Phyllobacterium loti sp. nov. isolated from nodules of *Lotus corniculatus*

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Strain S658^T was isolated from a *Lotus corniculatus* nodule in a soil sample obtained in Uruguay. Phylogenetic analysis of the 16S rRNA gene and *atpD* gene showed that this strain clustered within the genus *Phyllobacterium*. The closest related species was, in both cases, *Phyllobacterium trifolii* PETP02^T with 99.8 % sequence similarity in the 16S rRNA gene and 96.1 % in the *atpD* gene. The 16S rRNA gene contains an insert at the beginning of the sequence that has no similarities with other inserts present in the same gene in described rhizobial species. Ubiquinone Q-10 was the only quinone detected. Strain S658^T differed from its closest relatives through its growth in diverse culture conditions and in the assimilation of several carbon sources. It was not able to reproduce nodules in *Lotus corniculatus*. The results of DNA–DNA hybridization, phenotypic tests and fatty acid analyses confirmed that this strain should be classified as a representative of a novel species of the genus *Phyllobacterium*, for which the name *Phyllobacterium loti* sp. nov. is proposed. The type strain is S658^T(=LMG 27289^T=CECT 8230^T).

Lotus corniculatus L. (bird's-foot trefoil) is the most important and widely distributed crop from the *Lotus* genus (Díaz *et al.*, 2005). Although the species originated in temperate areas of Europe and North Africa, it has spread throughout the world as a result of cultivation (Díaz *et al.*, 2005). Nowadays *Lotus corniculatus* is the most widely used legume in sown pastures in Uruguay, where it establishes symbiosis with *Mesorhizobium* (Sotelo *et al.*, 2011).

In the present study, we characterized a novel strain, designated S658^T, which was isolated from *Lotus corniculatus* nodules, and showed that it represents a novel species of the genus *Phyllobacterium*. At the time of writing, the genus *Phyllobacterium*, described by Knösel (1962), contains nine species with validly published names

(Flores-Félix *et al.*, 2013), *Phyllobacterium myrsinacearum*, isolated from leaf nodules of Myrsinaceae (Mergaert *et al.*, 2002), *Phyllobacterium catacumbae*, isolated from a volcanic rock (Jurado *et al.*, 2005), *Phyllobacterium trifolii*, isolated from *Trifolium* nodules (Valverde *et al.*, 2005), four species isolated from the rhizoplane of different plants, *P. brassicacearum*, *P. bourgognense*, *P. leguminum* and *P. ifriqiyense* (Mantelin *et al.*, 2006), and *P. endophyticum*, a recently described species isolated from nodules of *Phaseolus vulgaris* (<http://dx.doi.org/10.1601/nm.3268>) (Flores-Félix *et al.*, 2013).

Strain S658^T was isolated from a nodule of *Lotus corniculatus* in Uruguay during a study of bacteria nodulating this legume. The root nodules were washed several times with sterile distilled water and surface-sterilized for 1 min in 90 % (v/v) ethanol followed by 3 min in 4 % (v/v) NaClO, then washed three times in sterile distilled water, as previously described (Sotelo *et al.*, 2011). The nodules were then crushed using a sterile glass

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *atpD* sequences of strain S658^T are KC577468 and KC577467, respectively.

A supplementary figure is available with the online version of this paper.

rod and the homogenized nodule tissue was inoculated on modified yeast mannitol agar (YMA; Vincent, 1970) (containing 1^{-1} : 10 g mannitol, 1 g yeast extract, 0.2 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 20 g agar) and incubated at 28 °C for 4 days. The cultures used in further phenotypic and molecular studies were purified from a single colony after incubation for 4 days at 28 °C on YMA. Colonies were white, mucoid, translucent and convex on this medium. Reinfection experiments were performed in *Lotus corniculatus*, as previously described (Sotelo *et al.*, 2011). Strain S658^T was unable to reproduce nodules in *Lotus corniculatus* and we failed to amplify the *nodC* gene with the primers described by Laguerre *et al.* (2001) that allowed the amplification of *nodC* genes of *Mesorhizobium* strains from *Lotus corniculatus* isolated in the same region as our strain (Sotelo *et al.*, 2011).

