic of alfalfa is the specificity of that legume in relation to its symbiotic partners, *Ensifer meliloti* and *Ensifer medicae*. Both of these bacterial species are extremely sensitive to low pH (Glenn & Dilworth, 1994), with their growth rates decreasing and even ceasing at pH 5.5 or below (Howieson *et al.*, 1992; Reeve *et al.*, 1993). Several studies focussing on the isolation and characterization of alfalfa-nodulating rhizobia from acid soils have demonstrated the presence of another group of strains that are able to nodulate alfalfa, represented by *Rhizobium* sp. LPU83 and *Rhizobium* sp. Or191, in such soils. The members of this group are acid-tolerant and have an extended host range, as they are able to nodulate *Leucaena leucocephala* and *Phaseolus vulgaris* among other legumes (Del Papa *et al.*, 1999; Eardly *et al.*, 1985; Wegener *et al.*, 2001) and are highly competitive for the nodulation of alfalfa in acid soils (Del Papa *et al.*, 2003).

To characterize this rhizobial group further, we undertook a series of experiments to classify finally these strains of rhizobia using the two representative strains, LPU83$^T$ and Or191. Based on the results of our polyphasic taxonomic study, both strains were considered to represent a novel

The complete genome of *Rhizobium favelukesii* strain LPU83 is available with the following accession numbers: HG916852 (chromosome), HG916853 (pLPU83a), CBYB010000001-58 (pLPU83b), HG916854 (pLPU83c) and HG916855 (pLPU83d). All sequence data were downloaded from public databases.

Two supplementary figures are available with the online Supplementary Material.

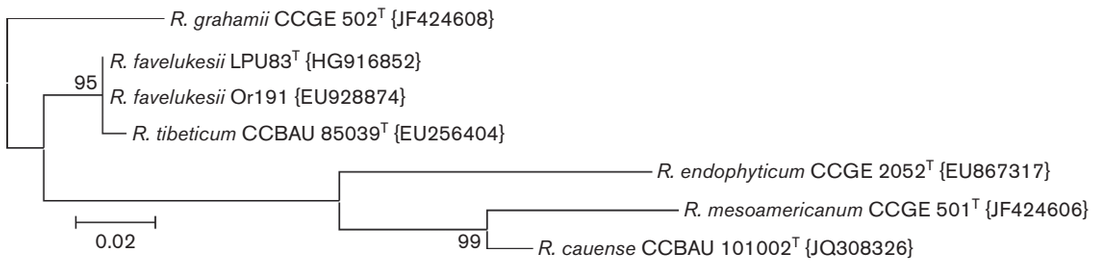


**Fig. 1.** Maximum-likelihood phylogeny reconstructed from 16S rRNA gene sequences of *Rhizobium favelukesii* and closely related rhizobia. Accession numbers are between curved brackets. Bootstrap values higher than 50 % are shown at the nodes. The bar indicates 2 substitutions per 100 nucleotide positions.

species of the genus *Rhizobium* and so should not be included in the species *Rhizobium tibeticum*, as stated by Reeve *et al.* (2015).

Strain LPU83$^T$ was isolated from a nodule of alfalfa grown in an acid soil (pH 6.08) from Castelar, Buenos Aires, Argentina (Del Papa *et al.*, 1999). This strain is able to nodulate *Medicago sativa*, *Medicago truncatula*, *Melilotus* spp., *Trigonella* spp., *Phaseolus* vulgaris and *Leucaena leucocephala*, although the biological nitrogen fixation of strains LPU83$^T$ and Or191 is inefficient (Del Papa *et al.*, 1999; Wegener *et al.*, 2001). LPU83 shared the same plasmid patterns, lipopolysacharide profiles, insertion-sequence fingerprints and PCR-fingerprinting patterns obtained with the ERIC primers, MBOREP1 and BOXC1, as strain Or191 isolated from acid soils in Oregon, USA.

