International Journal of Systematic and Evolutionary Microbiology (2005), **55**, 1985–1989 **DOI** 10.1099/ijs.0.63551-0

Phyllobacterium trifolii sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils

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Bacterial strain PETP02^T was isolated from nodules of *Trifolium pratense* growing in a Spanish soil. Phylogenetic analysis of the 16S rRNA gene sequence showed that this strain represents a member of the genus *Phyllobacterium*. However, divergence found with the 16S rRNA gene sequence of the single recognized species of this genus, *Phyllobacterium myrsinacearum*, indicated that strain PETP02^T belongs to a different species. The results of DNA–DNA hybridization, phenotypic tests and fatty acid analyses confirmed that this strain represents a novel species of the genus *Phyllobacterium*, for which the name *Phyllobacterium trifolii* sp. nov. is proposed. The type strain is PETP02^T (=LMG 22712^T=CECT 7015^T). This strain was strictly aerobic and used several carbohydrates as carbon source. It was not able to reduce nitrate. Aesculin hydrolysis was negative. It did not produce urease, arginine dihydrolase, gelatinase or β-galactosidase. The DNA G+C content was 56·4 mol%. The *nodD* gene of this strain showed a sequence closely related to those of strains able to nodulate *Lupinus*. Infectivity tests showed that this strain is able to produce nodules in both *Trifolium repens* and *Lupinus albus*.

Trifolium pratense is a common legume in temperate soils and establishes effective symbioses with *Rhizobium* strains. The most common endosymbiont of this legume is *Rhizobium leguminosarum* biovar trifolii, which induces the formation of indeterminate nodules (Jordan, 1984). This legume belongs to the natural plant cover of many soils in north-west Spain but there are no studies regarding the diversity of bacteria nodulating *Trifolium* in these soils. During a study of populations of bacteria nodulating *Trifolium* in several geographical locations we isolated a strain, designated PETP02^T, phylogenetically related to the genus *Phyllobacterium*. This genus was described by Knösel (1962) and currently contains one recognized species, since *Phyllobacterium rubiacearum* was recently reclassified as *Phyllobacterium myrsinacearum* (Mergaert *et al.*, 2002). The data obtained in the present study show that strain PETP02^T belongs to a novel species of *Phyllobacterium*.

Published online ahead of print on 6 May 2005 as DOI 10.1099/ijs.0.63551-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nodD* gene sequences of PETP02^T are AY786080 and AY786081, respectively.

Strain PETP02^T was isolated from *T. pratense* nodules according to the method of Vincent (1970) on YMA medium. Colonies are white, mucoid, translucent and convex following growth on this medium. This strain exhibits a growth rate in YMB (Vincent, 1970) medium similar to that of *Rhizobium* species (doubling time of 2 h).

Strain PETP02^T was grown on nutrient agar medium for 48 h to check for motility by phase-contrast microscopy. Cells were gently suspended in sterile water, stained with 0·2 % uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope (Peix *et al.*, 2003). Gram reaction of cells was ascertained by staining (Doetsch, 1981). Cells of strain PETP02^T were Gram-negative, rod-shaped, non-sporulating, motile by means of a polar flagellum and commonly observed as single cells.

Strain PETP02^T was re-isolated as a pure culture from nodules of *Trifolium repens* and a single colony was used for all molecular analyses. The nearly complete 16S rRNA gene sequence was analysed as described by Rivas *et al.* (2002). Comparison with sequences from GenBank using the BLAST program (Altschul *et al.*, 1990) indicated that this strain is phylogenetically related to members of the genus *Phyllobacterium*. Sequences of the new isolate and related bacteria