Strain S658^T was grown in nutrient broth (Difco, Becton Dickinson, BBL) for 48 h at 22 °C to check for motility by phase-contrast microscopy using the hanging-drop method. Gram staining was carried out by the procedure described by Doetsch (1981) after 24 h incubation at 28 °C. The flagellation type was determined by electron microscopy after incubation of strain S658^T for 48 h in nutrient agar (Difco, Becton Dickinson, BBL) at 22 °C, as previously described (Rivas *et al.*, 2007). Cells of strain S658^T were Gram-stain-negative, rod-shaped, non-sporulating, motile by peritrichous flagella and commonly observed as single cells (Fig. S1, available in the online Supplementary Material).

The 16S rRNA gene was amplified and sequenced as described by Rivas *et al.* (2007) and the *atpD* gene using the primers *atpD*-273F and *atpD*-771R, as described by Weir *et al.* (2004). The sequences obtained were compared with those from GenBank using the BLASTN program (Altschul *et al.*, 1990) and the 16S rRNA gene sequences were also compared with those from the EzTaxon-e server (Kim *et al.*, 2012). Sequences were aligned using CLUSTAL X software (Thompson *et al.*, 1997). The distances were calculated according to Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees of 16S rRNA and *atpD* genes were inferred using the neighbour-joining model (Saitou & Nei, 1987), and in the case of 16S rRNA gene, the maximum-likelihood model (Rogers and Swofford, 1998) was also used. MEGA5 software (Tamura *et al.*, 2011) was used for all analyses.

The resulting neighbour-joining tree from 16S rRNA gene analysis is shown in Fig. 1, and the results were congruent with those from maximum-likelihood modelling (data not shown) showing that strain S658^T belongs to the genus *Phyllobacterium*. Comparison of the 16S rRNA gene sequence of strain S658^T against those in the EzTaxon-e database showed that its closest relative was *P. trifolii* PETP02^T with 99.79 % sequence similarity without gaps, but strain S658^T contains an insert of 85 bp located between nucleotides 70 and 155 of the *rrs* gene sequence, which is absent in all species of the genus *Phyllobacterium* described to date.

Phylogenetic analysis of the *atpD* (ATPase synthase β subunit) gene (Fig. 2) showed that strain S658^T clustered within the *Phyllobacterium* clade, forming a separate branch within this genus, and related to a group formed by *P. brassicacearum* LMG 22836^T (STM 196^T) and *P. trifolii* PETP02^T, with the closest strain, *P. trifolii* PETP02^T, having 96.4 % sequence similarity, which was the same as that found between *P. brassicacearum* LMG 22836^T and *P. trifolii* PETP02^T. This suggested that the novel strain could also belong to a different species of the genus *Phyllobacterium*.

DNA–DNA hybridization experiments were carried out in triplicate using the method of Ezaki *et al.* (1989), following the recommendations of Willems *et al.* (2001). Strain S658^T was hybridized with *P. trifolii* PETP02^T showing 47 % (± 8 %) DNA–DNA relatedness. This value is lower than the threshold value of 70 % DNA–DNA relatedness for definition of bacterial species (Wayne *et al.*, 1987), indicating that the strain isolated in this study belongs to a new species of the genus *Phyllobacterium*.

DNA for analysis of DNA base composition was prepared according to the method of Chun & Goodfellow (1995). The DNA G + C content was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G + C content of strain S658^T was 57.8 mol%. This value is within the range of DNA G + C content reported for members of the genus *Phyllobacterium* (51–58 mol%; Mantelin *et al.*, 2006).