To establish the phylogenetic position of the strains within the genus *Rhizobium*, DNA sequences of rhizobia were collected from the database of the National Center for Biotechnology Information (NCBI) and aligned with the Clustal module implemented by MEGA5 software (Tamura *et al.*, 2011). The models of sequence evolution were selected with the jModelTest 2.1.7 program (Darriba *et al.*, 2012). For all the analyses (i.e. the 16S rRNA, *recA-atpD-rpoB*, and *recA-atpD* concatenated genes) the model used was GTR +I+G. Maximum-likelihood trees were reconstructed on the basis of the selected model by means of PhyML v3.1 software (Guindon & Gascuel, 2003). The robustness of the maximum-likelihood topologies was evaluated by bootstrap analysis (100 replicates). We employed the best of NNIs and SPRs algorithms to search the tree topology and used 100 random trees as initial tree reconstructions.

In Fig. S1 (available in the online Supplementary Material) a phylogenetic tree is presented, based on the 16S rRNA gene sequence of rhizobia, from which data we chose the nearest-neighbour rhizobia to reconstruct the tree shown in Fig. 1. The acid-tolerant alfalfa-nodulating strains LPU83$^T$ and Or191 clearly formed a clade with *Rhizobium tibeticum* CCBAU 85039$^T$. The 16S rRNA gene sequences of LPU83$^T$ and Or191 are 100 % concordant and, therefore, identical, while respective identities of 99.9 and 99.2 % were found with *R. tibeticum* CCBAU 85039$^T$ and *Rhizobium grahamii* CCGE 502$^T$. Moreover, in the GenBank-database, strains with 100 % 16S rRNA genetic identity with LPU83$^T$ and Or191 were found, four of these – *Rhizobium* sp. T136, *Rhizobium* sp. T1473, *Rhizobium* sp. T1155, and *Rhizobium* sp. T1470 – having been described by Bromfield *et al.* (2010) and another one, *R. tibeticum* strain 246–1, reported by Stajković-Srbinović *et al.* (2012). A well-supported clade was, therefore, reconstructed consisting of LPU83$^T$, Or191, *R. tibeticum*, *R. grahamii*, *Rhizobium endophyticum*, *Rhizobium mesoamericanum* and *Rhizobium cauense* (Fig. S1).

Three housekeeping genes, *recA, atpD and rpoB,* were concatenated for tree reconstruction to explore further the phylogenetic relationships between the two strains and

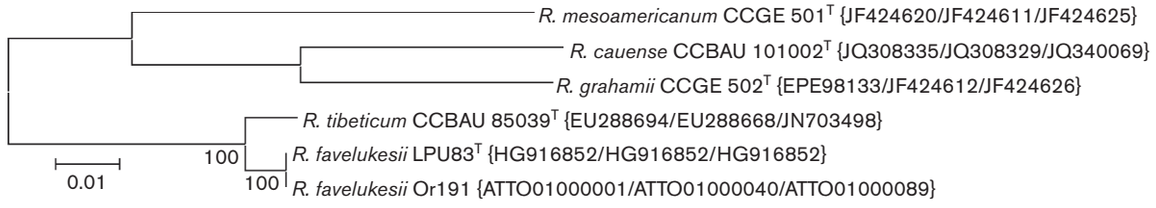


**Fig. 2.** Maximum-likelihood phylogeny reconstructed from concatenated *recA-atpD-rpoB* genes of *R. favelukesii* and closely related rhizobia. Accession numbers are indicated between curved brackets. Bootstrap values higher than 50 % are shown at the nodes. The bar indicates 1 substitution per 100 nucleotide positions.