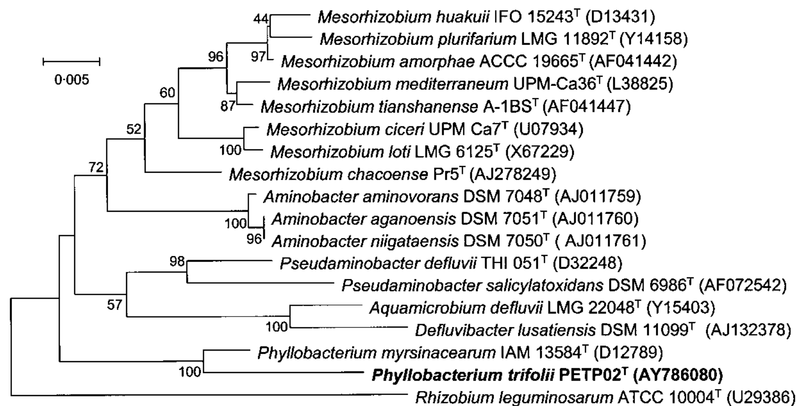

Fig. 1. Comparative sequence analysis of the 16S rRNA gene from *Phyllobacterium trifolii* PETP02^T and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. The analysis was based on 1478 nt. *Rhizobium leguminosarum* ATCC 10004^T was used as outgroup. Bar, 5 nt substitutions per 1000 nt.

were aligned using CLUSTAL W software (Thompson *et al.*, 1997). The distances were calculated according to Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses. The resulting neighbour-joining tree is shown in Fig. 1. The 16S rRNA gene sequence of strain PETP02^T showed 98·0 % similarity to that of *P. myrsinacearum*, suggesting that it belongs to a different species.

Strain PETP02^T was subjected to plasmid profile analysis according to Plazinski *et al.* (1985), except that electrophoresis was performed at 2 V cm^{-1} for 90 min, followed by 3 V cm^{-1} for 60 min and finally at 6 V cm^{-1} for 3 h. The 175 kb and 205 kb plasmids of *Sinorhizobium meliloti* GR4 (Toro & Olivares, 1986) were used as size markers and as a positive control for Southern analysis. Plasmid DNA was capillary-transferred to a nylon membrane according to Southern (1975) and immobilized by baking at 80 °C for 2 h. Oligonucleotide primers were designed to amplify a fragment of the *nodD* gene conserved among members of the family *Rhizobiaceae* as described by Rivas *et al.* (2002). The PCR-amplified fragments of the *nodD* gene were digoxigenin-labelled with the DIG DNA labelling kit (Roche Diagnostics Corp.) following the manufacturer's instructions and were used as probe. Hybridization was detected with the DIG nucleic acid detection kit (Boehringer Mannheim), using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as substrates for alkaline phosphatase, according to the manufacturer's instructions.

Results from the plasmid profile analysis and the Southern hybridization are shown in Fig. 2. The technique used revealed three plasmids in strain PETP02^T (Fig. 2, lane 2). The specific probe detected a *nodD* gene in the three plasmids of strain PETP02^T (Fig. 2, lane 4).

A partial sequence of the *nodD* gene of strain PETP02^T was obtained from genomic DNA using the method of Rivas *et al.* (2002). Phylogenetic analysis (Fig. 3) showed that the *nodD* gene of strain PETP02^T is closely related to that of a novel species of the genus *Ochrobactrum* that is able to nodulate *Lupinus albus* (*nodD* gene sequence similarity of 94·9 %; Trujillo *et al.*, 2005).

Taking into account the close phylogenetic relationship between the *nodD* gene present in strain PETP02^T and those from strains nodulating *Lupinus*, we suspected that this strain would also nodulate *Lupinus*. We therefore tested for nodulation using *T. repens* and *L. albus* as described previously (Velázquez *et al.*, 2001). The strain *R. leguminosarum* bv. trifolii ATCC 14480 and *Bradyrhizobium* sp. ISLU35 (Jarabo-Lorenzo *et al.*, 2003) were used as positive controls in *T. repens* and *L. albus*, respectively. As expected, strain PETP02^T was able to induce nodules in both plants (Fig. 4). The morphology of the nodules formed on *T. repens* was the same as that of those formed by *R. leguminosarum* bv. trifolii and they were formed along secondary roots in both cases. However, the nodules formed in *L. albus* were morphologically different from those formed by *Bradyrhizobium* sp. ISLU35. The nodules induced by strain PETP02^T were formed at the intersection of the main and secondary roots, whereas those induced by strain ISLU35 were formed along the secondary roots. Strain PETP02^T formed fewer nodules