Cellular fatty acids were analysed using the Microbial Identification System (MIDI; Microbial ID) Sherlock 6.1 and the RTSBA6 library, according to the technical instructions provided (Sasser, 1990). *P. trifolii* PETP02^T was included in this analysis together with strain S658^T. The strains were grown on TSA plates (Tryptic Soy Agar, Becton Dickinson; BBL) for 48 h (late-exponential growth phase) at 28 °C. The results of the fatty acid analysis are shown in Table 1. The major fatty acids (>10 %) present in strain S658^T were $C_{16:0}$ (20.7 %), $C_{18:1\omega7c}$ 11-methyl (16.5 %) and $C_{18:1\omega6c}/C_{18:1\omega7c}$ in summed feature 8 (48.5 %). Strain S658^T differed from the remaining tested strains in the amount of $C_{18:1\omega7c}$ 11-methyl, which was higher in the novel strain, in the amount of $C_{19:0}$ cyclo $\omega8c$, which was lower, and in the minor presence of $C_{17:0}$. It also differed from its closest relative *P. trifolii* PETP02^T in the amounts of $C_{16:0}$ and $C_{18:1\omega6c}/C_{18:1\omega7c}$ (in summed feature 8), in the presence of $C_{18:1}$ 2-OH and in the absence of $C_{16:0}$ 3-OH.

Strain S658^T was cultivated for 48 h in TSA at 28 °C to obtain the cell mass required for quinone analysis. Quinone analysis was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Ubiquinone Q-10 was the unique respiratory quinone.

Strain S658^T was grown on TY plates (containing 1^{-1} : 4 g tryptone, 3 g yeast extract, 0.9 g $CaCl_2$ and 20 g agar) for 24 h for catalase and oxidase production analysis. Catalase

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Phyllobacterium loti* sp. nov. S658^T, species of the genus *Phyllobacterium* and those from the closest genus *Mesorhizobium*. The significance of each branch is indicated by a percentage bootstrap value calculated for 1000 subsets. Bar, 1 nt substitutions per 100 nt.

production was assayed by using 0.3 % hydrogen peroxide with one colony taken from TY plates. Oxidase activity was detected using *N,N,N',N'*-tetramethyl-1,4-phenylene-diamine dihydrochloride. The ability to grow at different temperatures (4, 6, 10, 28, 37 and 39 °C) and in the presence of different NaCl concentrations (1, 1.5, 2 and 2.5 %) was determined on the YMA modified medium described above. The ability to grow at different pH (4.5, 5, 6, 7 and 8) was determined in yeast mannitol broth (YMB; containing 1⁻¹: 10 g mannitol, 1 g yeast extract, 0.2 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.5 g NaCl). PCA buffer

(0.4 M Na₂HPO₄ and 0.2 M citric acid) was used to adjust the pH from 4.5 to 6.5 and TE buffer (0.2 M Tris adjusted to pH 8 with HCl plus 1 mM EDTA) was used for pH 8. Other physiological and biochemical tests were carried out using API 20NE and API ID32GN systems (bioMérieux) following the manufacturer's instructions and the results were read after 72 h and 5 days incubation at 28 °C. To test natural antibiotic resistance the following antibiotics were used: ampicillin (2 µg), erythromycin (2 µg), ciprofloxacin (5 µg), penicillin (10 IU), polymyxin B (300 IU), cloxacillin (1 µg), oxytetracycline (30 µg), gentamicin (10 µg),

Fig. 2. Neighbour-joining tree based on partial *atpD* gene sequences of *Phyllobacterium loti* sp. nov. S658^T and related members of the family *Phyllobacteriaceae*. The significance of each branch is indicated by a percentage bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.