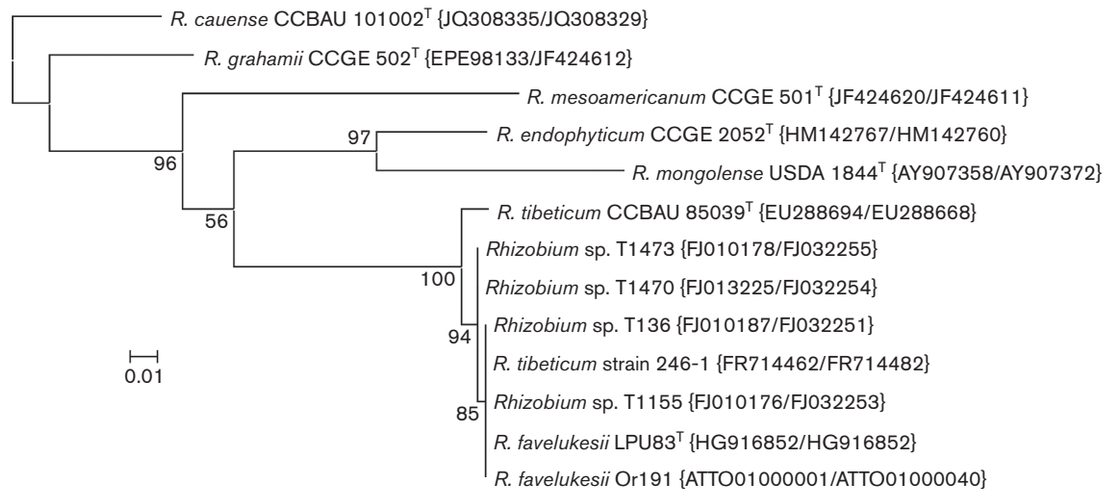


**Fig. 3.** Maximum-likelihood phylogeny reconstructed from concatenated *recA*-*atpD* genes sequences. Accession numbers are indicated between curved brackets. Bootstrap values higher than 50 % are shown at the nodes. The bar indicates 1 substitution per 100 nucleotide positions.

other rhizobia. Firstly, a tree with several strains of rhizobia was reconstructed (Fig. S2). We took the most closely related rhizobia, as shown in Fig. 2. These analyses clearly separated strains LPU83$^T$ and Or191 from *R. tibeticum*; nevertheless, these bacteria are closely related. The identity among *recA, atpD* and *rpoB* genes of LPU83$^T$ and *R. tibeticum* was 99 % for each gene. For the other strains sharing 100 % 16S rRNA gene identity with LPU83$^T$ and Or191, the *rpoB* gene was not available in the GenBank database. Accordingly, to include those strains in the study, the *recA* and *atpD* genes were concatenated to reconstruct the new phylogenetic tree shown in Fig. 3. Strains T136, T1155, and *R. tibeticum* 246–1 grouped with LPU83$^T$ and Or191. Strains T1473 and T1470 were closely related to the latter group, but strain *R. tibeticum* CCBAU 85039$^T$ was placed outside of the group. That Bromfield *et al.* (2010) described strains T136 and T1155 featuring the same plasmid pattern as Or191 is also highly relevant. Fig. 4 shows that the plasmid pattern of LPU83$^T$ is identical to that of Or191, but clearly different to that of *R. tibeticum* CCBAU 85039$^T$.

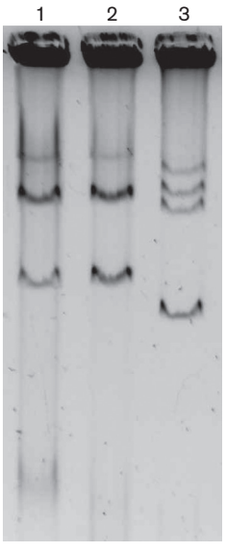


**Fig. 4.** Plasmid profiles in Eckhardt-like gels. Lane 1, LPU83$^T$; lane 2, Or191, lane 3, *R. tibeticum* CCBAU 85039$^T$.

The work by Stajković-Srbinović *et al.* (2012) did not include sequences from either LPU83$^T$ or Or191, while the DNA-DNA hybridization (DDH) between strains *R.*

**Table 1.** Average DNA–DNA hybridization values of *Rhizobium favelukesii* and related type strains

| | Strain | DDH (%±SE) |
|---|---|---|
| *R. favelukesii* | LPU83$^T$ | 100 |
| *R. favelukesii* | Or191 | 84±4 |
| *R. tibeticum* | CCBAU 85039$^T$ | 34±11 |
| *R. grahamii* | CCGE 502$^T$ | 16±1 |
| *R. endophyticum* | CCGE 2052$^T$ | 14±2 |
| *Rhizobium mesoamericanum* | CCGE 501$^T$ | 16±4 |
| *Rhizobium phaseoli* | ATCC 14482$^T$ | 10±3 |
| *Rhizobium fabae* | CCBAU 33202$^T$ | 14±3 |
| *Rhizobium etli* | CFN 42$^T$ | 12±3 |
| *Rhizobium tropici* | CIAT 899$^T$ | 13±4 |
| *Rhizobium mongolense* | USDA 1844$^T$ | 16±6 |
| *Rhizobium gallicum* | R602$^T$ | 10±7 |
| *Rhizobium mesosinicum* | CCBAU25010$^T$ | 10±8 |