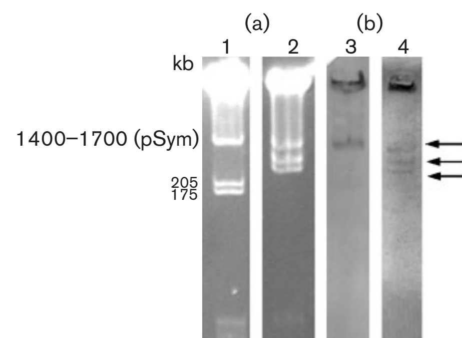

Fig. 2. (a) Plasmid profile in horizontal 0·7 % agarose gel: *Sinorhizobium meliloti* GR4 (lane 1) and strain PETP02^T (lane 2). (b) Results of hybridization (marked by arrowheads) using the *nodD* gene probe: strain GR4 (lane 3) and strain PETP02^T (lane 2).

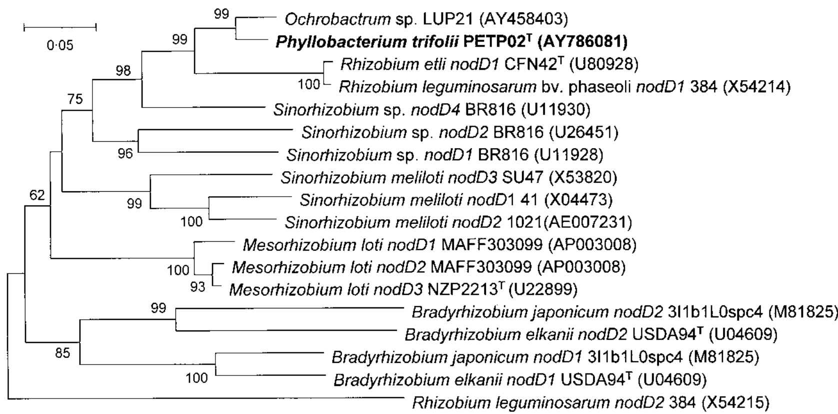

Fig. 3. Comparative sequence analysis of the *nodD* gene from *Phyllobacterium trifolii* PETP02^T and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. The analysis was based on 486 nt. *Rhizobium leguminosarum* 384 was used as outgroup. Bar, 5 nt substitutions per 1000 nt.

per plant in both *Trifolium* and *Lupinus* plants than did *R. leguminosarum* bv. trifolii ATCC 14480 and *Bradyrhizobium* sp. ISLU35, used respectively as positive controls (data not shown).

The DNA G+C content of strain PETP02^T as determined by HPLC (Rivas *et al.*, 2003) was 56·4 mol%. This value is lower than the range of 60·3–61·3 mol% reported for *P. myrsinacearum* (de Smedt & de Ley, 1977).

DNA–DNA hybridization was performed using a protocol described by Willems *et al.* (2001) and Rivas *et al.* (2004). Strain PETP02^T gave DNA–DNA hybridization levels of 12·0 % with two strains of *P. myrsinacearum*, LMG 1t1 and LMG 2t2^T.

Phenotypic characterization of strain PETP02^T was based on growth with different carbon sources (Bergersen, 1961) as described previously (Velázquez *et al.*, 2001). *P. myrsinacearum* LMG 2t2^T and LMG 1t1 (formerly *P. rubiacearum*) were used as reference strains. The temperature range for growth was determined by incubating cultures in YMA medium between 4 and 40 °C. The pH range was determined in YMA medium with a final pH between 5·0 and 10·0. Salt tolerance was studied in YMA medium containing 0–5 % (w/v) NaCl. Antibiotic resistance was tested by using the disc diffusion method with the following antibiotics: 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}. Strain PETP02^T and strains LMG 2t2^T and LMG 1t1 were also characterized by using API 20NE tests according to the manufacturer's instructions (bioMérieux).