cefuroxime (30 µg) and neomycin (5 µg), (Becton Dickinson, BBL). The disc diffusion method on YMA medium (Vincent, 1970) incubated at 28 °C was used. The results were read after 48 h incubation; the strain was considered to be sensitive if the inhibition zone was at least 1 cm in diameter. The type strains of species of the genus *Phyllobacterium* available in the LMG culture collection were included in the phenotypic study as reference strains. Phenotypic characteristics of the novel species are reported in the species description and the differences with respect to other species of the genus *Phyllobacterium* are recorded in Table 2. Our results were basically in agreement with those of Mantelin *et al.* (2006) for *P. brassicacearum* and *P. bourgognense*, although assimilation of gluconate by the type strains of these species was negative using the API 32GN system and that of L-alanine and maltose were negative for the type strains of *P. bourgognense* and *P. leguminum*, respectively. In the case of *P. trifolii*, the assimilation of sucrose was positive using the API 32GN system whereas it was not detected by Valverde *et al.* (2005) using the API 50CH system.

In summary, the phylogenetic, chemotaxonomic and phenotypic characteristics of strain S658^T showed that it represents a novel species of the genus *Phyllobacterium*, for which the name *Phyllobacterium loti* sp. nov. is proposed.

Description of *Phyllobacterium loti* sp. nov.

Phyllobacterium loti (lo'ti. L. n. *lotus* the African lotus and also a botanical genus name; L. gen. n. *loti* of lotus, isolated from *Lotus corniculatus*)

Gram-stain-negative, aerobic rods as for other species of the genus. Colonies are small (<2 mm in diameter) and pearl white on YMA after 48 h incubation at 28 °C. The temperature range for growth is 6–39 °C (optimal growth occurs at 28 °C). The pH range for growth is pH 5–8 (optimal growth occurs at pH 7). Grows in the presence of up to 2% (w/v) NaCl. The major cellular fatty acids are C_{16:0}, C_{18:1ω7c} 11-methyl and C_{18:1ω6c}/C_{18:1ω7c} (summed feature 8). The only quinone detected is Q-10. Catalase and oxidase activities are positive. Nitrate

Table 1. Cellular fatty acid composition of strain S658^T and type strains of the closest related species of the genus *Phyllobacterium*

Strains: 1, S658^T; 2, *P. trifolii* LMG PETP02^T; 3, *P. endophyticum* PEPV15^T; 4, *P. brassicacearum* LMG 22836^T; 5, *P. bourgognense* LMG 22837^T. All data are from this study. ND, not detected.

Fatty acid	1	2	3	4	5
Saturated straight-chain					
C _{13:1} at 12-13	ND	ND	ND	ND	1.6
C _{14:0}	0.79	ND	0.71	ND	1.4
C _{16:0}	20.68	12.3	22.6	15.8	22.1
C _{17:0}	0.83	ND	ND	ND	ND
C _{18:0}	2.78	1.8	1.9	1.5	6.4
Hydroxy fatty acids					
C _{16:0} 3-OH	ND	1.3	1.6	2.3	1.6
C _{18:1} 2-OH	1.03	ND	1.7	ND	ND
C _{18:0} 3-OH	ND	ND	0.6	0.7	ND
Cyclopropane fatty acids					
C _{17:0} cyclo	ND	ND	2.2	0.4	1.4
C _{19:0} cyclo ω8c	2.62	6.4	13.9	5.4	10.7
Unsaturated					
C _{20:2} ω6,9c	ND	0.7	ND	0.4	ND
C _{18:1} ω7c 11-methyl	16.53	10.7	9.2	9.7	9.9
Summed feature*					
2	0.97	1.5	3	3.1	1.4
3	5.73	2.5	3.6	2.6	4.3
8	48.05	62.9	39.1	58.3	39.3

*Summed features represent groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 2 contained C_{14:1}3-OH/ C_{16:1} iso 1; summed feature 3 contained C_{16:1}ω6c/C_{16:1}ω7c; summed feature 8 contained C_{18:1}ω6c/C_{18:1}ω7c.