**Table 2.** Distinctive features of the growth phenotype of the novel species in comparison to that of type strains of phylogenetically related species and *Rhizobium leguminosarum*

Taxa: 1, *R. favelukesii* LPU83$^T$; 2, *R. favelukesii* Or191; 3, *R. mesoamericanum* CGE 501$^T$; 4, *R. grahamii* CGE 502$^T$; 5, *R. tibeticum* CCBAU 85039$^T$; 6, *R. endophyticum* CCGE 2052$^T$; 7, *R. leguminosarum* USDA 2370$^T$. (+), Positive growth; (−), no growth; W, weak growth; ND, not determined.

$^{\#}$ Data obtained in the present study. $^{\nabla}$Data from López-López *et al.* 2012

| | 1$^{\#}$ | 2$^{\#}$ | 3$^{\nabla}$ | 4$^{\nabla}$ | 5$^{\#}$ | 6$^{\nabla}$ | 7$^{\nabla}$ |
|---|---|---|---|---|---|---|---|
| Utilization as sole carbon source : | | | | | | | |
| Acetic acid | + | + | − | + | + | − | + |
| L-Alanine | + | + | − | + | − | + | − |
| L-Alanyl glycine | + | + | − | + | − | − | + |
| L-Aspartic acid | + | + | + | − | − | + | − |
| Bromosuccinic acid | + | + | − | − | + | − | + |
| Citric acid | + | + | + | − | − | − | − |
| Fumaric acid | + | + | + | + | + | + | − |
| D-Galactonic acid-γ-lactone | + | + | − | − | + | − | + |
| D-Glucosaminic acid | + | + | + | − | − | − | + |
| L-Glutamine | + | + | − | + | + | + | + |
| Glycyl L-glutamic acid | + | + | − | + | − | − | + |
| Myo-Inositol | + | + | + | + | + | + | − |
| D-Malic acid | + | + | + | + | − | − | + |
| Maltotriose | + | + | + | + | + | − | + |
| *N*-Acetyl 3-D-mannosamine | + | + | − | + | + | + | + |
| L-Proline | + | + | + | + | − | + | + |
| D-Psicose | + | + | + | + | − | − | + |
| Pyruvic acid | + | + | − | + | − | − | + |
| Monomethyl succinate | + | + | − | + | − | − | − |
| L-Threonine | + | + | − | + | − | − | − |
| Resistance to tetracycline (5 mg ml) | − | − | ND | ND | + | ND | ND |
| Growth with/at: | | | | | | | |
| PY 37 °C | − | − | + | W | − | − | ND |
| LB | − | − | − | − | − | + | ND |
| β-Galactosidase | + | + | ND | ND | + | ND | ND |
| Urease | + | + | ND | ND | − | ND | ND |
| Oxidase | + | + | ND | ND | + | ND | ND |
| Nitrate reductase | + | + | ND | ND | + | ND | ND |

*tibeticum* 246–1 and *R. tibeticum* CCBAU 85039$^T$ was lower than 70 %. To advance in the positioning of LPU83$^T$ and Or191 in relation to closely related species, we performed DDH experiments using Southern-blot hybridizations, as described previously (Martínez-Romero *et al.*, 1991). The DDH values between strains LPU83$^T$ and Or191 were greater than 84 %, indicating that both were members of the same species. For all the members of the other taxa of *Rhizobium* tested, the DDH values were in the range of 10–34 % using DNA of strain LPU83$^T$ as a probe (Table 1). As the DDH value obtained for LPU83$^T$ and *R. tibeticum* CCBAU 85039$^T$ was too low in comparison with the identity of the housekeeping genes, *recA-atpD-rpoB,* we performed a new DDH experiment between both strains using the methodology described by Ezaki *et al.* (1989), according to a modification of the method (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998). In this case, a 62 % value of DDH was obtained, confirming the classification of LPU83$^T$ as a novel species.