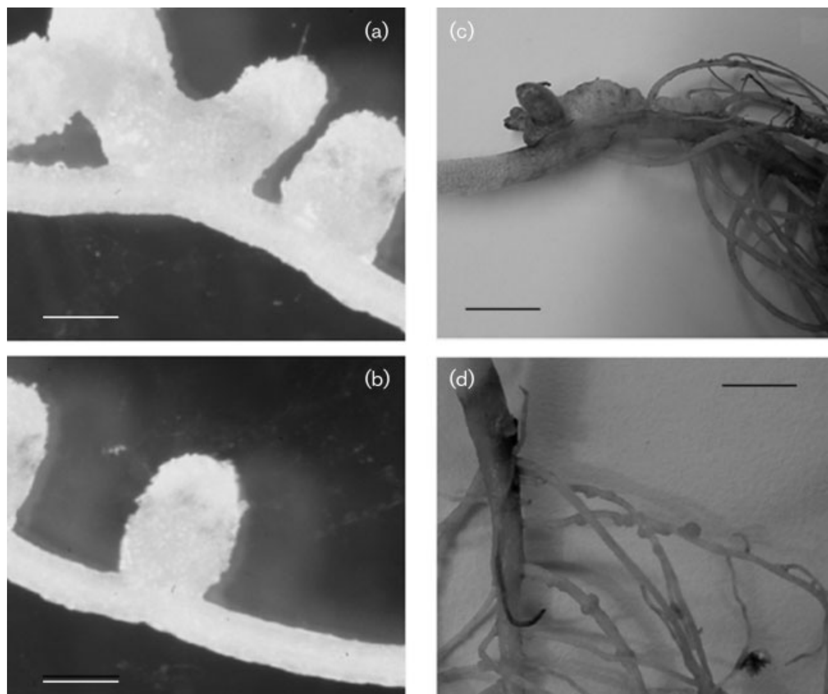

Fig. 4. Nodules induced in *Trifolium repens* by strain PETP02^T (a) and by *Rhizobium leguminosarum* bv. trifolii ATCC 14480 (b). Bars, 2 cm. Nodules induced in *Lupinus albus* roots by strain PETP02^T (c) and by *Bradyrhizobium* sp. ISLU35 (d). Bars, 0·2 cm.

Table 1. Differential phenotypic characteristics of *Phyllobacterium trifolii* sp. nov. PETP02^T and *P. myrsinacearum*

+, Positive; −, negative; W, weak.

Characteristic	*P. trifolii* PETP02^T	*P. myrsinacearum*
Acid from:		
Sucrose	−	+
Rhamnose	W	+
Trehalose	−	+
Adonitol	W	+
Raffinose	−	+
Citrate assimilation	−	+
Resistant to:		
Polymyxin B	−	+
Oxytetracycline	−	+
Neomycin	−	+

The results indicated that strain PETP02^T differs from strains of *P. myrsinacearum* in acid production (after 4 days of incubation) from sucrose, trehalose and raffinose, citrate assimilation, and resistance to polymyxin B, oxytetracycline and neomycin (Table 1). Acid production from rhamnose and adonitol was positive in *P. myrsinacearum* but weak in strain PETP02^T. Additional phenotypic characteristics of strain PETP02^T are given in the species description below.

We also compared the fatty acid composition of strain PETP02^T with those of *P. myrsinacearum* strains LMG 1t1 and LMG 2t2^T. Cells were grown for 48 h on TY medium (Jarvis *et al.*, 1996) and fatty acids were extracted and analysed in duplicate as described by Rivas *et al.* (2003). The results (Table 2) confirmed previous observations for *P. myrsinacearum* (Mergaert *et al.*, 2002). As in the case of *P. myrsinacearum*, the novel strain contains 18 : 1ω7*c* as the predominant fatty acid. It differs from *P. myrsinacearum* in that it contains more than 10 % 16 : 0, more than 15 % 18 : 1ω7*c* 11Me, small amounts of 17 : 0 and 20 : 2ω6,9*c* (neither of which was detected in *P. myrsinacearum*), less than 5 % 18 : 1 2-OH and no 18 : 0 3-OH.