reduction and β-galactosidase activity are negative and aesculin is hydrolysed in the API 20NE system. Arginine dehydrolase, indole, gelatinase and urease are not produced. In the same system glucose, L-arabinose, mannose, mannitol, N-acetylglucosamine, maltose and D-malate are used as carbon sources. Assimilation of gluconate, caprate, adipate, citrate and phenylacetate is negative. In the API ID32GN system the assimilation of N-acetylglucosamine, D-ribose, inositol, sucrose, maltose, D-mannitol, D-glucose, L-fucose, D-sorbitol, L-arabinose, L-histidine and 3-hydroxybutyrate is positive, but assimilation of DL-lactate, L-alanine, itaconate, suberate, malonate, glycogen, 3-hydroxybenzoate, L-serine, salicin, melibiose, propionate, caprate, valerate, citrate, 2-ketogluconate, 4-hydroxybenzoate and L-proline is negative. Assimilation of L-rhamnose, acetate and 5-ketogluconate is weak. Sensitive to ciprofloxacin (5 µg), neomycin (5 µg), polymyxin B (300 IU) and oxytetracycline. Resistant to ampicillin (2 µg), penicillin (10 IU), cefuroxime (30 µg), cloxacillin (1 µg), gentamicin (10 µg) and erythromycin (2 µg).

Table 2. Phenotypic differences among species of genus *Phyllobacterium*

Species: 1, *Phyllobacterium loti* sp. nov. (strain S658^T); 2, *P. trifolii* (Valverde *et al.*, 2005), 3, *P. endophyticum* (Flores-Félix *et al.*, 2013); 4, *P. brassicacearum* (Mantelin *et al.*, 2006); 5, *P. bourgognense* (Mantelin *et al.*, 2006); 6, *P. leguminum* (Mantelin *et al.*, 2006); 7, *P. ifriqiyense* (Mantelin *et al.*, 2006); 8, *P. catacumbae* (Jurado *et al.*, 2005); 9, *P. myrsinacearum* (Knösel, 1984; Valverde *et al.*, 2005; Mergaert & Swings, 2005; Mantelin *et al.*, 2006). Data for *P. ifriqiyense* are from Mantelin *et al.* (2006) and those from the type strains of the remaining species are coincident with those from the original descriptions of each one and from Flores-Félix *et al.* (2013) except in the cases marked in this table. +, Positive; −, negative; w, weakly positive; v, variable; ND, no data.

Characteristic	1	2	3	4	5	6	7	8	9
Growth at/in:									
37 °C	+	+	+	−	−	+	+	+	−
pH 5	+	−	+	+	+	w‡	+	+	+
2 % NaCl	w	+	+	w‡	−	+	+	−	+
Growth on:									
Maltose*†	+	+	−	+	+	−‡	+	+	+
Gluconate†	−	+	−	−‡	−‡	+	+	+	+
Sucrose*	+	+	−	+	+	−	ND	+	+
5-Ketogluconate*	w	+	+	+	+	−	+	+	+
D-Malate†	+	+	+	+	+	−	+	+	+
Malonate*	−	−	w	+	−	−	+	w	−
DL-Lactate*	−	+	+	+	+	+	+	+	+
Valerate*	−	+	−	+	+	−	v	+	+
L-Alanine*	−	w	+	+	−‡	−	+	+	+
4-Hydroxybenzoate*	−	−	−	+	−	−	−	−	+
3-Hydroxybutyrate*	+	+	+	+	+	−	+	+	+
2-Ketogluconate*	−	+	w	w	w	−	ND	+	+
Proline*	−	+	+	+	+	+	+	+	+
Resistance to:									
Cefuroxime	+	+	+	+	−	+	ND	+	+
Gentamicin	+	−	−	−	−	+	ND	+	+
Polymyxin B	−	−	−	+	−	+	ND	+	+

*Data from API ID32GN.

†Data from API 20NE.

‡Data differ from those of Mantelin *et al.* (2006).

§Sucrose assimilation was not detected using API 50CH (Valverde *et al.*, 2005).

The type strain is S658^T (=LMG 27289^T=CECT 8230^T), and was isolated from a nodule of *Lotus corniculatus* in Uruguay. The DNA G+C content of the type strain is 57.8 mol%.

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