The phenotypic features of the growth of the novel strains and other relevant taxa were studied in PM1 BIOLOG microplates, as previously described (López-López *et al.*, 2010). LPU83$^T$ and Or191 share the same carbon-source–utilization pattern (Table 2). In order to characterize more deeply the biochemical profile of the strains, different tests were performed such as production of urease, nitrate reductase, β-galactosidase, oxidase and catalase. The complete phenotypic properties of strains LPU83$^T$ and Or191 are given in the species description below, and characteristics that differentiate them from closely related species are provided in Table 2. The table also shows how the overall growth specificities of the two strains clearly differentiates

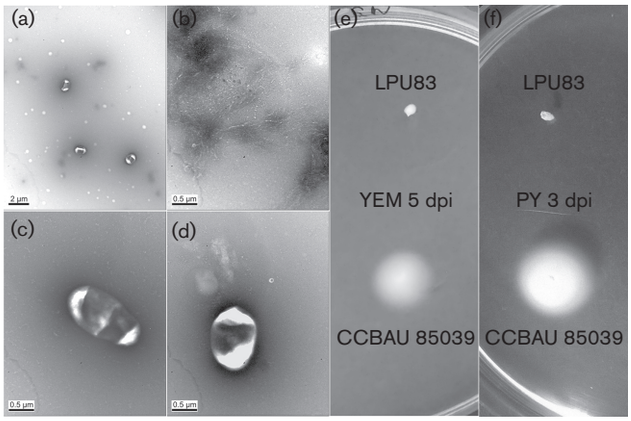


**Fig. 5.** Microscopical analysis of LPU83$^T$. Aliquots of cultures were observed by TEM after staining with 2 % (w/v) potassium phosphotungstate (pH 5.2; 2 % [w/v] KOH). a, c, and d, Different magnifications of LPU83$^T$ (no flagellum visible). b, No free flagella were observed in the preparations. e and f, Swimming test on 0.3 % (w/v) agar in YEM and PY, respectively.

them from the other closely related species analysed (López-López *et al.*, 2010, 2012). LPU83 and Or191 differed from its closest relative *R. tibeticum* CCBAU 85039$^T$ in terms of the metabolism of L-alanine, L-alanyl glycine, L-aspartic acid, citric acid, D-glucosaminic acid, glycyl L-glutamic acid, D-malic acid, L-proline, D-psicose, pyruvic acid, monomethyl succinate, L-threonine, urease activity and sensitivity to tetracycline (5 μg ml$^{-1}$).

Using transmission electron microscopy, we were not able to observe flagellum on LPU83$^T$ (Fig. 5). Swimming assays were performed as described previously (Althabegoiti *et al.*, 2008). LPU83$^T$ did not exhibit swimming motility in TY (Beringer, 1974), PY (Noel *et al.*, 1984), YEM (Vincent, 1970) and AG (Sadowsky *et al.*, 1987) with 0.3 % agar (w/v) in contrast to *R. tibeticum* CCBAU 85039$^T$ (Fig. 5).

The fatty acid profiles of strains LPU83$^T$ and Or191, as well as that of *R. tibeticum* CCBAU 85039$^T$, were determined with the MIDI system and the TSBA5 database after incubation for 24 h on YEM agar plates (Table 3). All the strains contained fatty acid profiles that were common to the *Rhizobium* genus-such as $C_{16:0}$, $C_{18:0}$ cyclo $\omega 8c$, $C_{18:1}\omega 7c$, and the second group of fatty acids that could not be separated by GLC with the MIDI system referred to as summed feature 2 [i. e. one or more $C_{12:0}$ aldehydes, the unknown equivalent-chain-length species 10.928, iso-$C_{16:1}$ I and $C_{14:0}$ 3-OH; (Tighe *et al.*, 2000)]. Strains LPU83$^T$ and Or191, produced a similar profile and could be distinguished from *R. tibeticum* by their synthesis of a summed feature 3 (i. e. $C_{16:1}\omega 7c$, and/or iso-$C_{15:0}$ 2-OH) along with $C_{18:0}$ 3-OH.

The phenotypic, chemotaxonomic and genotypic data from the present study indicate that strains LPU83$^T$ and Or191 represent a novel species of the genus *Rhizobium* that can be distinguished from the nearest phylogenetic neighbours phenotypically as well as genotypically. We, therefore, propose to classify these bacteria as a novel species, for which the name *Rhizobium favelukesii* sp. nov. is proposed.