Our results showed that strain PETP02^T is able to nodulate *Trifolium* and *Lupinus*, increasing the number of non-rhizobial species that are able to nodulate legumes. This strain can be differentiated genotypically and phenotypically from previously described species and we therefore consider it to represent a novel species, for which the name *Phyllobacterium trifolii* sp. nov. is proposed.

Table 2. Fatty acid methyl ester (FAME) profiles

Values are mean percentages of total FAMEs. Only fatty acids accounting for more than 1·0 % (mean) are indicated. tr, Trace amount (< 1·0 %); ND, not detected.

FAME	*P. myrsinacearum*		*P. trifolii* LMG 22712^T
	LMG 1t1	LMG 2t2^T	
14 : 0	ND	ND	tr
16 : 0	5·6	4·9	14·0
15 : 0 3-OH	1·3	1·9	tr
17 : 0 cyclo	ND	ND	tr
17 : 0	ND	ND	1·2
16 : 0 2-OH	tr	1·4	ND
16 : 0 3-OH	14·4	12·8	2·4
18 : 0	ND	tr	tr
18 : 1ω7*c*	30·9	36·3	35·8
18 : 1ω7*c* 11Me	3·0	3·8	16·8
19 : 0 cyclo ω8*c*	13·9	9·0	13·4
19 : 0 10Me	ND	1·0	ND
18 : 1 2-OH	13·5	14·3	1·2
18 : 0 3-OH	1·8	1·6	ND
20 : 2ω6,9*c*	ND	ND	1·6
20 : 0	8·7	5·8	3·8
Summed feature 2*	4·2	4·5	2·7
Summed feature 3*	1·9	2·2	4·3

*Summed features consist of one or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 2 consists of 12 : 0 aldehyde, iso-16 : 1 I and/or 14 : 0 3-OH; summed feature 3 consists of 16 : 1ω7*c* and/or iso-15 : 0 2-OH.

Description of *Phyllobacterium trifolii* sp. nov.

Phyllobacterium trifolii (tri.fo′li.i. L. gen. n. *trifolii* of clover).

Gram-negative rods, as for the other species of the genus. Colonies are small, pearl white in YMA at 28 °C. Temperature range for growth is 4–37 °C (optimal growth occurs at 28 °C). The pH range for growth is 6–8 (optimal growth occurs at pH 7). Grows in the presence of NaCl concentrations up to 3 % (w/v) although salt is not essential for growth. Isolated from *Trifolium pratense*, it is able to produce nodules on *Trifolium* and *Lupinus*. Nitrate reduction is negative. It does not produce indole gelatinase, β-galactosidase or arginine dihydrolase. Hydrolysis of urea and aesculin was weak. Produces acid from galactose and arabinose. Acid production from rhamnose and arabitol is weak. Uses glucose, L-arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose and malate as carbon sources. Gentiobiose is weakly used. It does not grow on caproate, adipate, citrate or phenylacetate. Resistant to cloxacillin, penicillin, erythromycin, cefuroxime and ampicillin. Does not grow in the presence of polymyxin B, ciprofloxacin, gentamicin, oxytetracycline or neomycin. The DNA G+C content is 56·4 mol%.

The type strain, PETP02^T (=LMG 22712^T=CECT 7015^T), was isolated from a *Trifolium pratense* root nodule.

Acknowledgements

This work was supported by Junta de Castilla y León and DGCYT (Spanish Government). A. W. is grateful to the Fund for Scientific

Research – Flanders for a position as Postdoctoral Researcher. We thank Renata Coopman and Ilse Vandecandelaere for excellent technical assistance.

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