## Description of *Rhizobium favelukesii* sp. nov.

*Rhizobium favelukesii* (fa.ve.lu.ke′si.i N.L. gen. n. favelukesii, of Favelukes, named in honour of Professor Gabriel Favelukes, who made valuable contributions to the development of rhizobiology in South America).

Aerobic, non-spore-forming, Gram-stain-negative rods that grow on TY, PY and YEM, but not on LB medium. The colonies on PY-agar plates are circular and convex with regular margins; they are pearly and appear within 3 days at 28 °C. Optimal growth occurs at 28 °C and at pH 7; can grow at pH 4.5, but not in any media at 37 °C. Does not exhibit swimming motility. The bacterium uses acetic acid, L-alanine, L-alanyl glycine, L-aspartic acid, bromosuccinic acid, citric acid, fumaric acid, D-galactonic acid-γ-lactone, D-glucosaminic acid, L-glutamine, glycyl L-glutamic acid, *myo*-inositol, D-malic acid, maltotriose, *N*-acetyl-3-D-mannosamine, L-proline, D-psicose, pyruvic acid, monomethyl succinate, L-threonine, succinic acid, D-galactose, D-trehalose, D-mannose, glycerol, D-glucuronic acid,

**Table 3.** Cellular fatty-acid composition (as a percentage) present in *R. favelukesii* strains LPU83$^T$ and Or191, and in *R. tibeticum* strain CCBAU 85039$^T$

Taxa: 1, LPU83$^T$ ; 2, Or191; 3, CCBAU 85039$^T$.

| | 1 | 2 | 3 |
|---|---|---|---|
| $C_{15:0}$ 2-OH | 9.1 | 10.94 | 10.69 |
| $C_{15:0}$ iso 3-OH | ND | ND | ND |
| $C_{16:0}$ | 1.68 | 2.21 | 3.74 |
| $C_{16:0}$ 3-OH | 3.65 | 1.86 | 2.55 |
| $C_{17:0}$ cyclo | ND | ND | ND |
| $C_{18:0}$ | 1.26 | 1.26 | ND |
| $C_{18:0}$ 3-OH | 2.93 | 1.42 | ND |
| $C_{18:1}$ 2-OH | 4.25 | 3.94 | 3.65 |
| $C_{11}$-methyl $_{18:1}$_7*c* | ND | ND | ND |
| $C_{19:0}$ cyclo $\omega 8c$ | 1.27 | 1.61 | 2.83 |
| $C_{19:0}$ 10-methyl | 0.2 | ND | ND |
| $C_{18:1}\omega 7c$ | 25.27 | 20.2 | 27.72 |
| Summed features* | | | |
| 2 | 49.63 | 55.66 | 48.83 |
| 3 | 0.78 | 0.9 | ND |

*Summed features are groups of two or more fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 contained one or more C12 : 0 aldehyde, unknown equivalent chain length 10.928, iso-$C_{16:1}$ I and $C_{14:0}$ 3-OH. Summed feature 3 contained $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH. ND: Not detected.

D-gluconic acid, L-lactic acid, formic acid, D-mannitol, D-fructose, acetic acid, $\alpha$-D-glucose and $\alpha$-D-lactose, but cannot use dulcitol, L-malic acid, uridine, D-serine, L-arabinose, 2-aminoethanol, phenylethyl-amine and glycyl-L-proline as carbon sources. Tests for urease, nitrate reductase, $\beta$-galatosidase and oxidase are positive, while catalase is weakly positive. The most abundant fatty acids are $C_{18:1}\omega7c$ and summed feature 2 (probably $C_{12:0}$ aldehyde, the unknown equivalent-chain-length species 10.928, iso-$C_{16:1}$ I and $C_{14:0}$ 3-OH). At the molecular level it can be differentiated from other species of the genus by sequence analysis of the *recA, atpD* and *rpoB* genes, and by DDH.

The type strain LPU83$^T$ (=LMG 29160$^T$=CECT 9014$^T$) was isolated from an alfalfa (*Medicago sativa*) root nodule in the course of a plant-trap experiment inoculated with soil from Castelar, Buenos Aires, Argentina (soil pH 6.08). The DNA G+C content of the type strain is 59.65 mol%, as determined from the 7.57 Mbp of total genomic sequence. The genome of strain LPU83$^T$ consists of one chromosome and four plasmids.

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